ARKINSON’S disease, a progressive neurodegenerative disorder characterized by dopamine deficiency, results from degeneration of the pigmented neurons in the substantia nigra. Although therapy with levodopa compensates for the deficiency through an interaction of dopamine with sensitive postsynaptic striatal receptors, this treatment fails as the disease progresses. A recent therapeutic approach uses implantation of autologous adrenal medulla or fetal mesencephalon, both catecholaminergic tissues, into the striatum. After implantation, the beneficial effects may be due to release of dopamine from the graft, synaptic connectivity of the graft with the host, alteration of the blood-brain barrier, or neurotrophic effects of the graft on the host brain.

Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces a severe parkinsonian motor deficit in humans and monkeys that closely resembles idiopathic Parkinson’s disease. Because of the extremely high total body clearance of MPTP, a large pharmacokinetic advantage is achieved by intra-arterial administration and unilateral infusion of MPTP into the internal carotid artery (ICA). This produces hemiparkinsonism with rigidity, tremor, and bradykinesia in the contralateral limbs and asymmetric motor activity.

Brain injury alone induces neuronal sprouting in mice and monkeys, although the responsible cell-to-cell interactions are not known. After implantation of fetal dopaminergic or nondopaminergic central nervous system tissue in MPTP-induced parkinsonian monkeys, we observed robust sprouting of dopaminergic fibers from the host brain and behavioral improvement, despite failure of the grafts to survive. Animals implanted with term amnion also had some sprouted dopaminergic fibers and behavioral improvement, but these were limited and were similar to the recovery, in prior experiments using the same primate model of parkinsonism, of animals that received surgical cavitation only.

Recovery after central nervous system grafting with fetal amnion, a fetal accessory tissue, does not require secretion of a deficient neurotransmitter(s) from the graft and occurs despite the failure of graft survival. Recovery after cerebral implantation of fetal tissues appears to depend more on the regenerative and recuperative processes of the host brain than on graft replacement of deficient neurotransmitters or development of functional synaptic connections between the graft and the host brain.

Key Words • Parkinson’s disease • amnion implantation • MPTP • dopamine • rhesus monkey
Materials and Methods

The experimental protocol was approved by the Animal Care and Use Committee of the National Institutes of Neurological Disorders and Stroke, and all animals were treated in accordance with National Institutes of Health guidelines for animal care and use.

Administration of MPTP

In 11 adult rhesus monkeys, MPTP-HCl was infused into the right ICA. The animals were anesthetized with ketamine (Vetlar, 7 mg/kg) intramuscularly followed by intravenous administration of pentobarbital (Somnifmer, 7.5 mg/kg). The common carotid artery (CCA) was exposed below the carotid bifurcation by means of a midline neck incision. Silk thread (2-0) was looped around the CCA, and the superior thyroid artery and external carotid artery were identified and temporarily clamped with vascular clips. A 27-gauge needle was inserted into the CCA retrograde to the direction of blood flow and 60 ml of saline containing 0.4 mg/kg of MPTP-HCl was infused at a rate of 4 ml/min. After the infusion was complete, the vascular clamps were removed from the superior thyroid and external carotid arteries, the needle was withdrawn from the CCA, and pressure was applied for 5 minutes. The wound was washed with hydrogen peroxide and closed in anatomical layers.

Assessment of Motor Function

Assessments, performed weekly for 2 to 6 months after MPTP infusion, included recordings of apomorphine-induced turning and assessment of the ability to use the arm contralateral to the lesioned striatum.

Hemiparkinsonian monkeys, when spontaneously active, turn predominately toward the affected side. Apomorphine (0.2 mg/kg) administered intramuscularly enhances the general level of locomotor activity and, because of increased sensitivity to apomorphine on the MPTP-treated side, reverses the direction of turning. The rate of turning after apomorphine administration was used as an index of striatal dopamine receptor sensitivity. Monkeys were videotaped during testing, and the rate and direction of turning were read from these tapes.

