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Cryopreservation, Culture, and Transplantation of Human Fetal Mesencephalic Tissue into Monkeys

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Studies in animals suggest that fetal neural grafts might restore lost neurological function in Parkinson's disease. In monkeys, such grafts survive for many months and reverse signs of parkinsonism, without attendant graft rejection. The successful and reliable application of a similar transplantation procedure to human patients, however, will require neural tissue obtained from human fetal cadavers, with demonstrated cellular identity, viability, and biological safety. In this report, human fetal neural tissue was successfully grafted into the brains of monkeys. Neural tissue was collected from human fetal cadavers after 9 to 12 weeks of gestation and cryopreserved in liquid nitrogen. Viability after up to 2 months of storage was demonstrated by cell culture and by transplantation into monkeys. Cryopreservation and storage of human fetal neural tissue would allow formation of a tissue bank. The stored cells could then be specifically tested to assure their cellular identity, viability, and bacteriological and virological safety before clinical use. The capacity to collect and maintain viable human fetal neural tissue would also facilitate research efforts to understand the development and function of the human brain and provide opportunities to study neurological diseases.

TRANSPLANTATION OF FETAL NEURAL tissue in rodents has been shown to alter host neurological, endocrine, cognitive, and motor functions; the alterations have been correlated with evidence of neuronal survival, axonal integration, synapse formation, appropriate neurochemical release, and electrophysiological interactions with host neurons (*1*). These studies raised the possibility that neural transplantation might benefit patients with neurological disorders, especially the neurodegenerative disorder Parkinson's disease, which involves progressive loss of striatal dopamine function (*2*). However, poor neural graft survival in monkeys (*3*) and ethical concerns about the use of human fetal tissue led instead to clinical trials with adrenal medullary autografts as a possible alternative source of replacement dopamine (*4*). The effectiveness of these adrenal autografts in patients with Parkinson's disease cannot yet be fully evaluated. Published reports are mixed, and a number of complications and problems appear to be emerging (*5*). Evidence of survival of identifiable adrenal tissue also is very limited (*6*).

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In contrast, survival and integration of transplanted monkey fetal substantia nigra (SN) tissue have been demonstrated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–treated monkeys, which have parkinsonian symptoms (*7*). After grafts were placed into regions of the caudate nucleus, there was improvement in behavioral and motor function and diminished dopamine-associated biochemical abnormalities in the regions of graft innervation. Persistent dopamine abnormalities in regions of caudate away from the grafts and in the substantia nigra (at an even greater distance away) suggest that recovery of host dopaminergic neurons is unlikely to be responsible for the behavioral and biochemical improvements. Control animals, in which grafts were placed outside the striatum or that had grafts of non–dopamine producing cells showed continued functional and biochemical abnormalities. Transplantation of fetal neural tissue, therefore, could probably replace lost function in parkinsonian patients, to the extent that MPTP-induced parkinsonism in monkeys is a model for the idiopathic human disease.

The use of fetal neural tissue from and for humans raises scientific, ethical, legal, and safety questions that are not confronted in studies of animals: (i) Can viable mesencephalic neural tissue be collected from human fetal cadavers in a manner that conforms to legal guidelines and considers ethical concerns? (ii) Can this tissue be safely implanted with appropriate confirmation of tissue and neuronal identity as well as bacteriological and virological safety? (iii) Can tissue collection and neurosurgical implantation be scheduled to minimize recipient risk while increasing the probable viability of implanted tissue? (iv) Is there evidence of graft failure or host rejection after transplantation?

