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Intracerebral xenografts of human mesencephalic tissue into athymic rats: Immunochemical and *in vivo* electrochemical studies

(human neuroblast maturation/transplantation/dopamine/Parkinson disease)

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ABSTRACT **Intracerebral allografts of fetal neurons have been studied in both rodents and nonhuman primates. Such research has been directed towards problems in developmental neurobiology and in animal models of neurological diseases. Whether intracerebrally transplanted human fetal neurons are capable of forming synapses and releasing neurotransmitters are key questions in any application of this approach to human brain development and dysfunction. We studied these questions by examining the immunocytochemical and *in vivo* electrochemical properties of xenografts of human mesencephalic dopaminergic neurons placed into athymic "nude" rats. The transplanted neurons survive, continue to express human-specific Thy-1 immunoreactivity, and extend neuronal processes into the host brain where morphologically identifiable synapses form. Potassium-evoked release of monoamines occurs in the vicinity of the graft but is absent in more remote areas of the host neuropil. These results indicate that human fetal tissue fragments can provide a source of viable neuroblasts for transplantation. Further, synapses form between pre- and postsynaptic elements expressing different species-specific cell surface markers; thus, these markers do not play a determining role in synaptogenesis.**

Fetal neurons are capable of functioning, forming synaptic connections, and releasing neurotransmitter even when removed from their normal site of development in the fetal brain and placed into the parenchyma of the brain in a mature animal of the same species (1–9). For example, grafted dopamine-containing central neurons have been found to reverse neurotoxin-induced deficits in an animal model of Parkinson disease, produced by 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (2–4). The grafted fetal cells in these lesioned animals differentiate, form synapses within the host brain neuropil (6–8), and release dopamine within the host striatum after stimulation (5, 10). To assess the capability of human fetal neuroblasts to act as donor material for transplants, we examined pieces of human basal mesencephalon placed into the dopamine-depleted striatum of immunodeficient "nude" rats.

MATERIALS AND METHODS

Six to 8 weeks prior to transplantation, the normal dopamine innervation of the host striatum was removed by unilateral injection of 6-hydroxydopamine into the nigrostriatal pathway. Human fetal material was obtained after termination of first trimester pregnancies. Healthy women with an apparently normal pregnancy in weeks 7 to 12 of gestation were admitted to the Karolinska Hospital for abortion. The pregnant women who had elected to terminate their pregnancy without prior knowledge of this study were informed orally and in writing about the aim of the study and the procedure to be used. The women gave their consent that the tissue fragments resulting from the abortion could be used in this study. These experiments, and the human subject protocols, were approved by the Regional Ethical Committee of the Karolinska Hospital and all proceaures conform to the guidelines of the Swedish Medical Association and the U.S. Public Health Service. The tissue fragments from the abortions were examined under a stereomicroscope, and pieces from the substantia nigra area were dissected out in cases where identifiable portions of the brainstem, including the mesencephalic flexure, were found. The resulting fetal tissue fragments were dissected in isotonic saline; solid tissue pieces were then stereotaxically implanted into the striatum (9, 11).

Three to 6 months after grafting, the animals were assessed immunocytochemically and electrochemically for viability of the grafted neurons. *In vivo* electrochemical studies were performed on urethane-anesthetized animals as described (5, 12, 13). Single-barrel micropipettes, filled with a solution containing 120 mM K^+ and 2.5 mM Ca^{2+}, were attached to the electrochemical recording electrodes. The potassium solutions were locally applied by pressure ejection. Electrochemical measurements were made using Nafion-coated graphite epoxy capillary (GEC) electrodes, which have been shown to be highly selective for the monoamine neurotransmitters (12, 13). High-speed chronoamperometric measurements (5 Hz, −0.2- to +0.45-V square-wave pulses) were recorded by using an IVEC-5 electrochemical instrument (Medical Systems Corp.). The high-speed chronoamperometric signals were digitally integrated during the final 40–100 msec of the oxidation and reduction phases. *In vivo* electrochemical experiments were initiated with the insertion of an electrode assembly into a selected region of the control or transplant reinnervated side of the rat caudate nucleus. Electrochemical measurements were repeated every 0.2 sec. Once a steady-state baseline response was established at a given recording site, the effects of locally applied K^+ were investigated. Signal magnitudes, expressed as micromolar change from baseline as well as the rise and half decay times, were used as indices to quantitate the electrochemical signals (13). At the conclusion of the electrochemical recording session or without prior recording, the rats were given an overdose of pentobarbital and perfused for light and/or combined light- and electron-microscopic immunocytochemistry (7). The host rats were perfused with 4% paraformaldehyde containing 0.1 or 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The tissue blocks from the three recorded animals were postfixed for 3–4 hr at 4°C and were

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Abbreviation: TyrOHase, tyrosine hydroxylase.

