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Catecholamine-Containing Biodegradable Microsphere Implants as a Novel Approach in the Treatment of CNS Neurodegenerative Disease

A Review of Experimental Studies in DA-Lesioned Rats

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Abstract

Biodegradable controlled-release microsphere systems made with the biocompatible biodegradable polyester excipient poly(DL-lactide-co-glycolide) constitute an exciting new technology for drug delivery to the central nervous system (CNS). Implantable controlled-release microspheres containing dopamine (DA) or norepinephrine (NE) provide a novel means to compare DA- or NE -induced restitution of function in unilateral 6-hydroxydopamine lesioned rats. A suspension of 3 μL of DA- or NE-containing microspheres or empty microspheres was implanted in 2 sites of the DA denervated striatum of rats previously unilaterally lesioned with 6-hydroxydopamine. Contralateral-rotational behavior induced by apomorphine was used as an index of lesion success and, following implantation of the microspheres, also as an index of functional recovery. Interestingly, both DA- and NE-microsphere-implanted rats displayed a 30–50% reduction in the number of apomorphine-induced rotations up to 8 wk postimplantation. Rats implanted with empty microspheres did not demonstrate significant changes in contralateral rotational behavior. Behavioral studies following implantation of a mixture of DA and NE microspheres revealed an 80% decrease in the number of apomorphine induced rotations up to 4 wk. On conclusion of the studies, immunocytochemical examination revealed growth of DA and tyrosine hydroxylase immunoreactive fibers in the striatum of DA and NE microsphere-implanted rats. Functional behavior appeared to correlate with the degree of fiber growth. Preliminary electron microscopic studies showed signs of axonal sprouting in the vicinity of the implanted microspheres. No growth was noted in rats implanted with empty microspheres. This report reviews the abilities of both microencapsulated NE and DA to assure functional recovery and to promote DA fiber (re)growth in parkinsonian rats. This novel means to deliver these substances to the central nervous system could be of therapeutic usefulness in Parkinson's disease.

Index Entries: Microencapsulated catecholamines; poly(DL-lactide-co-glycolide); experimental hemi-Parkinsonism in rats; striatum; apomorphine; 6-hydroxydopamine; dopamine fiber growth.

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Introduction

Drug-Delivery Systems for the Central Nervous System

The main neurochemical characteristic of Parkinson's disease (PD) is a marked lesion of the nigrostriatal dopamine pathway. In attempts to provide dopamine replacement therapy to Parkinson's patients, the current medication is L-DOPA *(1)*. Dopamine itself cannot be taken orally because it will not reach the brain. Unfortunately, L-DOPA can cause serious adverse reactions and its effectiveness decreases with time. For these reasons, there has been an increasing demand for and interest in novel techniques for site-directed delivery of substances into the central nervous system (CNS) *(2)*.

General Delivery Systems

It has been recognized for a long time that directing a drug to its therapeutic site of action within the CNS can be a very difficult task. Techniques used to deliver drugs to the CNS have to overcome chemical and physical barriers *(3,4)*. A number of methods have been designed in attempts to overcome some of these barriers. For instance, liposomes have been employed as a means to surmount the blood–brain barrier *(5)*. These small spheres, prepared from fatty molecules, will biodegrade, and can be made to entrap a variety of drugs. The disadvantage is their instability once in the body, low drug content, rapid entrapment, and degradation by the reticuloendothelial system.

Another approach consists of chemically modifying the active drug to a form (prodrug) that the body can absorb and will then convert to active drug. One such example is a prodrug that combines the neurotransmitter dopamine (DA) with a molecular mask derived from the fat-soluble vitamin niacin. The modified DA is taken up into the brain, where it is then slowly stripped from its prodrug mask to yield free DA *(6)*.

The most common approach to surmount some of the physical barriers is through the use of pumps. A variety of pumps have been designed for the purpose of drug delivery to the CNS *(7)*. For the most part, the drug is delivered through a catheter implanted in the lateral ventricles. In contrast to the prodrug approach, pump delivery can be externally controlled to a certain degree. The exact site of action of the drug within the CNS is largely beyond control, using either prodrug or pump approaches. It does not suffice just to get the drug within the CNS; it should be delivered at the intended site of action, at the required rate, and in proper therapeutic dosage *(8)*. The Alzet™ osmotic minipump has become a very useful and successful means of delivering drugs at a controlled rate and dose, for long periods of time—also within the CNS. However, adapting this technique to deliver drugs to discrete brain nuclei presents numerous difficulties. Also this minipump is not biodegradable.

Site-directed delivery of DA specifically aimed at damaged regions of the central nervous system would probably be more efficient with less adverse reactions. For this reason, there has been an enormous effort to find ways to deliver DA directly to the striatum.

Site-Directed Systems

Tissue Transplantation

Dopamine-producing cells (either of adrenal or fetal origin) transplanted into the striatum of animals with experimentally induced parkinsonism, survive, produce DA, and effect improved motor control *(9–15)*. These transplantation techniques surmount a number of the obstacles cited above. Viable neuronal tissue can be implanted directly within discrete brain nuclei. The duration of substance delivery from the transplanted tissue does not present a problem because the tissue may survive for a long time within the host CNS *(16)*. On the other hand, the action of the transplants once implanted is not easily controlled or terminated. Implantation of DA-producing cells has been shown to enhance the recovery of dopaminergic neurons or growth of DA fibers within the denervated striatum *(9,10)*. Transplantations of cell preparations like those described have even been performed in PD patients *(17–19)*. However, there are a number of practical as well as ethical concerns about the continuation of this cell-transplantation therapy.

Controlled-Release Polymeric Implants

Controlled release of encapsulated substances is not a new concept, but its application to the CNS is on the forefront. Quite recently, controlled-release polymeric implants have been examined as alternative sources for transmitter replacement in animal experimental models of parkinsonism. The section below describes some of these polymeric devices.

Nonresorbable Polymers

DA and DA-producing cells have been encapsulated in a device made with ethylene vinyl acetate

(EVAc) copolymers (20–23). In this device, a sustained release of DA occurs through a semipermeable membrane. Silicone polymers have also been employed to encapsulate DA (24). These polymeric devices are produced in a variety of shapes, including disks (21,22), rods (23), or pellets (24), and range in diameter from 500 μm to 3 mm (20–24). All the above devices have been shown to release DA for at least 2 mo and attenuate apomorphine-induced rotational behavior in rats with experimental parkinsonism (20–24). In spite of their success, the size of these polymeric devices is a serious drawback. Significant mechanical tissue damage results when they are implanted and removed (23); indeed, aspiration of parts of the overlying neocortex was required in order to put a disk-shaped DA-entrapping polymer in position for release into striatum (21).