Volitional responses to presentation of pieces of food were also recorded on videotape. Two pieces of food were offered sequentially; the second piece was presented when the first piece had been taken by one hand and brought to the animal’s mouth. In normal monkeys, the second piece of food is always taken with the other hand. In hemiparkinsonian monkeys, however, the first piece is held in the mouth while the same hand that was used initially is again used to obtain the second piece of food; the rigid and tremorous limb contralateral to MPTP infusion remains unusable. The ability to use the “parkinsonian arm” to obtain the second piece of food was used as an index of improvement in volitional motor function. Monkeys were scored according to the following rating scale. A score of 100: unable to use affected limb; 300: arm use only when intact arm used, unable to grasp objects; 500: can grasp large objects but only with assistance of intact limb; 700: can grasp only large objects without assistance from intact limb, tremor present; 900: can grasp small objects, independent use; and 1000: normal use. A rating of greater than 500 denoted effective arm use.

Precavitation and Amnion Implantation Surgery

Four hemiparkinsonian monkeys were left unoperated and served as control animals. Each of the other seven animals underwent two brain operations. To enhance graft survival,36 cavities were prepared in the heads of the left and right caudate nuclei 3 to 6 weeks before tissue was implanted. The animals were anesthetized with pentobarbital (7.5 mg/kg), and surgery was performed with sterile technique. A diamond saw was used to cut and remove a right frontal bone flap (5 × 3 cm) extending just across the midline. The dura was incised and retracted medially to expose the interhemispheric fissure, and the hemispheres were gently separated. Using an operating microscope and microsurgical technique, the surgeon made a small incision through the body of the corpus callosum and the most dorsal aspect of the septum was resected exposing both lateral ventricles. Pituitary rongeurs were used to make two small cavities on the mediodorsal aspect of the head of each caudate nucleus anterior to the foramen of Monro. Each cavity was filled with trypan blue-stained sponge (Gelfoam). The dura was closed, the bone flap was replaced, and the scalp incision was closed.

After the rate of turning in response to apomorphine returned to within 80% of the precavitation level (3 to 6 weeks), the gelatin sponge in both caudate nuclei was replaced by amnion tissue. In four monkeys the gelatin sponge in both caudate nuclei was replaced by pieces of fetal amnion; in the other three animals, the gelatin sponge was replaced by term rhesus monkey amnion.

Amnion Tissue Harvesting

Fetal rhesus monkey amnion was obtained from fetuses aborted by Caesarean section at the end of the second and beginning of the third trimester. Term rhesus monkey amnion was obtained from Caesarean section at birth.

The amniotic membrane was dissected, cut into approximately 5 × 8-mm pieces, washed, and stored on ice-cold phosphate-buffered saline (PBS) until implantation. The delay between tissue harvesting and implantation ranged between 3 and 8 hours.

Examination of Brain Tissue

The seven implanted animals were sacrificed 5 to 9 months after implantation; two control monkeys were sacrificed 12 months after MPTP lesioning. Animals were killed by an intravenous overdose of pentobarbital (460 mg) and were perfused through the ascending aorta with 500 ml of ice-cold PBS followed by 2 L of ice-cold 10% formalin in PBS (pH 7.0). The brains were rapidly removed, cut into 6-mm coronal slices in brain matrix, and postfixed for 30 minutes in the same fixative. The tissue slices were rinsed for 48 hours in 20% sucrose in PBS, frozen on dry ice, cut into 20-μm coronal sections in a cryostat, mounted on chrome-alum-coated slides, and processed for immunocytochemistry using antibodies against tyrosine hydroxylase immunoreactivity (TH-IR) at a dilution of 1:1000, dopamine beta-hydroxylase immunoreactivity (DBH-IR) at a dilution of 1:1000, laminin immunoreactivity (L-IR) at a dilution of 1:200, and glial fibrillary acidic protein immunoreactivity (GFAP-IR) at a dilution of 1:50* as previously described.36,37 Adjacent sections were stained with hematoxylin and eosin and with thionin.

The TH-immunoreactive cells were counted in the substantia nigra and ventral tegmental area in the most representative sections of the midbrain. The number of cells in the two sides in each of the structures was compared.

Results

Control Monkeys

The four control monkeys remained hemiparkinsonian during the 10 to 12 months of observation after

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MPTP administration. They had no decrease in apomorphine-induced turning and were not able to use their bradykinetic arm effectively (Figs. 1 and 2 upper).

Animals Undergoing Surgery

Within 1 week of the first operation (surgical cavitation only), contralateral apomorphine-induced turning diminished in all seven animals (Fig. 1). This response was transient and turning returned to 80% of baseline within 6 weeks.