Physicians in Sweden, Mexico, England, and Spain have now reported preliminary results of clinical attempts at direct transplantation of human fetal tissue into patients with Parkinson's disease (*8, 9*). The details of timing of neural tissue collection, holding or preservation methods, and evidence of cell viability in humans have not been clear in most of these studies. Collections have been made from spontaneous and elective abortions at various gestational intervals, and tissues may not have been held long enough to demonstrate specific tissue viability or biological safety. The absence of procedural details related to the use of spontaneously aborted fetuses, such as definitions of death and the necessity for rapid scheduling of transplantation procedures, raises questions about tissue viability. The two collaborating Swedish research teams with

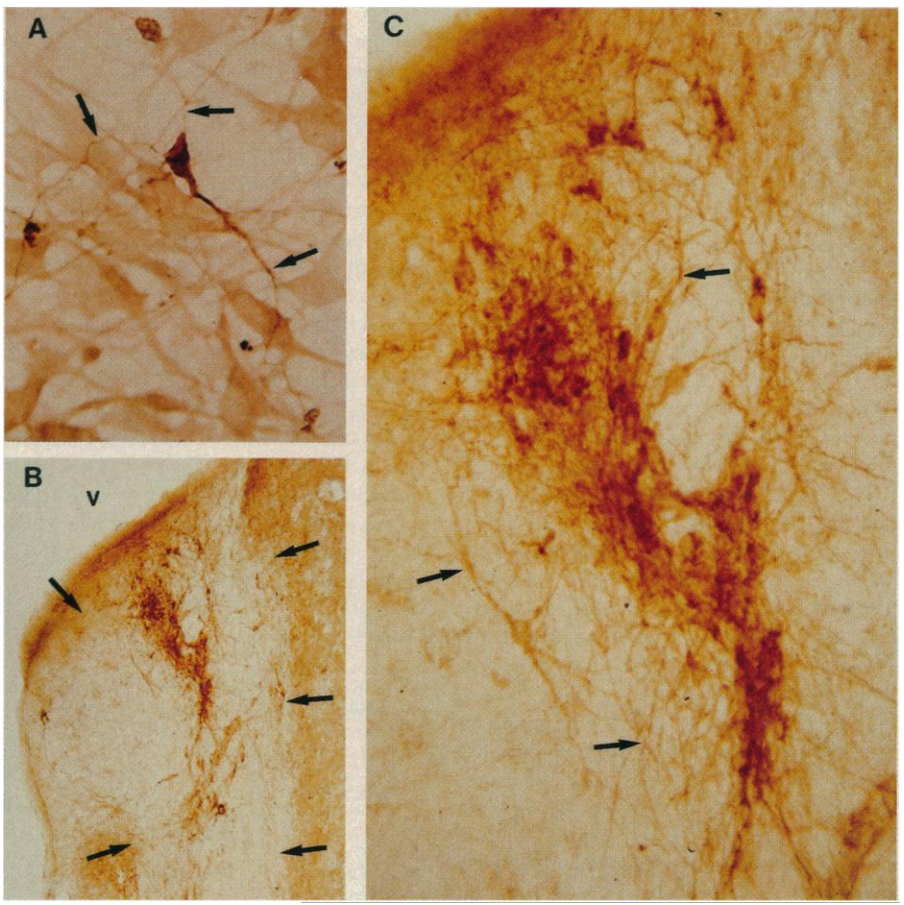

Fig. 1. (**A**) Photomicrograph of a cell culture of human mesencephalic tissue collected from a first trimester fetal cadaver; the tissue was cryopreserved, stored for approximately 2 months, thawed, and then cultured for 14 days. After immunohistochemical reaction with an antibody to tyrosine hydroxylase, this culture shows neurons containing this key enzyme for synthesizing dopamine, confirming the identity of the mesencephalic tissue. The extensive neuritic arborization (arrows) indicates that the fetal midbrain dopaminergic neurons grew and developed in culture. (**B**) Photomicrograph of tyrosine hydroxylase staining of cryopreserved and thawed human fetal cells 70 days after implantation into the caudate nucleus of a normal monkey. This scanning view shows a clearly identifiable plug of grafted material (arrows) along the lateral border of the lateral cerebral ventricle (V). (**C**) A higher power view includes detail of several neurons typical of nigral dopaminergic neurons with branching neuritic extensions (arrows). (A) ×150; (B) ×40; (C) ×160.

the most completely developed and elaborated methods for collection and preparation of fetal neural tissue have reported little clinical improvement 1 year after transplantation of fetal neural tissue into two patients, suggesting that cells may not have survived (*9*). It is possible, therefore, that techniques that produce graft survival in rodent brain (*10, 11*) must be altered for human fetal neural tissue to be successfully transplanted into humans.