Resorbable Polymers

Researchers at Southern Research Institute (SRI) in Birmingham, AL, have developed injectable, microencapsulated, controlled-release delivery systems in which substances can be encapsulated with biocompatible and biodegradable poly (DL-lactide-co-glycolide) (DL-PLG) copolymers (25,26). Drugs, neurotransmitters, vaccines, growth factors, this list seems limitless, can be microencapsulated in these formulations—so-called microspheres. The microspheres are tiny spheres, so small that over 1000 would fit inside a grain of common table salt. The microsphere excipient serves two functions: protecting the contents from preterm degradation, and releasing substances at a controlled rate for desired time periods extending for weeks or months. The polymeric material is of the same class used for biodegradable, synthetic sutures and has a long history of safe use in humans. Also, the drugs are dispersed within the DL-PLG matrix of the microspheres in a dry state, thus providing extended shelf life without the need for stabilizers.

After administration to the body, DL-PLG induces only a minimal inflammatory response, and biodegrades through the hydrolysis of its ester linkages to yield the biocompatible lactic and glycolic acids (25,26). The rate at which DL-PLG biodegrades is a function of the ratio of lactide-to-glycolide. Thus, microspheres with different lactide-to-glycolide ratios, when blended prior to administration, allow a single injection to release one or more programmed booster doses at predicted intervals (25,26), to afford a constant release of drug.

The microsphere product is a free-flowing powder. The amount of drug inside the microsphere can be varied as desired ranging from very small amounts to as high as 95% of the microsphere composition. The diameter of the microsphere can also be varied as desired, ranging from <1 μm to as large as 3 mm. The microspheres are easily suspended in aqueous vehicles and may be injected through conventional hypodermic needles or cannulaes. The microspheres are sterilized with gamma radiation or prepared aseptically.

To date, a variety of substances have been microencapsulated, including steroids, fluoride, peptides, antibiotics, and vaccines (26,27). Extensive testing of these microencapsulated substances in rodents—and in some cases, also in humans—support the feasibility of this system for drug delivery (28). The technique also allows a major reduction in the total dose administered into the organism; microencapsulation will thereby in effect enhance substance "potency" dramatically.

The numerous advantages of the microsphere technology suggests that it has an application as a means to deliver transmitter molecules to the CNS. The potential use of microspheres as sources of transmitter replacement has been examined in the unilaterally hydroxydopamine-lesioned rat. Even though DA is considered as the main target in PD, there are reasons to consider that deficiencies in norepinephrine (NE) may also participate in the symptomatology and progression of PD (29). It therefore appeared pertinent to compare the ability of implanted DA- and/or NE-containing microspheres to correct motor function in experimental parkinsonism rats.

Our previous findings indicate that a single administration of microencapsulated DA directly into the denervated striatum provides prolonged release of this neurotransmitter into striatal tissue, to substitute for the experimentally induced subnormal levels of the endogenous transmitter (30–32). Immunocytochemical investigations of the tissue from these rats unexpectedly revealed the growth of DA and tyrosine hydroxylase (TH) immunoreactive fibers in the predenervated striatum. Interestingly, there was an apparent correlation between functional recovery and the degree of fiber growth in the DA microsphere-treated animals (33). The microsphere implant technology may therefore be considered useful not only in PD, but potentially also in other neurodegenerative diseases (e.g., Alzheimer's disease). The object of this report is to review behavioral and immunocy-

tochemical results from rats with experimental parkinsonism implanted with DA- and/or NE-containing microspheres.

Methods

Polymeric Excipients

The DA or NE microspheres consist of 40% (by weight) DA or NE and 60% (by weight) of the copolymer. They consist of spherical particles approx 5–45 μm in diameter. DA was encapsulated in two types of copolymer excipients. One copolymer had a 50:50 mole ratio of lactide-to-glycolide (referred to as 50:50). The other copolyner had a 65:35 molar ratio of lactide-to-glycolide (65:35). It is known that, because of its higher lactide content, the 65:35 copolymer will take longer to biodegrade than the 50:50 copolymer, thus potentially affording a longer duration of delivery of DA in vivo. To ensure that similar amounts of DA would be released per unit time the quantity of DA 65:35 microspheres (30 mg for about 2 mo) employed was therefore twice that of the DA 50:50 microspheres (15 mg for about 1 mo). NE was only encapsulated in the 50:50 copolymer. Total vehicle, saline, was 50 μL in all cases.

DA Denervation Procedure

Male Sprague-Dawley rats (200–250 g) were unilaterally lesioned, under ether anesthesia, in the ascending median forebrain bundle (MFB) of monamine neurons (coordinates A-P –4.3, L +1.4, D-V- 8.7 from bregma, midline, and top of skull, respectively) *(34)* using 6-hydroxydopamine HCl (6-OHDA; Sigma, St. Louis, MO); 8 μg/4 μL saline vehicle containing 0.1% ascorbate. It is well established that this treatment results in an upregulation of postsynaptic DA receptors in the denervated striatum, functionally manifested as contralateral rotational behavior after parenteral DA-agonist administration *(35)*. Two weeks after lesion, rats were challenged with the classical DA-agonist apomorphine HCl (0.1 mg/kg SC) and rotational responses were monitored in a computerized rotometer setup *(32)*. Animals responding to apomorphine with <400 contralateral-rotations per 60 min within the first 2 wk of testing were eliminated from the study.

Microsphere Implantation

Rats selected for the studies had responded for 6 wk after the 6-OHDA lesion with a typical "two-peak" contralateral-rotation pattern to apomorphine challenge, a reliable indicator of ≥95% DA lesion success *(36,37)*. They were stereotaxically injected under light ether anesthesia with a suspension containing microspheres (prepared immediately prior to injection). Results are reported from groups of rats receiving DA 50:50, DA 65:35, NE 50:50, an equal mixture of DA and NE 50:50, or empty (sham) microspheres. The microspheres were infused into two sites (3 μL-deposits, 2 levels/site) in the striatum (A-P +0.7, L 2.3, D-V 4.5 and 5.5; A-P + 0.2, L 2.3, D-V 4.5 and 5.5 *(34)*. The injections were performed with a 10-μL glass capillary tube (calibrated at 3-μL intervals) connected to a 50-μL Hamilton syringe via standard polyethylene tubing. The entire duration of injection was 3 min/site. On completion of the second infusion, the injection cannulae was left *in situ* for an additional 60 s before being slowly retracted. The skin wound was closed with surgical clips and the animals were allowed to recover from anesthesia.