Behavioral Effects. Within 2 months of implantation, two of the four monkeys implanted with fetal amnion regained use of their bradykinetic arm, and by 4 months, three implanted monkeys had functional arm use (Fig. 2 center); two of them (AMN-1, AMN-2) were using both arms almost equally well and practically as well as normal animals. Monkey AMN-3 regained independent use of the bradykinetic arm (with some tremor), but still showed preference for the normal arm. Monkey AMN-4 did not regain arm use. Three monkeys (AMN-1, AMN-2, and AMN-4) had decreased apomorphine-induced turning when compared to preimplantation testing (Fig. 1 center). These behavioral improvements persisted during the 7-month observation period after implantation (Figs. 1 and 2 center).

Two of the three monkeys implanted with term amnion had no return of function of the affected limb. Within 2 months of implantation of term amnion, the other monkey (AMN-6) regained functional use of the bradykinetic arm; however, at 6 months after the implantation its recovery declined to only marginal functional arm use (Fig. 2 lower). By 3 months after implantation, this monkey (AMN-6) also began to demonstrate recovery of apomorphine-induced turning, but by 7 months the rate and direction of rotation in response to apomorphine had started to return to presurgical levels. One of the term amnion-implanted monkeys (AMN-5) had an improvement in apomorphine-induced turning by 4 months after the implantation that continued until the end of testing (Fig. 1 lower).

Histochemical Observations

Control Animals. In the control monkeys, TH-immunoreactive fibers were depleted in the caudate nucleus and putamen on the MPTP-treated side (Fig. 3A), whereas the nucleus accumbens and the area ventral to it contained nearly normal TH-immunoreactive staining (not shown). The number of dopaminergic cells in the substantia nigra pars compacta on the MPTP-treated side was estimated to be reduced by more than 96% when compared to the untreated side. In contrast, the dopaminergic cells medial to the substantia nigra (the ventral tegmental area) were reduced by no more than 50% compared to the untreated side. The histology of the dopaminergic areas of the midbrain in the control hemiparkinsonian monkeys was similar to that in the implanted monkeys.

![Graphs depicting apomorphine-induced turning in monkeys with MPTP-induced hemiparkinsonianism.](image)

**Upper:** Untreated monkeys' turning remained stable or increased during 12 months of observation. **Center:** Within 4 months of surgical cavitation followed by allogeneic fetal amnion implantation into the head of the caudate nucleus, three of four monkeys decreased contralateral apomorphine-induced turning. For statistical analysis, baseline (preoperative) turning in response to apomorphine was compared with the results of the last four apomorphine tests after the amnion implantation (AMN-1, 64% decrease \( p < 0.001 \); AMN-2, 56% decrease \( p < 0.001 \); AMN-4, 59% decrease \( p < 0.02 \); Student’s t-test). **Lower:** After surgical cavitation followed by term amnion implantation there was a persistent decrease in apomorphine-induced turning in one of three animals (AMN-5, 53% decrease \( p < 0.02 \)).
Amnion-Implanted Monkeys. In one fetal amnion-implanted animal and in one term amnion-implanted monkey (AMN-6), laminin-immunoreactive basal membrane of the amnion graft was found at the graft site (Fig. 3B and C). In AMN-3, the implants were more caudal than in the other monkeys; they were posterior to the foramen of Monro on the dorsal aspect of the body of the caudate nucleus. The TH-immunoreactive fibers were present in the mediolateral portion of the caudate nucleus only in AMN-6. As in amnion-implanted monkeys, these fibers were DBH negative and were oriented toward the implant (Fig. 3D).

The sites of implantation in all term amnion-implanted animals were identified in the head of caudate nucleus, rostral to the foramen of Monro. They were at the same level or rostral to the sites of implantation seen in the fetal amnion-implanted monkeys. The fetal amnion grafts abutted the nucleus accumbens, whereas the term amnion grafts did not touch the nucleus accumbens. In three of the four fetal amnion-implanted animals (AMN-1, AMN-2, and AMN-4), numerous TH-immunoreactive-positive, but DBH-immunoreactive-negative, fibers were observed in the portion of the caudate nucleus surrounding the implants. These fibers were oriented from the ventral striatum and the nucleus accumbens toward the implants. The GFAP immunoreactivity, present in the area of the caudate nucleus surrounding the implant, was overlaid with sprouted dopaminergic nerve fibers. Dopaminergic neuronal processes (TH-immunoreactive positive, but DBH-immunoreactive negative) also coursed from the region surrounding blood vessels toward the implant (Fig. 4).

