Morphological and Electrophysiological Studies of Human Hippocampal Transplants in the Anterior Eye Chamber of Athymic Nude Rats

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Human fetal hippocampal tissue from normal women was obtained following elective abortion in the 8th to the 11th week of gestation. The hippocampal tissue was transplanted to the anterior chamber of the eye of adult athymic nude rats, where it was allowed to develop for up to 9 months before histological and electrophysiological evaluation. The transplants were revascularized from the host iris and many grew extensively in oculo. Large neurons were present in all transplants. Immunohistochemical studies revealed glutamic acid decarboxylase-containing terminals and clusters of γ-aminobutyric acid-positive nerve cell bodies within the transplants, as well as scattered tyrosine hydroxylase-positive and acetylcholinesterase-containing fibers. Single neurons recorded extracellularly from transplants 4–9 months in oculo showed a slow spontaneous discharge, with both complex and single action potentials. Stimulation of the transplant surface evoked a small initial wave followed by a larger and longer-lasting field potential, similar to that seen in hippocampus in situ. A conditioning–testing paradigm was used to evaluate the presence of inhibitory circuitry in the hippocampal transplants. Significant suppression of the evoked test response was seen with interstimulus intervals ranging from 20 to 500 ms. Superfusion of enkephalin (100–300 nM) or penicillin (1600 U/ml) increased slow-wave activity, as did tetanic electrical stimulation. These treatments appeared to generate ictal-like activity, which in some cases persisted as interictal spikes. Illumination of the retina also increased neuronal activity, presumably by reflex activation of cholinergic afferents from the parasympathetic innervation of the iris. Taken together, our data suggest that fragments of hippocampus from aborted first trimester human fetuses, grafted to the eye chamber of rodent hosts, develop many organotypic histological and physiological features. This preparation may provide a unique means for the study of neurobiological properties of human brain in both normal and disease states. © 1989 Academic Press, Inc.

INTRODUCTION

The hippocampus has been implicated in several major nervous and mental illnesses, including schizophrenia (12) and Alzheimer's disease (15). Both disturbances in sensory processing, typical of psychoses (18), and disturbances in memory, typical of dementia (41), have been linked to hippocampal dysfunction. Autopsy specimens show anatomical pathology in neurons in the hippocampus in both illnesses (5, 3), but a clear neurobiological understanding of the pathophysiology has yet to be elucidated for either condition. A major obstacle to such an understanding is the paucity of techniques that can be used to study the neurobiology of human brain. Techniques such as single neuron recording and immunohistochemistry, which have become mainstays of modern neurobiology, are not possible in most human subjects. This paper describes a technique for the study of human hippocampus in an animal host, in which invasive and destructive protocols are ethically possible. The present study demonstrates that small fragments of hippocampus from aborted first trimester fetuses can be grafted into the anterior chamber of the eye of a rat host, where they continue their development and express anatomical and physiological features typical of the hippocampus in situ. Since many of the neural abnormalities of schizophrenia and Alzheimer's disease may be genetically determined (19, 13), abortuses derived from women with family histories of these illnesses might be more likely to carry the neurobiological defects associated with these illnesses. The in oculo transplant preparation may thus represent an approach in which the techniques...
of modern neurobiology can be used to study inherited brain illnesses.

The intraocular transplant preparation has been used for some time to study the development and function of isolated portions of the brain. When small defined areas of fetal rat brain, such as nucleus locus coeruleus (33) or the cerebellar cortex (22), are grafted syngeneically to the anterior chamber of the eye of an adult rat host, these tissues attach to the iris, from which they receive vascularization, as well as innervation from the autonomnic adrenergic (20) and cholinergic (14) fibers. The grafts increase in size and develop many of the regional anatomical and electrophysiological features characteristic of rat brain in situ (35, 31). The anterior chamber of the eye was chosen for the recipient site in these studies because it allows continuous visualization of the graft and easy access for electrophysiological experiments (22). Recently the technique has been expanded to encompass human tissue derived from aborted first trimester fetuses. In our first studies, the hosts were treated with cyclosporin A to suppress immunological rejection of the graft (37). Later, an immunologically compromised mutant, the athymic nude rat, was used to obviate the need for daily cyclosporin treatment (4). Cerebral and cerebellar cortices grew well for up to 4 months in these immunocompromised animals and spontaneous electrical activity could be recorded.