Following implantation, all microsphere-implanted rats were challenged with apomorphine and their contralateral-rotatory behavior was recorded on a weekly basis *(30–32)*. An attenuated responsiveness to DA agonist challenge in this experimental model is likely explained by reversal of the 6-OHDA lesion-induced postsynaptic DA receptor upregulation, and can therefore be used as an index of functional normalization (recovery) after implantation of the in vivo DA-releasing microspheres *(36,37)*.

Immunocytochemistry

Light Microscopy

At the termination of the behavioral studies, the rats were overdosed with sodium pentobarbital and perfusion-fixed with 5% glutaraldehyde. Following perfusion, the brains were left overnight in a solution containing 10% sucrose in Tris-meta bisulfite (pH 7.6). The brains were then frozen using compressed CO_2, and 10-μm sections of the striatum incubated overnight with anti-DA antiserum (1/500) *(38)*. The immunocytochemistry was performed with the avidin-biotin peroxidase technique, and DA immunoreactivity was visualized with diaminobenzidine (DAB) enhanced with nickel ammonium sulfate *(39)*.

For tyrosine hydroxylase (TH) immunocytochemistry rats were overdosed with sodium pentobarbital and perfusion-fixed with 4% paraformaldehyde (PFA). The brains were postfixed for

4 h in the PFA solution and then immersed overnight in phosphate-buffered saline (PBS) containing 10% sucrose. Cryostat sections were then incubated overnight with anti-TH antibody (1/800) and further processed with the avidin-biotin peroxidase method (VECTOR). TH immunoreactivity was visualized using the method described above. All sections were photographed with a Nikon (Tokyo, Japan) Optiphot microscope using Agfa (100 ASA) film.

Immunoelectron Microscopy

In these experiments rats were perfused with 4% paraformaldehyde/0.2% glutaraldehyde in a phosphate buffer. After 4 h of postfixation the brains were rinsed in PBS and vibratome sections were incubated with anti-TH antiserum overnight. After the glucose oxidase procedure *(see above)* sections of the striatum were rinsed in 1*M* cacodylate buffer. A dissecting microscope was used to visualize striatal regions where the microspheres were implanted. Small tissues pieces were cut from this region and postosmicated in 1% osmium tetroxide for 30 min. Dehydration was carried out in a graded series of alcohols followed by embedding in an Araldite® mixture. Ultrathin sections were prepared of the striatal tissue, contrasted with lead citrate, and examined in a Jeol (Tokyo, Japan) 1200 EX microscope.

Results and Discussion

Dopamine-Containing Microspheres

Effect of Different Polymer Excipients on the Apomorphine-Induced Rotational Response

DA was microencapsulated in two copolymer excipients (*see* description of polymer excipients) having different lactide-to-glycolide mole ratios. The object was to compare these 2 different copolymer formulations. This was accomplished by comparing the duration of time that these intrastriatally implanted DA microspheres were able to reduce the extent of apomorphine-induced rotational behavior in unilaterally 6-OHDA-lesioned rats.

DA 50:50 Implants

When compared to the average premicrosphere rotational baseline, the DA 50:50 implanted rats displayed a 29% decrease in the total number of apomorphine-induced contralateral rotations 4 wk postimplantation. During the subsequent 2 wk the response to apomorphine challenge slowly approached the premicrosphere baseline (Fig. 1A).

DA 65:35 Implants

Compared to their premicrosphere rotational baseline, DA 65:35 implanted rats displayed a 37% decrease in the total number of apomorphine-induced contralateral rotations 2 wk postimplantation. From there on and up to 8 wk following implantation there was a significant average reduction corresponding to a 26% decrease in the total number of apomorphine-induced contralateral rotations (Fig. 1B).

Sham Implants

During the 6-wk testing period postimplantation, rats that received empty microspheres did not display any significant change in the number of apomorphine-induced contralateral rotations when compared to the preimplantation baseline (Fig. 1C).

First, these results demonstrate that implantation of microencapsulated DA in the striatum can counteract, over respectable periods of time, apomorphine-induced rotational behavior in rats with chronic unilateral 6-OHDA lesions of ascending (nigrostriatal) dopaminergic neurons. The attenuated responsiveness to DA agonist challenge in this experimental model is likely explained by a downregulation of denervation-sensitized striatal postsynaptic DA receptors, following in vivo implantation of the DA-releasing microspheres. Furthermore, the microsphere-induced attenuation of apomorphine-induced rotation is comparable to that reported for implanted chromaffin tissue in the same rodent model *(15)*.

Second, the results show that 65:35 PLG microspheres afford drug release longer than 50:50 PLG microspheres. Implanted 65:35 DA microspheres resulted in a rather constant reduction in the apomorphine response from wk 3 through wk 8. Implanted 50:50 DA microspheres resulted in a reduction in the apomorphine response through wk 4. This result agrees with the resorption time typically seen for microspheres made with 50:50 and 65:35 PLGs having inherent viscosities of about 0.7 dL/g. In microsphere form, 50:50 PLG typically resorbs in about 5 wk; 65:35 in about 8 wk. At constant molecular weight, increasing the lactide content of PLG increases the resorption time. We anticipate DA and NE is initially released from microspheres through water-filled pores in the PLG excipient, with subsequent release as PLG resorbs *(25,26)*.

Importantly, the data equally support the biocompatibility of the microsphere formulations. It

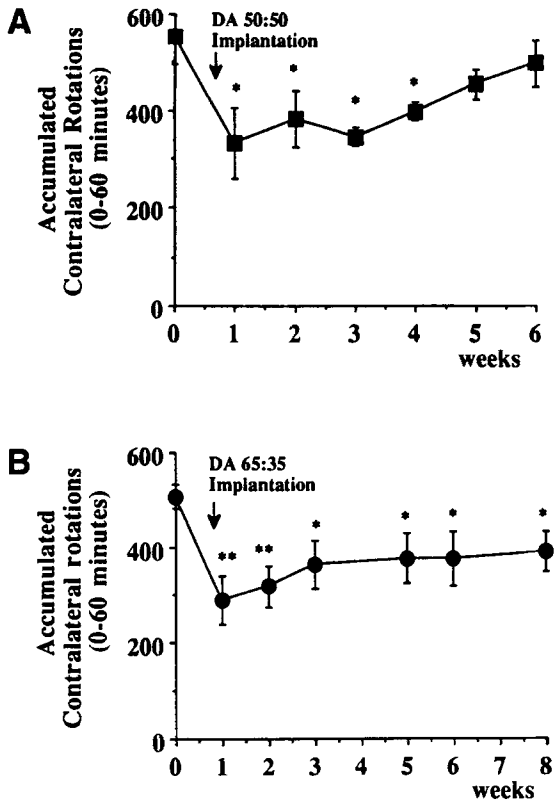
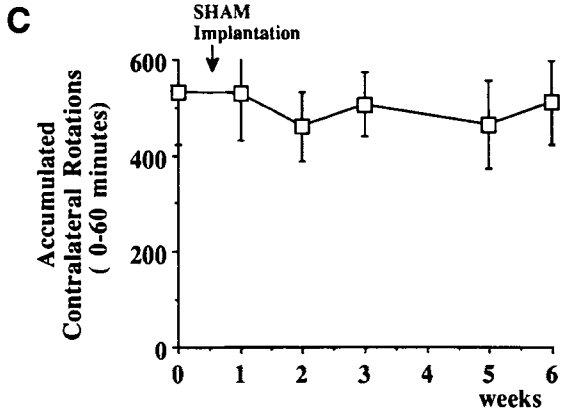