To evaluate this possibility, we have transplanted human fetal neural tissue into monkeys. In earlier work, we preserved fetal dopaminergic cells from monkeys by freezing them in liquid nitrogen prior to further study or transplantation (*12*). This allowed us to have longer intervals to test identity and safety of specific samples for later scheduled use. We applied a modification of this cryopreservation procedure to fragments of human fetal nervous system tissue, collected from cadavers from first trimester human abortions. Consent for the use of the fetal cadaver tissue was obtained from the gravidae, under a protocol approved by the Yale University School of Medicine/Yale New Haven Hospital institutional review board for human research (*13*). After routine suction abortions, the disrupted products of conception were collected in a sterile container, rinsed, chilled, and dissected on ice until cryopreservation. Fragments from individual samples were placed in liquid nitrogen for storage and transport (*14*).

As a test of viability and cellular identity, some tissue fragments were rapidly thawed in a 37°C water bath and then were moved to a sterile environment for washing, cell dispersion, and seeding onto tissue culture dishes. Primary cell cultures were obtained and allowed to develop for 14 days before fixation (*15*) to assess the presence and viability of probable dopamine neurons. Identifiable neurons and glial cells attached to the culture substrate and developed typical morphology. Neurons extended processes and stained positively for tyrosine hydroxylase (TH) and neuron-specific enolase (NSE) after fixation (Fig. 1A).

Other thawed tissue fragments were implanted stereotaxically into the caudate nucleus of three normal male *Cercopithecus aethiops sabaeus* to test cell viability after transplantation (*16*). To distinguish possible graft failure due to cryopreservation or transplantation techniques from that caused by immune rejection of a xenograft (cross species), the monkeys were treated with cyclosporine A (15 mg/kg) just before surgery until 1 week before they were killed (*17*). The monkeys recovered quickly from surgery, and remained behaviorally normal and healthy until they received pentobarbital anesthesia (at doses sufficient to eliminate the corneal reflex) prior to perfusion and brain removal at approximately 70 days after implantation (7).

The brains were processed with standard immunocytochemical techniques. Each brain revealed clear evidence of survival of grafted dopamine neurons. Considering that the chronological age of these neurons was mid-gestational (<12 weeks prior to collection plus 10 weeks after transplantation), their level of development was remarkable. Clusters of TH-positive neurons were identified at graft sites (Fig. 1B). Dense networks of neuritic profiles within the grafts provided a morphological appearance reminiscent of the dendritic bundles that characterize the zona reticulata of the substantia nigra in situ. Neurites did not appear to extend from the grafts into the host brain, and no evidence was seen for dopamine fiber ingrowth from the host striatum. This is consistent with our previous studies with monkey tissue transplants; neuritic ramification outside the graft is not usually seen in normal recipients, but is extensive in MPTP-treated dopamine-deficient recipients (7). Cellular shapes also were consistent with neurons of the substantia nigra. These neurons often were seen in clusters of 25 to 30 in each histological section and were observed bilaterally at several implantation sites over several sections (Fig. 1C). One graft was seen continuously on 50 tissue sections, encompassing a distance of 2.5 mm. Individual neurons appeared robust with no evidence of pyknosis or other signs of cellular degeneration. Graft sites were free of signs of rejection. Very

minor lymphocytic accumulations were seen at the periphery of some levels of the grafts and along the penetration tracks in the cerebral cortex overlying the grafts. A few lymphocytes and macrophages were present in the tracks, the latter probably as a result of minor trauma induced by the implantation needle. Macrophages were not seen within the confines of the grafts, which suggests the absence of rejection response at this time after xenografting and withdrawal of immunosuppression. Astrocytes and microglia were present in the host neuropil surrounding the grafts, but the grafts did not appear to be encapsulated by astrocytes.

The absence of rejection in these xenografts may have been a result of the use of cyclosporine A, but more studies are needed to address the necessity for immunosuppression. Fetal monkey tissue transplanted into adult parkinsonian monkeys has generally not produced evidence of rejection, even without the use of immunosuppressants (*18*). Thus, while the present results suggest that transplants from other primates to humans might also be possible with adequate immunosuppression, they cannot provide evidence regarding the probability of rejection of human allografts implanted into the human brain.