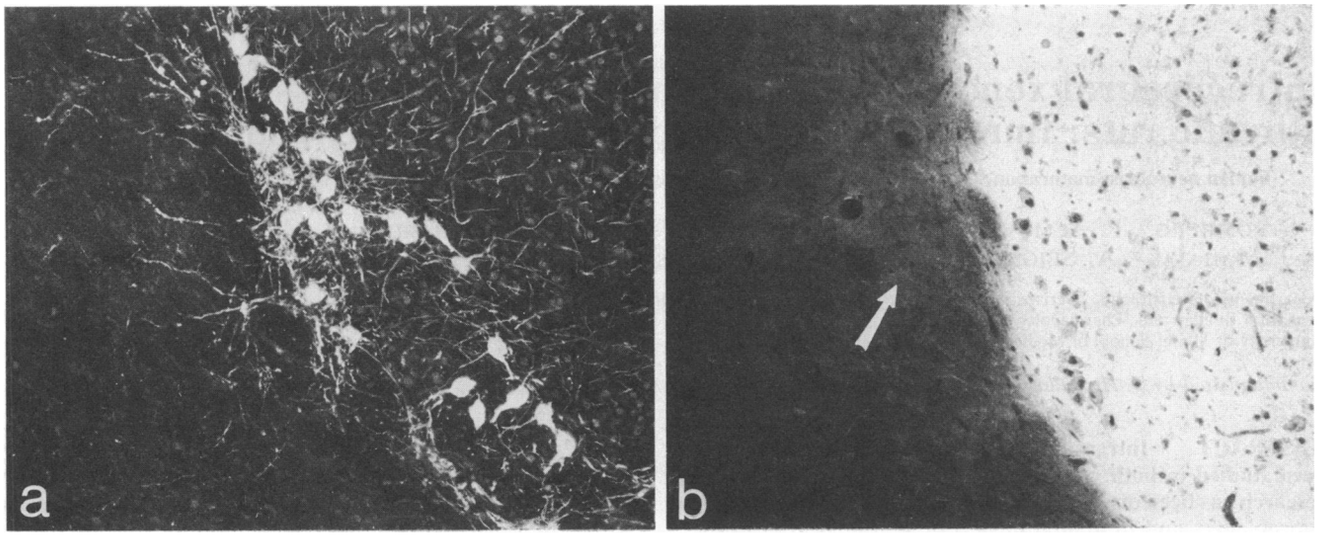

FIG. 1. Human fetal nigral grafts in striatum of nude rats. (*a*) TyrOHase immunoreactivity in a transplant from a 7-week-old human fetus 4 months after grafting. TyrOHase-immunoreactive neuronal processes coming from cells in the graft are seen both in the graft (brightly fluorescent area to the right) and entering the host rat striatum (left). (*b*) Same graft as in *a*, identified by human specific Thy-1 antibodies. Graft neuropil is strongly immunoreactive (right). Fine weakly stained Thy-1-positive fibers (arrow) extend into the host striatal neuropil (left). (×95.)

stored overnight in phosphate-buffered saline (PBS). Tissue from the four other animals was postfixed overnight at 4°C in 4% paraformaldehyde at pH 10.4. After the different postfixation protocols, tissues were rinsed in PBS and cryoprotected in a graded series of sucrose/glycerin. Frozen sections, 14–80 μm, were collected in PBS and incubated for 1 hr in a 1% solution of sodium borohydride. The thinner sections were used for light microscopy, whereas the thicker sections were prepared for electron microscopy.

Sections for light microscopic studies were incubated overnight in primary antisera directed against either tyrosine hydroxylase (TyrOHase; 1:500) or human-specific Thy-1 (1:500). All antibodies were diluted in PBS containing 1% normal goat serum and 0.3% Triton X-100. The sections were rinsed in PBS and incubated with fluorescein-conjugated secondary antisera of the appropriate species specificity. In addition to sections through the striatum, serial sections through the substantia nigra were treated with TyrOHase antisera to assess the degree of the initial neurotoxin-induced lesion. For ultrastructural immunocytochemistry, the tissue was fixed and sectioned as described above. Free-floating sections were then washed in PBS and incubated in PBS containing either 5% powdered milk or 2% normal goat serum. The sections were subsequently incubated in the primary antisera, without the Triton X-100 detergent for 24–48 hr at 4°C. The peroxidase-antiperoxidase technique was used to localize the primary antisera with 3,3′-diaminobenzidine as the chromogen. After this reaction, the sections were rinsed, postfixed in 2% OsO_4, dehydrated, and flat-embedded in Epon. The flat-embedded sections were examined with the light microscope for the presence of electrode tracks and for immunoreactive cells and processes. Appropriate areas of the tissue were then cut out of the plastic sheet, glued to a Beem capsule blank, and resectioned for electron microscopy. The ultrathin sections were collected on copper grids and stained with lead citrate before examination in a Philips CM-10 electron microscope.