Fig. 1. **(A)** Total number of apomorphine-induced contralateral rotations in 6-OHDA-treated rats before and for 6 consecutive wk following implantation of DA 50:50 microspheres. Bars represent the mean ± SEM (n = 5). The preimplantation baseline value (= obtained in the last apomorphine test session before implantation) was 553 ± 58. This value is shown at time 0. Abscissa, time in weeks; ordinate, accumulated contralateral rotations recorded for 60 min. Statistics: paired t-test $^*p < 0.05$ compared to the preimplantation baseline value. **(B)** Total number of apomorphine-induced contralateral rotations in 6-OHDA-treated rats before and for 8 consecutive wk following implantation of DA 65:35 microspheres. Bars represent the mean ± SEM (n = 9). The preimplantation baseline value (= obtained in the last apomorphine test session before implantation) was 506 ± 26. Statistics: paired t-test $^*p < 0.05$, $^*p < 0.001$ compared to the preimplantation baseline value.



(C) The number of apomorphine-induced contralateral rotations in 6-OHDA-treated rats before and for 6 consecutive wk following implantation of empty microspheres. Bars represent the mean ± SEM (n = 4). The preimplantation baseline value (= obtained in the last apomorphine test session before implantation) was 532 ± 108. This value is shown at time 0. There was no statistical difference in the number of contralateral rotations postimplantation compared to the preimplantation baseline value.

might be suggested that biodegradation through hydrolysis of the excipients used was harmful to the brain tissue. If so, it could be expected that the striatal tissue would be damaged during the process and thus the rats would no longer be able to respond to apomorphine after termination of the biodegradation period. Our results clearly demonstrate that this is not the case. First, the DA 50:50 animals returned to preimplantation baseline values, and second, animals implanted with "sham" microspheres (without DA, but of the same DL-PLG composition) did not display any reduction in their apomorphine-induced rotational behavior over the entire period.

Apomorphine-Induced Rotation Correlates with the Degree of Fiber Growth

Even though, as a group, the DA 65:35 implanted rats showed significant reductions in apomorphine-induced rotational behavior there were differences in response amplitude among the individual rats. These differences could be related to the placement of microspheres, or the amount of DA released from the microspheres. In order to investigate these possibilities, an experiment with rats implanted with DA 65:35 was run for 8 wk and followed by immunocytochemical investigations. Variations in the reduction of apomorphine-induced rotation ranged between 10 and 50%. The immunocytochemical investigations unexpectedly revealed the presence of DA-immunoreactive fibers growing in the predenervated striatum. Fiber growth was rated blindly by an observer on a graded scale: sparse, moderate, or intense. One immediate observation was that there were clearcut differences in the degree of fiber growth among the rats. The fiber growth ratings were there-