The hippocampus was chosen for transplantation in the present study because of our interest in this area as a site of pathology in a number of human illnesses. In addition, our previous studies with rat fetal transplants had shown that the hippocampus would develop well as an isolated in oculo transplant (23, 32, 34, 43). In this study we were also able to maintain the transplants in oculo for longer periods, up to 9 months, compared to the shorter times in our previous work, to allow further time for organotypic development. This additional time for development may be important, because the human hippocampus reaches considerable maturity by birth (16).

MATERIAL AND METHODS

Donor material. Fetal hippocampal tissue was obtained following termination of first trimester pregnancies. Healthy women with an apparently normal pregnancy in the 8th to the 11th week of gestation, admitted to the hospital for elective abortion, were informed both orally and in writing about the aim of the study and the procedure to be used and gave their consent. Anonymity was strictly maintained. The abortion was performed using paracervical blockade following premedication. After standard dilatation of the cervical canal, the abortion was carried out using vacuum aspiration. The fetal tissue was collected and kept in isotonic saline until further processed. The study was approved by the Regional Eth-
were immersed in the same fixative for 2 hr. Cryostat sections (14 μm) were incubated with antisera to the following antigens: tyrosine hydroxylase (TH, 1:50 solution, Eugene Tech. International Corp.), neurofilament (NF, 1:500 solution, (10)), glial fibrillary acidic protein (GFAP, 1:100, (9)), glutamic acid decarboxylase (GAD, 1:500, (50)), γ-aminobutyric acid (GABA, 1:2000, (39)), and laminin (LAM, 1:500, (11)). Antibodies were dissolved in a 0.3% Triton X-100 solution in phosphate buffer, and the indirect immunohistochemical technique of Coons (8) was performed. Secondary antibodies were IgG directed against the appropriate species and conjugated with fluorescein isothiocyanate (FITC, (11)). As a control, the first antibody was omitted.

Acetylcholinesterase (AChE) staining of individual sections from 13 transplants was also performed (24, 7). Briefly, sections were incubated at room temperature for 2 h, in a medium containing acetylthiocholine iodide, sodium citrate, copper sulfate, and potassium ferricyanide in sodium acetate buffer. In addition, representative sections from all transplants evaluated with histochemistry were stained with cresyl violet.

Electrophysiological recordings. Extracellular recordings were carried out in seven transplants by means of a single-barrel glass micropipet filled with 5 M NaCl, sodium glutamate (50 mM) being added to the pipot solution in four transplants to increase the neuronal firing rate. The recipient rats were anesthetized with urethane (1.25 g/kg ip), intubated, and placed in a stereotaxic frame. The cornea overlying the transplant was removed, and 37°C Earle’s balanced salt solution (EBSS) was continuously superfused through a plexiglass superfusion chamber, as previously described (23, 21). Following amplification by an emitter–follower preamplifier, single unit activity was displayed directly on a strip chart recorder. The single unit activity was further amplified, separated from background activity by a window discriminator, and integrated over 1-s intervals to indicate discharge rate. The output of the window discriminator was led to a digital computer to construct post-stimulus time histograms and to average slow-wave activity following bipolar surface stimulation. Single square-wave pulses of 0.1 ms in duration and currents ranging from 10–100 μA were applied at 50- to 500-ms intervals (14).

Enkephalin (d-ala²-methionine⁵-enkephalinamide, 100–300 nm) and sodium penicillin-G (1600 U/ml) were dissolved in EBSS and superfused over the transplants for a minimum of 10 min, to allow for the drug concentration to reach equilibrium. We have found that this time period is required in our superfusion system for drugs to reach a steady-state concentration (17).