Previous studies have suggested that low-temperature cryopreservation may lead to decreased tissue viability and diminished graft survival because of the weakening of neurons during cryopreservation or storage (*11, 12*). There was no suggestion of adverse effects in the transplants examined, and neuronal survival and appearance compared favorably with those seen previously with fresh fetal monkey neural tissue transplanted into monkeys. Additional studies with this method, however, will be required to quantitate effects of this collection and cryopreservation procedure and prolonged storage on viability.

This study utilized procedures that allow the collection, preservation, transportation, and safety testing of neural tissues obtained from human fetal cadavers. Neurons containing TH were dissected accurately from first trimester fetal cadavers after routine suction abortion and identified by immunohistochemistry. The tissues remained viable after cryopreservation, thawing, and transplantation into another primate species. Other studies in progress are determining further the optimum parameters for collection and cryopreservation techniques and for assessing the characteristics and safety for transplantation of human fetal mesencephalic cells collected under these conditions (*19*). The methods and results of the present study support the possibility of fetal neural grafting of appropriately informed and consenting Parkinson's disease patients under conditions that conform to existing statutes and guidelines in several countries and most state laws in the United States. The methods might also improve the safety and reliability of procedures being attempted or contemplated by providing a necessary interval for identification and safety testing of implantable tissues, demonstration of cell viability for each implantation, and scheduling of the clinical implantation procedure.

In addition to possible applications for "cellular replacement therapies" for neurological diseases or trauma, the methods described may aid in the development of models for studying other conditions that affect human neural tissue. Further studies with human fetal neural tissue may also lead to the development of cultured cell lines that might have functional or immunological advantages over cadaver allografts.

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11. A number of studies by these two groups of Swedish researchers have investigated the transplantation of human fetal mesencephalic tissue into immunosuppressed or immune-deficient rodents. Human fetal neural tissue after 9 to 11 weeks of gestation was found to be the most uniformly viable and effective in these transplant models. Potential sources of tissue injury and loss of viability have been investigated, such as delays between tissue collection and implantation. To investigate the impact on graft viability, rodent fetal neural tissue was transplanted after being held in culture media for 6 days, or in a balanced salt solution at 4°C for 5 days prior to transplantation. [P. Brundin, O. Isacson, A. Björklund, *Brain Res.* **331**, 251 (1985); F. H. Gage, P. Brundin, O. Isacson, A. Björklund, *Neurosci. Lett.* **60**, 133 (1985); P. Brundin *et al.*, *ibid.* **61**, 79 (1985)]. The fraction of surviving cells was relatively low after transplantation into rats by these procedures, but it was within the range these investigators previously obtained with freshly prepared and transplanted material. However, 6 days may be insufficient for more extensive safety or viability testing, and longer holding times with these methods diminish transplant viability.
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13. The methods used to obtain fetal neural tissue comply with all federal guidelines for fetal research and with the Uniform Anatomical Gift Act, as adopted in the state of Connecticut. The approved protocol for this study provided specific protections including, but not limited to, the following: (i) Only fetal cadavers were used, and investigators were not involved in determinations of fetal status, viability, or fetal death. (ii) Tissue was obtained from first trimester abortions only, also further avoiding the issue of fetal viability or possible influences from research use. (iii) Consent for the donation of the tissue was obtained from the gravida, according to the Uniform Anatomical Gift Act. (iv) Consent for donation was explained and sought only after the gravida entered the clinic and signed consent for the abortion on the day that it occurred. She was told that she could change her mind up until research utilization of the tissue actually began, with no adverse consequences to anyone. (v) No investigator or individual involved in the research procedures was involved in any aspect of the decision for, timing, procedures, or method of the abortion. (vi) Confidentiality of donor family and specific use of cells was and will be maintained. (vii) No payment was made for donating the fetal tissue or for terminating the pregnancy. There were no inducements or incentives for abortion from the research or any clinical use proposed. (viii) Since there was no alteration in the methods or timing of pregnancy termination, there was therefore no added risk to the fetus or gravida. (ix) With appropriate and necessary risk disclosures, consent was obtained for tests of a single blood sample from the gravida for human immunodeficiency virus (HIV), cytomegalovirus, and other viruses in order to maximize the biological safety of the fetal tissue utilized. If a woman refused HIV testing or did not want to know results of an HIV test, the fetal tissue was not used.
14. Brain fragments were identified with the aid of a dissecting microscope. The region of the substantia nigra was identified by surface landmarks, dissected, and held in a phosphate-buffered saline solution at 4°C for 25 minutes and then transferred to a Hanks balanced salt solution (HBSS) on ice. Fragments were minced with iridectomy scissors into pieces of less than 2.5 mm and then placed into HBSS containing 7% dimethyl sulfoxide (DMSO) at 4°C. The tissue was aspirated into small glass vials under sterile conditions and frozen 1°C per minute until −60°C was reached, at which point the vials were transferred into liquid nitrogen for storage and transport.
15. Fixation utilized a 5% acrolein solution and treatment with sodium metaperiodate and sodium borohydrate. Fixed cultures were processed for tyrosine hydroxylase (TH) and neuron-specific enolase (NSE) immunocytochemistry by means of the Vectastain ABC method (Vector Laboratories, Burlingame, CA) with the avidin-biotin method [S. Hsu, L. Raine, H. Fanger, *J. Histochem. Cytochem.* **29**, 557 (1981)].
16. Graft procedures were as previously described (*7, 12*). The grafts shown in Fig. 1, B and C, are from a single monkey. A second monkey transplanted at the same time, but with different samples of human neural tissue showed no surviving TH-positive cells that could be attributed to the grafts. Another monkey transplanted subsequently and examined