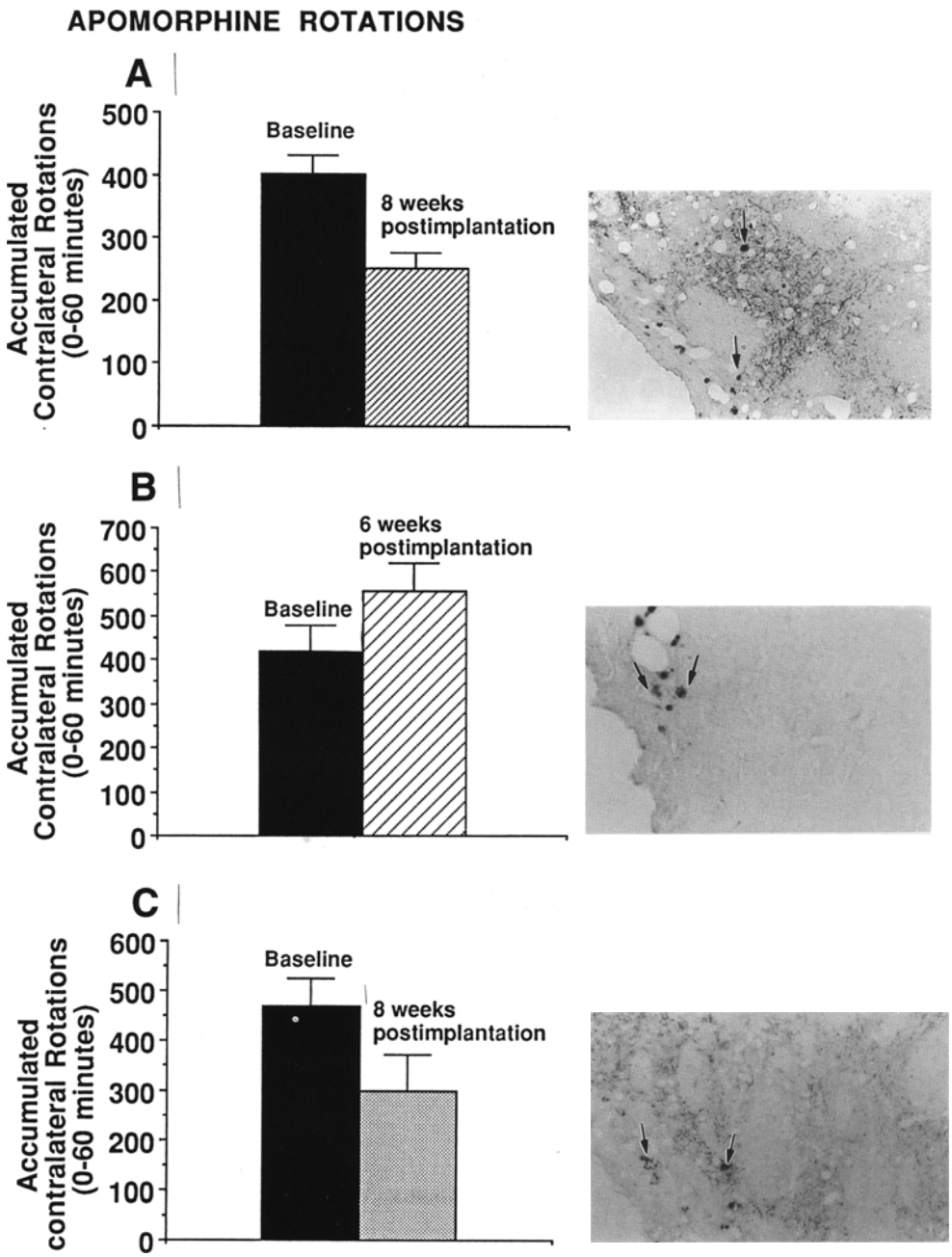


Fig. 2. The left panel of the figure gives accumulated contralateral rotations for 3 DA 65:35 implanted rats following 6–8 wk after implantation of microspheres. The right panel shows fiber growth or the absence in the striatum of the individual rats. The fibers were visualized with anti-DA antiserum (1/500, *see text* for description). **(A and C)** Eight weeks postimplantation there was a 46 and 37% decrease in the total number of contralateral rotations, respectively. The fiber growth in the striatum was considered as being intense. Small arrows show the microspheres and numerous immunoreactive fibers can be observed growing in the vicinity of the microspheres. **(B)** There were no reductions in contralateral rotational behavior and the striatum was devoid of immunoreactivity.

fore compared to the functional data obtained. As indicated in Fig. 2 there seems to be an apparent correlation between functional recovery and the degree of fiber growth. However, the fiber growth could be related to a number of factors, such as wounded tissue, uptake of DA in other fiber sys-

tems (e.g., serotoninergic), or possibly the weekly repeated administration of apomorphine.

An investigation was then carried out that included DA-containing and sham microsphere implanted rats, as well as a group of DA-microsphere-implanted rats that did not receive the weekly apomorphine challenge. Some of the DA microsphere-implanted rats were then processed for tyrosine hydroxylase immunoreactivity to answer questions about uptake of DA in non-DA fibers. A comparison of DA and TH-immunoreactive fibers growing in the striatum of DA-microsphere-implanted rats is depicted in Fig. 3. The same figure shows the absence of immunoreactive fibers in rats implanted with empty microspheres. Observations of DA fiber growth in rats not treated with apomorphine have been reported *(33)*.

Ultrastructural investigations confirmed the presence of TH-immunoreactive processes growing in the vicinity of implanted microspheres up to 3 mo following implantation (Fig. 4).

Our observations demonstrate that implanted DA microspheres may induce growth of DA fibers in the denervated rat striatum. The claim that the DA immunoreactive fibers in the lesioned striatum represent new growth as opposed to sprouting of partially denervated fibers is supported by the fact that rats employed in this study had responded with the typical "two-peak" contralateral rotation pattern when initially challenged with apomorphine, a reliable indicator that the 6-OHDA lesion had resulted in a near-total (≥95%) nigrostriatal DA denervation *(36,37)*. However, further studies are warranted, with antibodies aimed at establishing growth, such as growth-associated protein 43 (GAP-43) or *in situ* hybridization techniques.

Fibers, demonstrable both by DA and by TH immunocytochemistry, appear to grow mainly around the DA microspheres, hence clearly contrasting with the remaining denervated parts of the striatum. Also, if the visualized immunoreactivity were a result of an uptake of DA in, e.g., tryptaminergic fibers, one would have expected a distinctly denser innervation because there is a vast serotonin innervation of the striatum *(40)*. Even though the source of the fibers is presently unknown, it is of interest to note that the fibers appear to emanate from the ventral striatum, analogously to what has been reported in the nonhuman primate brain following adrenal medullary transplantation *(9,41)*. The distribution of fiber growth following DA microsphere implantation is comparable to previous observations on growth of TH-positive fibers in the striatum after adrenal medulla transplants *(10)*.

It might be suggested that the DA fiber growth results from effects of the microsphere excipient *per se*, or from surgical tissue injury *(9,41)*. However, the absence of fiber growth in the sham microsphere implanted rats argues against local effects of the poly(DL-lactide-co-glycolide) microsphere excipient, the surgical procedure, gliosis, or macrophage invasion as factors involved in the presently observed DA-fiber growth process. Neither does the repeated apomorphine challenge regimen explain our findings, since DA-fiber growth was equally observed also in rats that had not received apomorphine over the 8-wk period after microsphere implantation *(33)*.

The absence of fiber growth in some DA-microsphere-implanted rats may be tentatively attributed to insufficient DA delivery during the 8 wk, or to the placement of the microspheres in the striatum, or to unknown mechanisms that govern fiber growth.

Norepinephrine-Containing Microspheres

Apomorphine-Induced Rotational Behavior

As shown in Fig. 5, the attenuation of apomorphine-induced rotational behavior by implanted NE microspheres is comparable to that of DA. The mechanisms through which NE influences DA receptors is still unclear. However, there are indications for a noradrenergic interaction with DA receptors in the denervated striatum. First, direct injections of NE to the denervated striatum induce rotational behavior *(42)*. Second, implanted adrenal medulla tissue, which releases amounts of NE and epinephrine that far exceed that of DA, elicits an immediate rotational response in rats *(42)*. Third, pharmacological studies have shown that α2-adrenoceptor agonists inhibit amphetamine-induced rotation *(43)*, whereas α2-adrenoceptor antagonists enhance both amphetamine- and apomorphine-induced rotational behavior *(44)*. Of particular note in this context are the results of Richardson and Heath *(45)*, who implanted catethers into the striatum of PD patients as a means to deliver NE. Intermittent injections of NE were carried out for 6 wk at which time the catethers were removed. The "on"-time and PD symptoms, including tremors, rigidity, and oculogyric crisis, were improved for extended periods of time—actually up to 12 yr in one of the 5 patients. These results add significant support to the hypothesis that noradrenergic mechanisms may play an important role in the