RESULTS

Intraocular growth. Fetal human hippocampal tissue was transplanted into a total of 20 eyes of adult, nude athymic rats. These transplants remained viable for up to at least 9 months in oculo. There was a large variability in individual graft growth (Fig. 1). Some grafts grew extensively, while others remained at approximately the same size until sacrificed. There appeared to be no correlation between growth and fetal donor stage or size of the transplanted tissue fragments.

Morphology. Routine histological evaluation with cresyl violet revealed a large number of nerve cell bodies scattered between smaller cells, presumably glial cells, in all transplants (Figs. 2A and 2D). No clear cell layers, such as a pyramidal cell layer, were found but strands of elongated bi- and multipolar cells were observed. No signs of rejection were observed in any of the transplants, even after 9 months in oculo. The human hippocampal transplants evaluated with GFAP-immunohistochemistry exhibited a marked gliosis with a dense pattern of immunoreactive cells and processes throughout the transplants (Fig. 3A). The neuronal fiber network, shown with NF-immunohistochemistry, was distributed in patches of parallel fibers and some positive cell bodies were observed as well. GAD-positive terminals were found in parts of the transplants (Fig. 3C). Some transplants contained GABA-positive nerve cell bodies as well (Fig. 3D).

The hippocampal transplants became vascularized from the host iris within 6 weeks after transplantation. Laminin immunohistochemistry revealed a few, apparently thick-walled, blood vessels near the attachment to the host iris (Fig. 3B). The vessels sometimes sprouted toward the surface and inner parts of the transplants. A patchy ingrowth of AChE- and TH-positive fibers, presumably from collaterals of the host iris autonomic nerve plexus, could also be demonstrated in most transplants (Figs. 2B and 2C).

Electrophysiological recordings. Seven transplants were studied after 4–9 months in oculo using electrophysiological techniques. Neurons in all transplants (15 cells in total) exhibited spontaneous electrical activity, with discharge rates ranging from 0.5 to 7 Hz prior to glutamate application. Neurons fired with both single and complex spike patterns. An example of a complex

FIG. 2. Microphotographs of human hippocampal transplants. An overview of a cresyl violet-stained 9-month-old transplant is shown in D. Note the migration of heavily stained pigment-containing cells into the entire transplant from the pigmented rat host iris (left, arrow). Detail from a somewhat younger transplant, also stained with cresyl violet, is shown in A. Large neurons are scattered throughout this transplant (arrow), as well as strands of smaller cells, presumably glial elements. Scattered fibers containing AChE (B) as well as TH (C) were found in several transplants, especially close to the attachment of host iris. Scale bars in A and C represent 50 μm; in B and D, 100 μm.
FIG. 3. Microphotographs of sections of intraocular hippocampal transplants processed for immunohistochemistry. In (A), a section from a 3-month-old transplant incubated with GFAP antibodies, is shown. There is an abundance of astrocytic processes and cell bodies throughout the transplant, suggestive of glialosis. The blood vessels in host iris (lower left) and in a 3-month-old hippocampal transplant are visualized with laminin immunohistochemistry in B. Note the unusually thick-walled vessels sprouting along the transplant surface as well as in the inner part of the graft. In C, a GAD-immunoreactive network of thin fibers and terminals, surrounding larger, dark nerve cell bodies, is shown in a section from an intraocular hippocampal transplant. Transplant surface is to the right. GABA-immunoreactive cell bodies and processes are found at the surface of a 9-month-old transplant in D. Note that strands of GABA-positive thin, varicose fibers extend along the graft surface (lower right). Scale bars in A, C, and D = 50 μm; in B, 100 μm.

spike is shown in Fig. 4. Also shown in Fig. 4 is the effect of bipolar electrical stimulation of the transplant surface with currents ranging from 10–100 μA. Such stimulation elicited a reproducible series of waves in all of the five transplants studied. The first was a small field potential which occurred 5 to 7 ms after stimulation (Fig. 4A). A series of larger waves followed over the next 100 ms (Fig. 4A). In two transplants, cell discharge could be observed in synchrony with the initial small wave. As the electrode was driven through the tissue in three transplants, the waves inverted polarity, suggesting the generation of this activity within these transplants (Figs. 4B and 4C).