after similar time periods also showed evidence of graft survival (two of the three monkeys studied had surviving grafts). Very careful observation of the grafted monkeys failed to reveal any neurological, infectious, or other problem. We believe that the initial failure to achieve graft survival in one of the monkeys was due to early difficulties in tissue collection and cryopreservation methods that have been eliminated with subsequent efforts. All animal work was carried out according to *The NIH Guide for the Care and Use of Animals* at the St. Kitts Biomedical Research Foundation, which maintains an assurance of compliance with these guidelines with the Office for Protection from Research Risks, U.S. Public Health Service.

17. In an earlier attempt at cross-species transplantation, we found that cryopreserved rat fetal mesencephalic cells placed into the striatum of a monkey had failed or been rejected, consistent with work previously cited in which human fetal cells were implanted into rodents (*10*). In those studies, immunosuppression with cyclosporine was effective in preventing or reducing rejection. In the first documented transplantation of human embryonic brain tissue, two Yale researchers found that tissue implanted into the anterior chamber of the eye in guinea pigs survived without rejection for up to 2 years in 50 of 55 attempts, without immunosuppression [H. S. N. Greene and H. Arnold, *J. Neurosurg.* **2**, 315 (1945)]. It is not clear, therefore, whether immunosuppression would have been necessary. Further studies of the effects of immunosuppression on transplants of human fetal tissue into the brains of monkey would be of scientific interest, but might not be predictive of the need for immunosuppression of human fetal tissue allografts implanted into human brains.
18. Previously reported fetal neural grafts in St. Kitts green monkeys (*Cercopithecus aethiops sabaeus*) may have had reduced immunogenicity due to decreased genetic heterogeneity in the St. Kitts monkey population, which is descended from a relatively small number of individuals imported from Africa 200 to 300 years ago [F. E. Poirer, *Folia Primatol.* **17**, 20 (1972)]. Whether the extent of this heterogeneity might affect neural tissue compatibility factors is not known. Successful transplants into rhesus monkeys from more heterogenous populations have been reported in a preliminary fashion [R. A. E. Bakay and F. A. King, *Lancet* **ii**, 163 (1986); K. S. Bankiewicz, D. M. Jacobowitz, R. J. Plunkett, E. H. Oldfield, I. J. Kopin, *Soc. Neurosci. Abstr.* **13**, 163 (1987); K. S. Bankiewicz *et al.*, *Soc. Neurosci. Abstr.* **14** (1), 3 (1988)]. One report has suggested that a second transplant of fetal tissue in a bonnet monkey induced rejection in an earlier graft on the contralateral side [C. Freed, J. B. Richards, K. E. Sabol, M. L. Reite, in *Pharmacology and Functional Regulation of Dopaminergic Neurons*, P. M. Heart, G. Woodruff, D. M. Jackson, Eds. (Macmillan, New York, in press)].
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Brain Stem Neurons in Modified Pathways for Motor Learning in the Primate Vestibulo-Ocular Reflex