Discussion

Recovery After Striatal Grafting

We previously demonstrated that hemiparkinsonian monkeys implanted with fetal mesencephalon show dramatic behavioral improvement. Apomorphine-induced turning nearly stops and they recover use of the bradykinetic arm. Histological examination of the brains of these monkeys demonstrated survival of implanted fetal dopaminergic neurons as well as host dopaminergic nerve fibers that appeared to be sprouted from MPTP-resistant mesolimbic neurons. In these animals either of these two sources of dopamine, the graft or the host brain, could theoretically account for the behavioral improvement noted.

The other dopaminergic tissue that has been extensively studied for grafting in parkinsonian models is the adrenal medulla. However, in hemiparkinsonian monkeys we observed no difference in recovery of apomorphine-induced rotation or arm function when animals that received adrenal medulla autografts were compared to animals that received only surgical cavitation. Neither group had improvement of arm function, in contrast to the animals that received fetal dopaminergic grafts and the fetal amnion-implanted...
most animals with cavitation, with or without a graft, including the animals with term and fetal amnion investigated here, have some degree of recovery of apomorphine-induced turning within 6 months of cavitation. Although the reduction of rotation after apomorphine was greater in the animals that received fetal amnion than was the reduction in animals implanted with term amnion, it was similar in extent to the recovery that we saw previously in the same model after cavitation alone. Because the interval from cavitation to implantation was too brief to examine the recovery that may have occurred with cavitation alone in the animals in this study, interpretation of the results between the two sets of implanted animals using this measure of recovery may be misleading.

Although histological analysis is also widely used to measure the success of tissue grafting and regeneration in animal models of parkinsonism, the degree to which local changes in dopaminergic sprouting occur after grafting is difficult to quantify reliably and a close correlation of local or regional dopaminergic recovery with functional recovery is not solidly established in large-brained animals. In this study some animals had either apomorphine-induced rotational responses or recovery of arm use that was discordant with the histological findings. For example, one fetal amnion-implanted monkey (AMN-4) had histological evidence of dopaminergic sprouting similar to the other animals that received fetal amnion yet had no recovery of arm use, one animal that received fetal amnion (AMN-3) had some recovery of arm use despite the absence of histological evidence of sprouting, and one animal that received an implant of term amnion (AMN-5) had recovery from apomorphine-induced turning but no histological signs of sprouting. Thus, although there were differences in the recovery of apomorphine-induced rotation and the degree of sprouting of dopaminergic fibers in the striatum between the term amnion-implanted and the fetal amnion-implanted monkeys, these measures of recovery may not be concordant in an individual animal and may be discordant with the recovery of arm use. However, arm use, the only measure of recovery that can be related to human function, was the most consistent and obvious difference between the two implanted groups.

The dramatic recovery of arm function in the fetal...
Implantation of fetal amnion resulted in sprouting of neural processes from the region of blood vessels in the caudate toward the implant site. Montage of tyrosine hydroxylate immunoreactive (TH-IR) fibers in the implant area, nucleus accumbens, and ventral striatum. Dopaminergic fibers arise from the region of blood vessels (closed arrow) lateral to the nucleus accumbens and from the rostral portion of the nucleus accumbens (open arrow) in A (TH-IR immunocytochemistry, original magnification × 12). Since they are TH-IR positive in B (TH-IR immunocytochemistry, original magnification × 125) and dopamine beta-hydroxylase-immunoreactive (DBH-IR) negative in C (DBH-IR immunocytochemistry, original magnification × 125), these fibers are dopaminergic. However, some of the sprouted fibers from the nucleus accumbens are adrenergic, as they are TH-IR positive and DBH-positive in D (DBH-IR immunocytochemistry, original magnification × 125). E: Section from a fetal amnion-implanted animal (AMN-1). The outlined area represents the part of the striatum shown in A (H & E, original magnification × 12).