RESULTS

Human tissue grafts were readily found within the host striatum. In five of seven animals, TyrOHase-immunoreactive somata could be seen within the graft. Thick and thin processes extended from the graft into the host striatum (Fig. 1) for a distance of 1–3 mm. Regions of the host striatum remote from the graft were virtually devoid of TyrOHase-immunoreactive elements. In the three cases prepared after

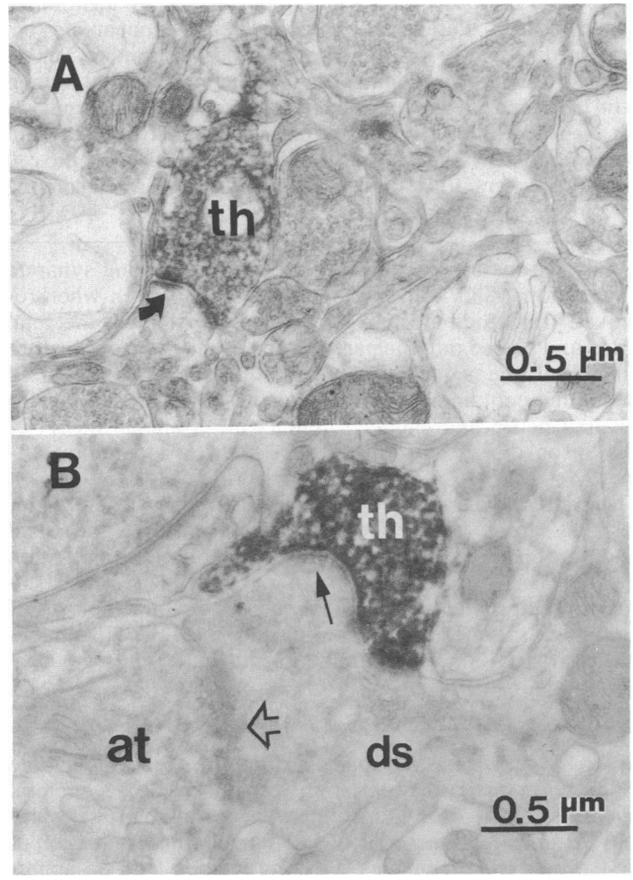

FIG. 2. (*A*) A TyrOHase-immunoreactive terminal making asymmetric contact with a dendritic spine. Note postsynaptic specialization (arrow). (*B*) A TyrOHase-immunoreactive terminal makes an asymmetric contact (closed arrow) with the neck of an unlabeled spine (ds); an unlabeled axon terminal (at) forms an additional asymmetric synaptic contact (open arrow) on the head of the spine.