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The vestibulo-ocular reflex (VOR) stabilizes retinal images by generating smooth eye movements that are equal in amplitude and opposite in direction to head turns. Whenever image motion occurs persistently during head turns, the VOR undergoes motor learning; as a result image stability is gradually restored. A group of brain stem neurons that are in the modified pathways has now been described. The neurons express changes in firing in association with motor learning in the VOR and receive monosynaptic inhibition from the flocculus of the cerebellum. The changes in firing have an appropriate magnitude and are expressed at the correct latency to account for the altered VOR. The response properties of the neurons point to their brain stem vestibular inputs for further investigation of the site of motor learning.

MOTOR LEARNING PLAYS A CRUcial role in establishing and maintaining the excellent performance of the vestibulo-ocular reflex (VOR). Normally, the VOR generates smooth eye movements that are equal in amplitude and opposite in direction to head movement (*1*). As a result, the eyes are stabilized in space and the retinal images of the surroundings remain stable during head turns. Any deterioration in the performance of the VOR causes retinal image motion during each head turn. The combination of visual and vestibular inputs causes learning, which, over a time course of several days, restores the performance of the VOR so that image stability is reestablished (*2*).

Although the site of motor learning has not been located, much is known about the neural basis for motor learning in the VOR. The VOR is subserved by at least two parallel pathways; only some of those pathways are subject to modification (*3, 4*). The cerebellar flocculus must be intact for motor learning to occur (*5*). The output from the flocculus changes in association with motor learning (*6*).

To investigate the site of learning we have identified neurons that receive monosynaptic inputs from the flocculus and analyzed their firing during the VOR before and after motor learning. Seven rhesus monkeys were trained to fixate and track a small, movable target. They were then anesthesized with Halothane, and sterile procedures were used to prepare each monkey for monitoring eye movements and for chronic single unit recording (*7*). We recorded from the brain stem in five of the monkeys and from the flocculus in the other two monkeys. Before beginning brain stem recordings, we cemented stimulating electrodes in the flocculus at a site where stimulation evoked smooth eye movement toward the side of the stimulated flocculus.

Motor learning was induced by fitting monkeys with spectacles that provided magnified (2.2×) or miniaturized (0.25×) vision (*4*). These spectacles require large changes in the amplitude of the VOR to restore image stability during head turns. Monkeys wore the spectacles in their home cages and underwent motor learning during active head turns. The performance of the VOR was then measured by imposing passive head rotation in the dark and computing the gain of the VOR, defined as smooth eye speed divided by head speed. Before motor learning, the gain of the VOR was between 0.9 and 1.0. We began recordings from adapted monkeys after they had worn the spectacles for 1 week, when the gain of the VOR had increased to values above 1.5 or decreased to values below 0.4.

We identified flocculus target neurons (FTNs) by the fact that they were completely inhibited for 10 to 20 ms after stimulation of the flocculus (Fig. 1A). The latency of inhibition, estimated by superimposing sweeps on an oscilloscope, ranged from 1.0 to 1.9 ms. FTNs discharged in relation to the VOR, smooth pursuit, saccades, and steady fixations (*8*). Before motor learning, their firing was modulated by head rotation when the eyes were driven by the VOR, but not when the monkey kept his eyes stationary in the orbit by tracking a target that moved exactly with his head (*9*). Although we found FTNs that preferred upward, downward, ipsilateral, or contralateral eye movements, we report only on those that increased their firing for eye movements toward the side contralateral to the recording, since they formed the majority of our sample. Reconstruction of electrode pene-

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