Inhibitory neuronal circuits were demonstrated in a total of four transplants by the use of a paired pulse or conditioning-testing paradigm. Two identical stimuli were presented. Decrement of the response to the second
of the paired stimuli was observed over a considerable range of interstimulus times in these hippocampal transplants. Decremental of 50% were observed at intervals between 20 and 500 ms (Fig. 5).

As an alternative demonstration of the integrity of intrinsic inhibitory and excitatory neuronal circuitry, increased electrical activity was induced in seven transplants by tetanic stimulation, by superfusion with so-

FIG. 4. Potentials evoked after bipolar stimulation to the transplant surface (arrow). Each wave form is the average of 25 responses to stimuli at 10-s intervals. Note that the small initial wave (*), as well as later waves (**), becomes smaller and then reverses polarity as the electrode is lowered through the transplant. Depth of electrode from the transplant surface is indicated on the y-axis. The inset shows a complex action potential which was recorded 100 μm below the surface, at the same location as waveform B.

FIG. 5. Potentials recorded in a conditioning-testing paradigm. A shows the conditioning response and the test response after a 50-ms interstimulus interval. The asterisk marks the initial response as in Fig. 4. This wave is significantly reduced in the test response. The lower field potentials (B) are from the same transplant, but the interstimulus interval has been extended to 500 ms. Note that the response to the test stimulus is also decremented at this interval. The asterisk in the test response in both instances is placed at the same distance from the stimulus as in the conditioning response, to facilitate comparison of the responses. Each waveform is the average of 25 paired stimulus trials, delivered at 10-s intervals. The trace duration and amplitude scale bars are to the right, below record.
Penicillin 1600 U/ml

FIG. 6. Response of a transplant to penicillin superfusion (solid bar over records B and C). In A an oscilloscope tracing of a single action potential from a cell at the site of recording prior to penicillin is shown. B Epileptiform slow-wave activity which occurred during superfusion with penicillin and continued after cessation of drug administration. C Ratemeter recording which shows the increasing discharge of the single recorded neuron during penicillin superfusion. Horizontal bar under the record in C represents time in seconds; vertical bar represents firing rate in events per second (Hz).

dium penicillin (Fig. 6), or by superfusion with enkephalin (Fig. 7). These treatments caused an increase in slow-wave magnitude in all transplants tested and, in six of seven transplants, even led to seizure-like activity. In addition, the discharge rate of single neurons was increased. In most cases the ictal activity became periodic, with brief periods of increased activity, followed by relative silence. This interictal-like activity outlasted the period of treatment of the transplant with the epileptogenic agent.

Brief illumination of the ipsilateral retina produced an increase of slow-wave activity and of single unit discharge in three of five neurons studied. The effect appeared to be more prominent in the oldest graft, studied at 9 months after transplantation (Fig. 8). Both slow-wave activity and cell discharge rate returned to control levels several minutes after cessation of illumination.

DISCUSSION

The object of this study was to initiate neurobiological assessment of human hippocampal xenografts derived from first trimester abortuses. If the grafts could be demonstrated to continue their growth and development over many months, then the preparation could serve as a valuable tool for studies of human hippocampal anat-

FIG. 7. Response of a transplant to enkephalin superfusion (solid bar over the upper record). Slow-wave activity (top trace) is shown together with the ratemeter record from a single neuron recorded simultaneously. Enkephalin (100 nM) elicited periods of epileptiform slow-wave activity, interspersed with interictal periods. The increased slow-wave activity, as well as the elevated neuronal discharge rate, reversed after the cessation of drug superfusion.
HUMAN HIPPOCAMPAL TRANSPLANTS

FIG. 8. Ratemeter record of the excitatory response of a single neuron in a transplant to illumination of the retina (light). The extracellularly recorded action potentials of this neuron are shown in the inset oscilloscope trace.