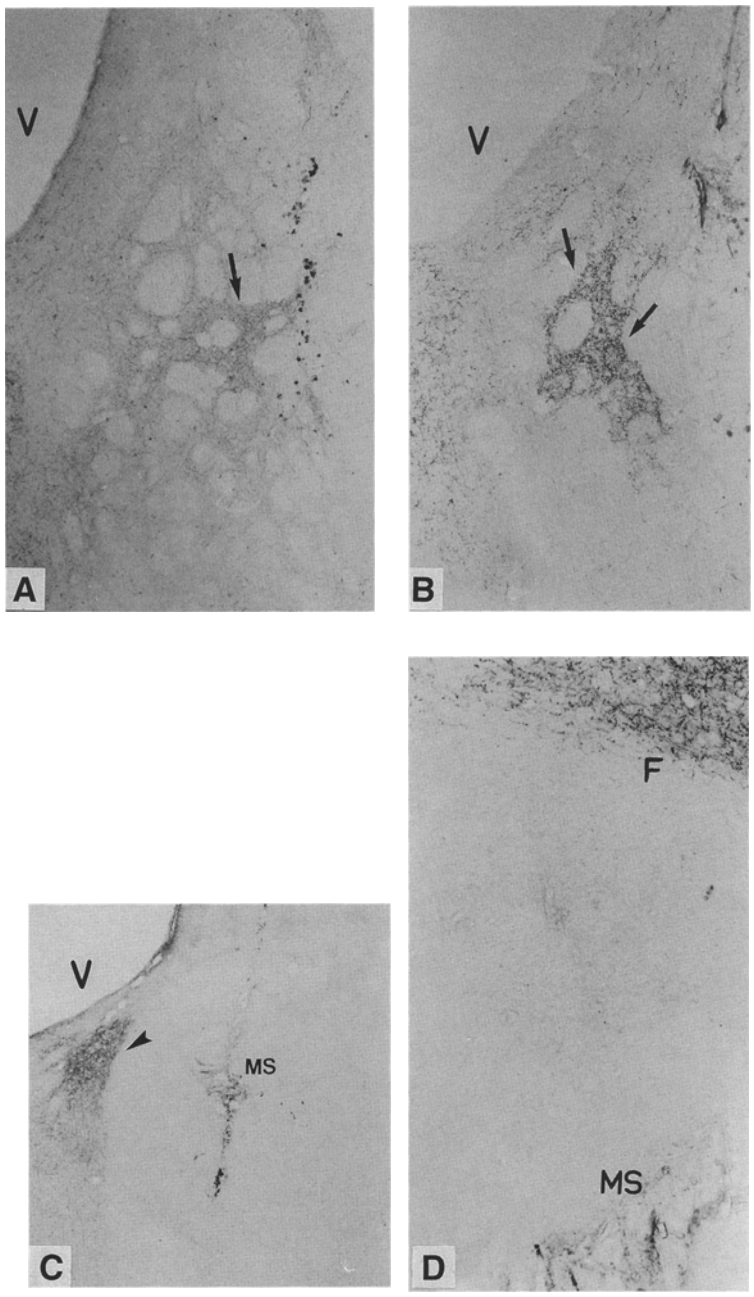


Fig. 3. Photomicrographs of DA **(A)** or TH **(B–D)** immunoreactivity in frontal cryostat sections (10 μm) of the striatum 8 (A) or 15 wk (B–D) after implantation of DA microspheres (A, B) or empty (C, D). This figure illustrates how fibers grow in mass toward DA microspheres but not toward empty microspheres. In addition, the entire photomicrograph demonstrates that implantation of the microspheres does not cause any noticeable damage to the striatal tissue. (A–B) Typical examples of placement of DA microspheres implants in the predenervated striatum. The sections show DA (A) or TH (B) immunoreactive fibers in the previously 6-OHDA lesioned striatum. The DA microspheres are readily observed in the striatum (arrowhead) and numerous DA or TH immunoreactive fibers (arrows) are growing toward and around them (×80). (C–D) Photomicrographs of the striatum 15 wk following following implantation of *empty microspheres*. This section is devoid of TH immunoreactivity and there is no outward fiber growth from the remaining TH positive fibers (arrowhead) (×32). (D) is an enlarged view of the space between remaining TH immunoreactive fibers (F) and the placement of the microspheres (MS) and shows the absence of fibers (×320).

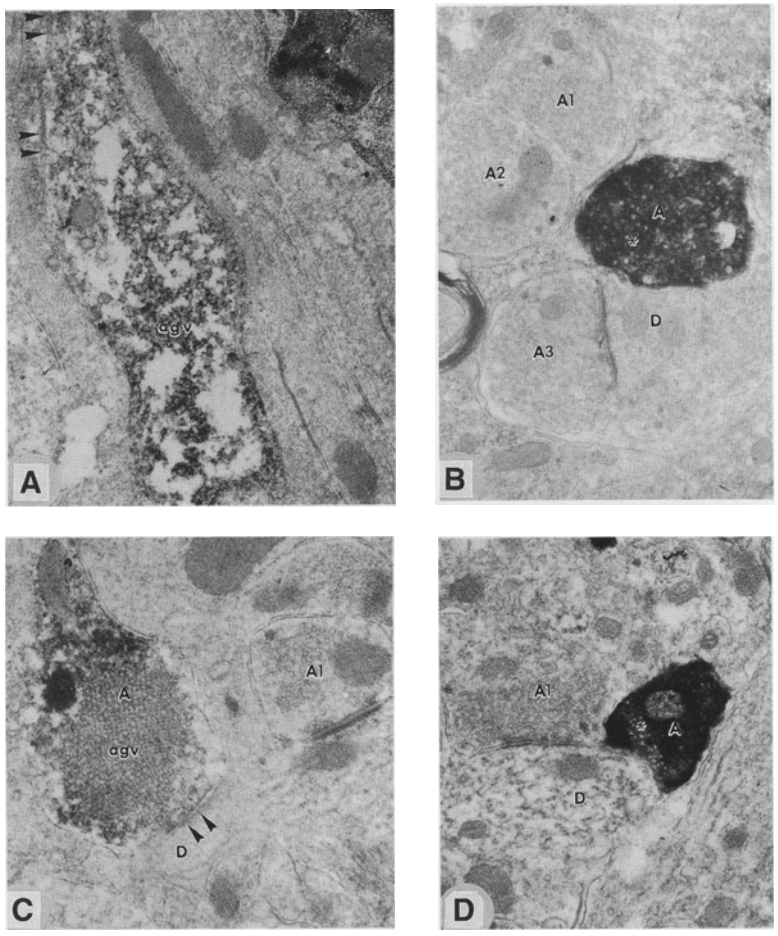


Fig. 4. Immunoelectron micrographs of TH immunoreactivity in the striatum of a rat 4 mo following DA 65:35 microsphere implantation. This particular rat displayed a 47% decrease in the total number of contralateral rotations at the time of sacrifice. **(A)** An immunoreactive axonal profile contains numerous agranular synaptic vesicles (agv) at the site of microsphere injection. Note that the immunoreactive products as black deposits are between the vesicles. Arrowheads indicate neurotubules in the axon ×29,750. **(B)** An intensely stained axonal terminal containing agranular vesicles (asterisk) near the site of microsphere injection. The terminal appears to contact a dendrite that is postsynaptic to a nonimmunoreactive axon terminal (A3). Two other axon terminals (A1, A2) remained unstained ×29,750. **(C)** An immunoreactive axon terminal (A) containing closely packed (lattice-like arrangement) agranular synaptic vesicles (agv) is presynaptic to a dendrite **(D)**. Arrowheads indicate a possible postsynaptic membrane thickening. On the right is an axon terminal (A1) that is nonimmunoreactive ×38,500. (D) A dendrite (D) appears to be postsynaptic to a nonimmunoreactive (A1) and an intesely stained immunoreactive axon terminal (A). Asterisk shows synaptic vesicles ×29,750.

pathogenesis of PD. Even more, these results suggest that a single administration of NE microspheres could be sufficient to improve symptoms in PD.