Implants of adrenal autografts or term amnion tissue in this primate model of Parkinson’s disease. The reduced effectiveness of the term amnion implants may be due to the later developmental stage of the tis-
Mechanism of Recovery After Striatal Grafts

The following are produced by amnion: epidermal growth factor,25 which stimulates proliferation of murine adult striatal neurons in vitro;27 transforming growth factor,25 which reduces apomorphine-induced turning in hemiparkinsonian rats;33 insulin-like growth factor,17 interleukin-1,317 which when imbedded in a slow-releasing polymer and implanted into the striatum of hemiparkinsonian rats induces behavioral (rotational) recovery and dopaminergic sprouting from the nucleus accumbens and area olfactoria30 and may produce other unidentified neurotrophic materials. Human and rat amnion membranes, which contain laminin, serve as a substrate for axonal growth in vitro and in vivo.28,31 Laminin elicits rapid and prolific neurite outgrowth from peripheral and central types of neurons. Glial processes, which provide scaffolding to direct and support the elongation and orientation of neuronal process in embryonic neural development, produce laminin after injury to the adult brain.2,20 Whether astrocytes can provide similar functions in the adult primate brain has not yet been demonstrated, but staining for laminin and GFAP in the brains of the animals in this study was strongly positive in the region surrounding the site of the implant, and the longitudinal orientation of the staining was in the same direction as the sprouted host dopaminergic nerve processes. Elaboration of growth factors, neurite-promoting factors, cytokines, laminin, and other identified or as yet unknown neurotrophic factors may alone, or in combination, underlie the enhanced recovery seen in the animals that received fetal tissue.15

The results of this study support a neurotrophic hypothesis of implant-induced recovery. A trophic and behavioral response follows cavitation alone or term amnion implantation, but a greater response occurs after fetal tissue (dopaminergic tissue or amnion) implants. None of the four fetal amnion-implanted animals described here had surviving graft at autopsy (only nonviable L-immunoreactive basal membrane was present). Despite this, these animals had induction of formation of TH-positive neuronal processes and behavioral recovery, as measured by return of the functional use of the affected extremity and reduction in apomorphine-induced turning. Sprouted nerve fibers from the recipient brain appear to be responsible for the behavioral improvement that occurs after surgical implants in MPTP-induced parkinsonian monkeys and for the limited but measurable recovery in patients who receive such grafts.14,18

Grafting into the medial portion of the caudate nucleus, in the vicinity of the nucleus accumbens, may provide an optimum site for inducing the dopaminergic sprouting reaction in MPTP-lesioned primates. The ventral tegmental area, in addition to projecting to the nucleus accumbens, also contributes to the area olfactoria in the dorsal aspect of the putamen. It is possible that sufficient ventral tegmental area innervation of that region would permit a similar mechanism of recovery originating from the area olfactoria or the external capsule. It is also noteworthy that, compared to the MPTP-lesioned hemiparkinsonian monkeys, there are more dopaminergic cells preserved in the substantia nigra in idiopathic parkinsonian patients (our unpublished observations), cells that may provide a substrate for sprouting to occur in other regions of the striatum. Because of this residual dopaminergic source, a robust sprouting reaction may occur in patients after caudate or putaminal implants,21,22,28,31

These results suggest that the fetal tissue provides factors that stimulate functionally significant sprouting of host dopaminergic neurons. The results did not, however, indicate whether the amnion was the direct source of the substance(s) for recovery, or if the amnion, alone or combined with the surgical trauma of cavitation and implantation, stimulated the expression of the responsible factor(s) from the host brain. However, the persistence of these beneficial effects not only suggests that the recovery is related to a regenerative process by the host brain, but implies that once the effect occurs it no longer requires the trophic effects of the graft (at least for the several months of recovery observed in our animals).

Conclusions

Refinement of the capacity to induce formation of a new neuronal process might bring several benefits that would be superior to the experimental approach using tissue grafting. Limited tissues are suitable for CNS grafting if replenishment of a specific neurotransmitter by the graft is required to replace a singular neurotransmitter deficiency. In contrast, induction of neuronal sprouting from the host brain by neurotrophic factors might require less specificity, inducing recovery of various neuronal systems, and might be applicable to neurological disorders other than Parkinson’s disease. Furthermore, if the neurotrophic factor(s) responsible for recovery can be identified and produced, tissue grafts would be unnecessary, problems with immune rejection and requirements for immunosuppressive therapy would be eliminated, and the controversy associated with procurement of fetal tissue would be avoided. Last, elimination of the space-occupying tissue of a graft would permit larger volumes of the brain to be targeted for treatment and would allow access to anatomical regions of the brain or spinal cord that could not safely receive placement of a solid tissue graft. Thus, induction of recovery by stimulating neuronal process regeneration in situ, as occurred in the brains of the primates described in this study, might offer more widespread therapeutic application than tissue grafting to replace missing or deficient neurotransmitters.

References

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