Omy and physiology. In this study, we have successfully maintained human grafts in athymic nude rats for over 9 months. No dense neuronal cell layers such as the pyramidal cell layer earlier demonstrated in rat allografts (32, 23, 21) were observed in the human hippocampal transplants even after 9 months in oculo. However, such layering is less pronounced in the adult human hippocampus than in other species (25). It is evident, both from this study and previous studies on intraocular transplants of human fetal brain fragments (37, 4) that the isolated human brain tissue develops according to a human rather than the host rat time schedule.

Immunohistochemistry showed the presence of several neurotransmitter systems in the grafts. The presence of immunoreactivity for the GABA-synthesizing enzyme GAD, as well as for GABA itself, suggests that the grafts contain intrinsic GABAergic interneurons. Fibers containing immunohistochemical markers for catecholamines and AChE were also visualized. In our previous studies of rat allografts, we have shown that such fibers derive from the autonomic ground plexus of the host iris, which invades the transplant (20, 14). While these sympathetic and parasympathetic fibers are quite large in diameter in the iris itself, in the transplant they become morphologically more similar to the thinner varicose fibers seen in the central nervous system (20, 14). Electrophysiologically, the transplants showed several properties of mammalian hippocampus. One of the most extensively studied characteristics of hippocampus is the response to stimulation of the alveus or hippocampal surface. Within 10 ms, a small wave occurs, which is the population spike, composed of synchronous action potentials (2). This small wave is followed by larger and longer lasting slow waves, thought to represent later synaptic currents. A population spike and field potential have been observed in rat transplants in oculo (14) and a similar sequence of waves was also recorded here in the human hippocampal transplants. The initial wave was associated in some cases with action potential discharge and reversed in the transplant at the depth at which individual spikes could be recorded. Further characterization, however, is necessary before concluding definitely that the initial small wave is really a population spike.

A second important electrophysiological characteristic of hippocampus is the presence of local inhibitory mechanisms (1). The conditioning-testing paradigm, which showed suppression of the test response at a 20 ms interstimulus interval, provides evidence for such mechanisms. A recurrent basket cell synapse would be a likely candidate for the inhibitory mechanism. These circuits are usually GABAergic, which correlates well with the previously discussed findings of GAD-positive terminals and GABAergic nerve cell bodies in the transplants. Suppression of the test response was also shown at conditioning-testing intervals as long as 500 ms. This longer interval exceeds the usual parameters of either GABA-A or GABA-B inhibitory neurotransmission in the hippocampus, but it is consistent with the potassium-mediated afterhyperpolarization (AHP), which is a long duration inhibitory mechanism in hippocampal pyramidal neurons. This potential was first demonstrated in hippocampus in vitro (27) but has also been demonstrated in rat hippocampal syngeneic transplants in oculo (29).

Another test of the integrity of intrinsic excitatory and inhibitory mechanisms is the ability to generate epileptiform activity. Seizure-like activity was present in the human hippocampal grafts, in response to a variety of stimuli. The seizures in response to enkephalin are of particular interest, since enkephalin is thought to act primarily by inhibiting the inhibitory interneurons, and thus disinhibiting pyramidal cells (44, 26). The epileptiform activity is also of interest because its generation requires interconnection between many of the neurons in the transplants, and its presence is thus further evidence for neuronal organization within the transplants.

Preliminary suggestion of innervation of the hippocampal transplants by cholinergic fibers from the rat host iris was obtained from both immunohistochemical and electrophysiological data. In previous studies of syngeneic rat transplants, we have also obtained pharmacological evidence for the reflex excitation of transplant pyramidal neurons by acetylcholine after illumination of the host retina (21, 45). These previous studies, involving specific nicotinic and muscarinic antagonists, need to be repeated in human hippocampal transplants to
provide further evidence for the cholinergic nature of the excitation described here. In summary, fragments of fetal human hippocampus develop several organotypic anatomical and physiological properties 4 to 9 months after grafting in oculo. The transplant preparation may thus serve as a unique excitation described here.

versed disease states as epilepsy (28), Alzheimer's disease (15), and schizophrenia (12), among others, an approach to allow invasive study of transplanted human hippocampal tissue may be of value in understanding the cellular basis of these disease states, as well as providing insight into human brain development.

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