Fiber Growth

Implanted NE-containing microspheres as depicted in Fig. 6 induce growth of DA fibers in the denervated rat striatum. The fiber growth following implantation of NE microspheres is comparable to that noted after DA microspheres. Ultrastructural results equally confirmed the presence of TH immunoreactive fibers growing in the striatum up to 4 mo following NE microspheres implantation (Fig. 7).

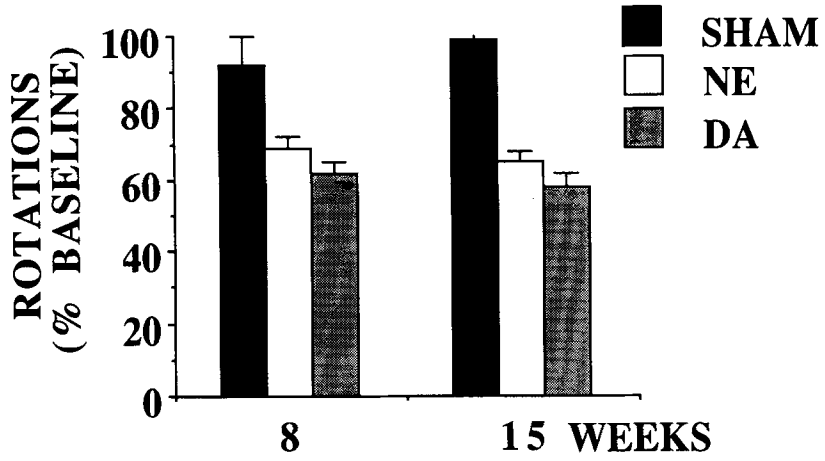


Fig. 5. Apomorphine-induced rotational behavior 8 and 15 wk following empty, DA, or NE microspheres. The data were averaged from 9 DA, 9 NE, and 5 sham-microsphere-implanted rats. Sham implanted rats do not show modifications in rotational behavior. There are similar decreases in apomorphine induced rotational behavior following DA or NE implanted microspheres at 8 and 15 wk postimplantation.

The induction of fiber growth by NE may be related to the established trophic actions of this transmitter. NE is known to exert trophic actions during development *(46–48)*. Trophic actions of noradrenergic fibers have also been demonstrated in the adult rat by the induction of collateral sprouting of uninjured target noradrenergic fibers in the cerebellum *(49)*, and uninjured mesocortical dopaminergic neurons *(50)*. Recently, the normal recovery of DA fibers in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned nonhuman primates *(52)* has been shown to be prevented by lesioning the major NE cell body-containing area in the CNS, the locus ceruleus (LC) *(51)*. The LC lesion in combination with MPTP causes drastic reductions in the DA content of the striatum and in cell loss of the substantia nigra *(51)*, in comparison to MPTP alone. These results suggested to the investigators that LC projections control trophic processes in the nigrostriatal pathway.

The mode of delivery of DA and NE with microspheres seems to be crucial for the induction of fiber growth in the striatum. Thus, no DA fiber growth, in animals or humans, has been reported in response to other means of assuring functional levels of DA in the striatum, such as systemic L-DOPA injection, intrastriatal infusion of DA *(53)*, or implantation of other DA-releasing polymeric systems *(20–24)*. One important difference between the present and other modes of DA delivery is the continuous release of DA or NE from the microspheres in a specific brain region, for prolonged periods of time. It is possible that maintaining a discrete and continuous, regionally restricted release of DA or NE (by means of microspheres, at least 25–100× smaller in size than other polymeric delivery systems), as opposed to intermittent dosing and/or flooding the entire striatum with the transmitter (by other means of delivery), is critical for the induction of fiber outgrowth. Finally, interestingly, low concentrations of DA and other catecholamine moiety-containing agents have been shown to stimulate the production in vitro of nerve growth factor *(54)*, possibly suggesting a direct neuronotrophic role for DA and structurally related substances.

Our results with DA- and NE-containing microspheres suggest that they may have clinical potential as an alternative source of neurotransmitter restitution in the striatum, tentatively by two mechanisms: by acutely releasing microencapsulated neurotransmitters, thereby counteracting deficits in motor control, and by eliciting fiber growth of DA fibers in the brain, which in turn would produce sufficient endogenous levels of the neurochemical to alleviate motor disturbances. Given the encouraging results obtained by injecting DA- and NE-containing microspheres to rats with experimental parkinsonism, we next examined the effects of the administration of a mixture containing both DA and NE.