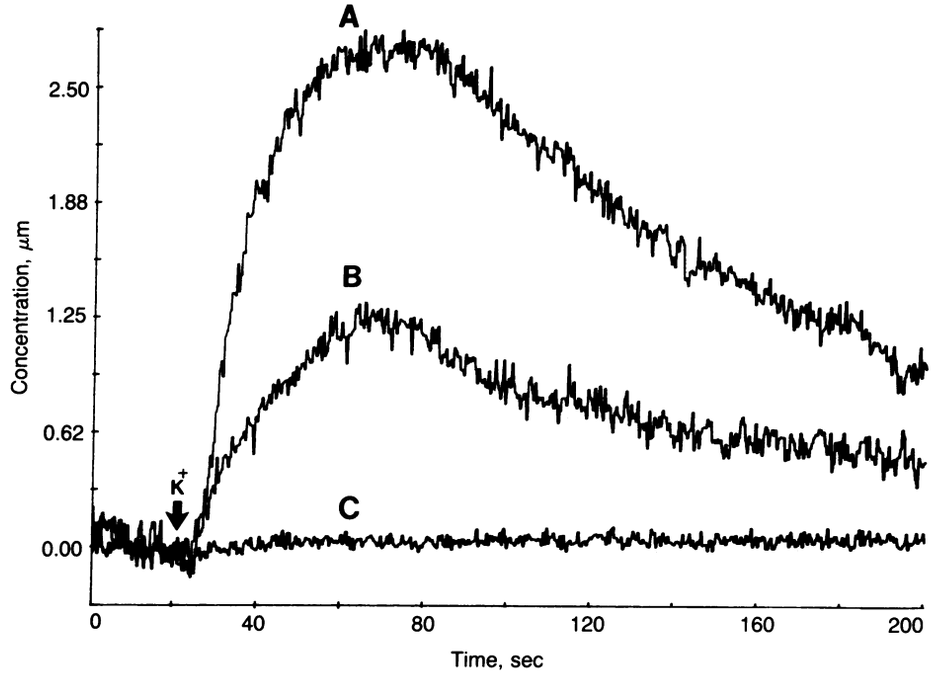

FIG. 3. High-speed chronoamperometric recordings (5 Hz) of K^+-evoked releases from a single animal in: control caudate nucleus contralateral to a transplant-reinnervated striatum (trace A), histologically confirmed electrode placement within 0.5 mm of a fetal substantia nigra graft in a 6-hydroxydopamine-denervated striatum (trace B), and 6-hydroxydopamine-denervated striatum remote (>3 mm) from the graft (trace C). The relatively flat line in trace C is indicative of virtually complete absence of host dopamine fibers on the lesioned side.

electrochemistry, TyrOHase-positive processes were found adjacent to positive electrochemical release sites (see below). At the ultrastructural level, TyrOHase-immunoreactive processes were found in host brain within a few millimeters of the graft. These processes often contained vesicles and formed symmetric and asymmetric synapses with unlabeled dendritic spines and shafts, presumably from host neurons (Fig. 2).

The anti-human Thy-1 antibody reacted with all grafts but did not react with any portion of the host brain. The Thy-1 immunoreactivity appeared relatively diffuse throughout the graft, but a few faintly labeled processes could be seen radiating from the graft into the host striatum (Fig. 1).

Local application of K^+ near the graft in the transplant-reinnervated striatum yielded electrochemical signals with amplitudes more than double those of denervated striatum and about 50% of that found in the intact striatum (Fig. 3). The magnitudes of the electrochemical signals in striatal areas remote from the graft (i.e., farther than 3 mm away) were similar to those in dopamine-denervated striatum (5). These electrochemical findings correlate well with the immunochemical data, which demonstrate destruction of approximately 95% of the dopaminergic neurons of the substantia nigra and virtual absence of native catecholaminergic innervation on the side of the transplant. Thus, we conclude that the electrochemical signals detected near the transplants were due to transmitter release from the catecholaminergic neurons in the transplant.

DISCUSSION

The present data demonstrate that human fetal nigral tissue grafted into the dopamine-denervated striatum of athymic nude rats survives and develops many features typical of nigral allografts in rats. The fact that processes from the human cells extend into the host brain, form morphologically identifiable synapses, and release neurotransmitters suggests that no major barriers to transplant–host neuronal interactions exist in this xenograft system.

In two previous studies (11, 14), human neuroblasts were transplanted into the dopamine-depleted striatum of rats immunosuppressed with cyclosporin A. Both of these studies utilized only behavioral and light microscopic indices of transplant function and survival. Since nonsynaptic diffusion of catecholamine from transplanted chromaffin cells is sufficient to ameliorate behavioral dysfunction (15), we felt it was critical to utilize ultrastructural and electrochemical measures of synapse formation and function in the present report.

Recently, *in vivo* electrochemical techniques have been developed which not only detect monoamine release but also can potentially discriminate between various molecular species (16). Results from the present experiments are consonant with the interpretation that within a few mm of the graft, significant release of monoamine occurs upon local K^+ application to the tissue. Release of dopamine *in situ* is regulated in a complex manner, but dopamine autoreceptors are believed to play a prominent role (17). Such receptors, when activated, elicit decreased excitability and transmitter release. The extent to which there is autoregulation in transplanted human dopamine neuroblasts is obviously an important issue in terms of normalizing function in the host brain.

Parkinson disease involves a characteristic motor disorder and a concomitant loss of neurons of the substantia nigra, which provide the major dopamine input to the striatum. A potentially new mode of treatment, involving intracerebral grafting of dopamine neuroblasts, has been suggested by studies on toxin-induced animal models of this disease. Taken together, the present results show the feasibility of utilizing tissue fragments from aborted fetuses as sources of neuronal transplant donor material. The human cells are capable of growing, differentiating, forming synapses, and releasing transmitter in response to stimuli after maturation in an appropriate environment.

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