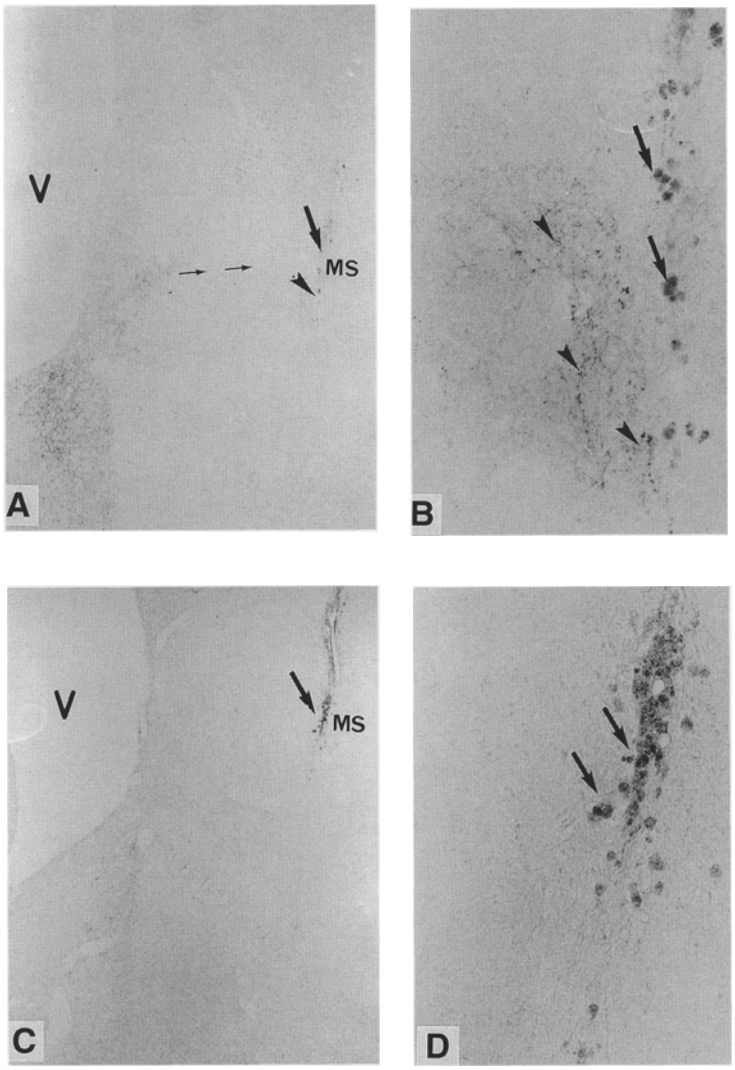


Fig. 6. Photomicrographs of TH immunoreactivity in frontal cryostat sections (10 μm) of the striatum 4 mo after implantation of NE microspheres **(A, B)** or empty **(C, D)**. (A) The NE microspheres (MS, arrow) are at a distance from the ventricle. The small arrows show the pathway that the fibers (F) grow toward the microspheres and the arrowhead indicate fiber growth at the level of the microspheres ×32. (B) An enlarged view of the immunoreactive fiber growth (arrowhead) at the level of the NE microspheres (arrows) ×160. (C) Sham implanted microspheres (MS, arrow) at approx the same distance from the ventricle as the NE microspheres in A. There is no growth of fibers toward the sham implanted microspheres ×32. (D) An enlarged view of the absence of fiber growth in the striatum of a sham microsphere (arrows) implanted rat ×160.

Mixed Dopamine/Norepinephrine-Containing Microspheres

This investigation is still in its preliminary stages, and at the writing of this manuscript most of the rats were still undergoing rotational testing. Therefore, immunocytochemical data is not available. The rotational behavior is summarized in Fig. 8. In comparison to implanting DA or NE microspheres alone, implanting a mixture of DA/NE-containing microspheres produced a dramatic reduction in the number of apomorphine-induced rotations 4 wk after implantation. These data suggest that enhanced therapeutic efficacy may be achieved by administering microspheres containing mixtures of substances whether it be transmitters, growth promoting factors, or the two in combination.

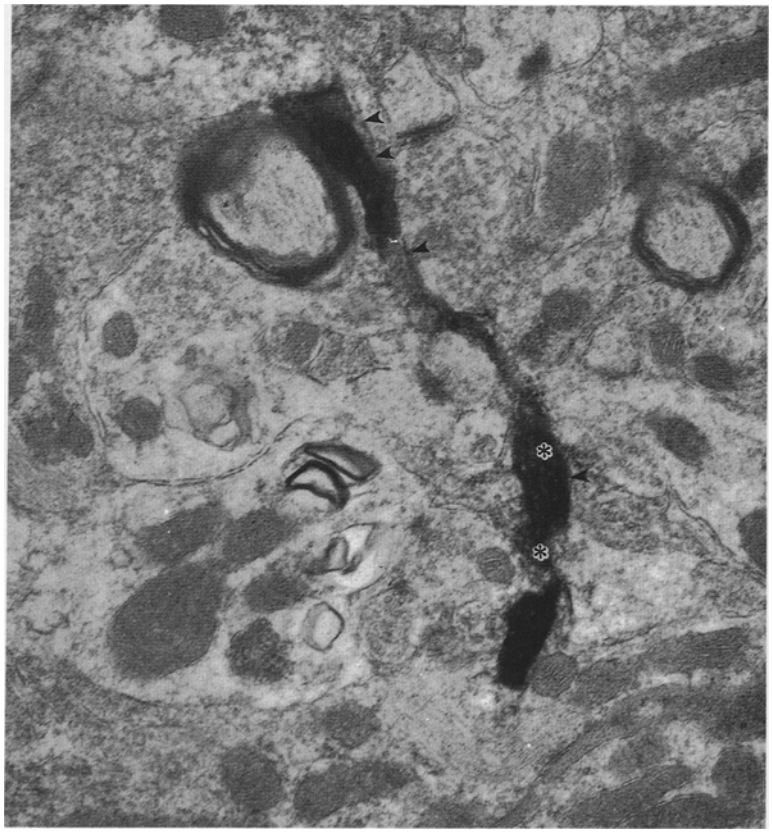


Fig. 7. Immunoelectron micrograph of a TH reactive fiber growing in the striatum 4 mo following implantation of NE microspheres. Arrowheads indicate the varicosities of the fiber. Small pale vesicular profiles (asterisk) are considered to be synaptic vesicles in the axonal dilation ×29,750.

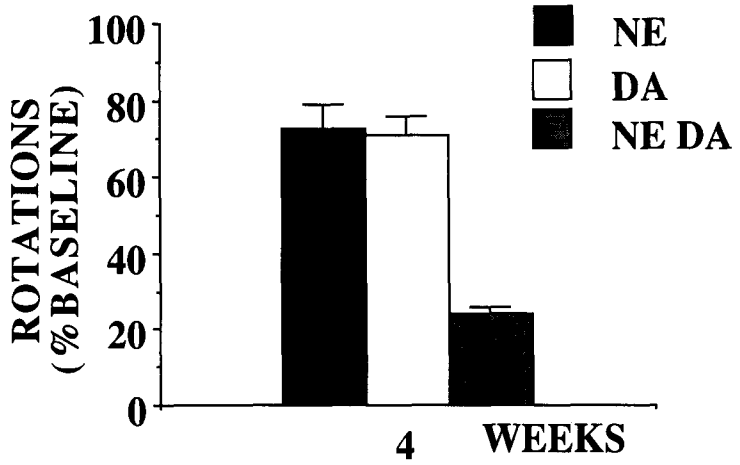


Fig. 8. Attenuation of apomorphine induced rotational behavior 4 wk following DA, NE, or a mixture of DA–NE microspheres. DA and NE data are the average of 5 rats, whereas DA and NE are from 2 rats.

Conclusions

It appears that synthetic DA and NE microspheres may have clinical potential as alternative sources of neurotransmitter restitution in the striatum. Most important is the fact that a single injection of synthetic microspheres into the striatum stimulated growth of DA fibers. From a more basic neuro-

scientific point of view, the fact that synthetic DA- or NE-containing microspheres stimulate growth of DA fibers raises some interesting questions about the role of these neurotransmitters as putative trophic factors.

Finally, because it is possible to microencapsulate other drugs or active substances, the microencapsulation technology could potentially be exploited to develop novel therapeutic means for treating a number of other neurological or neurodegenerative disorders.

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