Integration of Fetal Neocortical Transplants with the Damaged Host Brain

Mette Katrine Schulz
Loyola University Chicago

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INTEGRATION OF FETAL NEOCORTICAL TRANSPLANTS WITH THE
DAMAGED HOST BRAIN

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

NEUROSCIENCE PROGRAM

BY

METTE KATRINE SCHULZ

CHICAGO, ILLINOIS

MAY 1995
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<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>C</td>
<td>crus cerebri</td>
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<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CPU</td>
<td>caudate putamen</td>
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<tr>
<td>DDT</td>
<td>dethiothreitol</td>
</tr>
<tr>
<td>DY</td>
<td>diamidino yellow</td>
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<tr>
<td>FB</td>
<td>fast blue</td>
</tr>
<tr>
<td>FOS</td>
<td>fos and fos related antigen protein</td>
</tr>
<tr>
<td>FR</td>
<td>frontal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma\text{-amino-butyric-acid})</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotrophin releasing hormone</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxy tryptamine</td>
</tr>
<tr>
<td>IC</td>
<td>internal capsule</td>
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<tr>
<td>IEG</td>
<td>immediate early gene</td>
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<tr>
<td>KA</td>
<td>kainic acid</td>
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<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
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<td>LV</td>
<td>lateral ventricle</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PHA-L</td>
<td>phaseolus-vulgaris-leucoagglutinin</td>
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<td>PO</td>
<td>posterior thalamic nucleus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium citrate buffer</td>
</tr>
<tr>
<td>TEA</td>
<td>triethanolamine-HCl</td>
</tr>
<tr>
<td>3V</td>
<td>third ventricle</td>
</tr>
<tr>
<td>TH</td>
<td>thyrosine hydroxylase</td>
</tr>
<tr>
<td>VHC</td>
<td>ventral hippocampal commisure</td>
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<tr>
<td>VL</td>
<td>ventrolateral thalamic nucleus</td>
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<tr>
<td>VM</td>
<td>ventromedial thalamic nucleus</td>
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<tr>
<td>VPL</td>
<td>ventroposterolateral thalamic nucleus</td>
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<td>VPM</td>
<td>ventroposteromedial thalamic nucleus</td>
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CHAPTER I

INTRODUCTION

The subject of this dissertation concerns the transplantation of fetal neocortical tissue into neocortical lesions of adult rats. Transplantation of fetal neural tissue into the developing or mature CNS is a commonly used experimental tool in basic neuroscience research. Neural tissue transplantation can be used to address questions regarding mechanisms of promotion or prevention of neuronal regeneration. Transplantation techniques have also been employed as a therapeutic tool in the treatment of injury-induced lost function. Accordingly, the grafting of neocortical tissue into neocortical lesions of adult rats may be an important way to answer questions regarding the neuronal death that occurs following neurodegenerative diseases particularly affecting the neocortex. One common cause of neocortical neurodegeneration is cerebrovascular disease which is often referred to as stroke.

Stroke is a disease that is especially prevalent in the elderly population. Due to an overall increase in the 65 year and older age group, stroke and stroke related illnesses pose an increasing health care burden. Because no curative therapy is currently available for this debilitating disease, basic science research on neuronal regeneration may aid in the development of new treatment strategies.

The most commonly used experimental models for stroke injuries are vascular interfering models in which the blood supply to certain regions of the brain is transiently or permanently arrested. Chemical models using excessive amounts of
glutamate agonists or excitatory amino acids are reported to resemble the pathological changes observed following cerebral ischemia; due to the ease of applying and controlling the extent and location of the lesions in these so-called excitotoxic models, they are commonly used as well.

The experiments in this dissertation are based on the hypothesis that fetal neocortical neurons transplanted into ischemia-like excitotoxin-induced lesions of the sensorimotor cortex of adult rats can become functionally integrated with the host brain. Therefore the following studies were designed to examine the ability of such grafts to survive and become functionally integrated with the host brain. An important prerequisite for functional integration of neuronal transplants is related to the establishment of appropriate host-graft axonal interconnections. Accordingly, the main focus of this work has examined the anatomical/connective features of the fetal neocortical transplants as compared to the normal sensorimotor cortex.

The specific aims of these experiments are: (i) to clarify the host brain origin of transplant afferents, (ii) to determine the distribution patterns and densities of transplant afferents, (iii) to examine the distribution patterns of transplant efferent projections into the host CNS and (iv) to examine if transplant neurons can become activated following stimuli that are known to activate neurons within the normal sensorimotor cortex.

As an extension of these specific aims, additional work has been initiated to examine transplant integration with the damaged neonatal brain. These studies were undertaken to compare the regenerative capacities of developing versus mature host neurons.
CHAPTER II

REVIEW OF RELATED LITERATURE

Anatomy of the rat cerebral cortex: a brief review

The mammalian cerebral cortex can be subdivided into a neocortex (conventionally defined as the phylogenetically youngest part of the brain) and an allocortex. The cortical regions that show a roughly comparable lamination into six layers are termed neo- or isocortex, while the remaining components of the cerebral cortex are termed allocortex. The neocortex, which is the main area of interest in this dissertation, demonstrates a considerable degree of differentiation into functionally and anatomically specialized subunits. Functionally, the neocortex can be divided into sensory, motor and associative regions that are further characterized by their laminar and cellular composition. The sensory areas show a prominent layer IV (inner granular layer) which is the major target of ascending thalamocortical fibers originating from specific thalamic relay nuclei. Motor and associative areas are characterized by a small, less developed or almost absent (agnanular motor cortex) layer IV. Functionally specific areas of the neocortex may be demonstrated by a variety of methods including cellular stains (62, 129, 225), degeneration stains (129), anatomical tracing techniques (128), electrophysiological methods (236), metabolic activation detected by the $[^{14}\text{C}]$-deoxyglucose technique (205), the activation of cellular immediate early genes
and other histological and histochemical methods. The frontal and parietal neocortical areas are the main focus of this dissertation, and therefore a more detailed description of the anatomy of these areas is presented.

**Frontal cortex**

The frontal cortex is represented by three architecturally distinct areas, Fr1, Fr2 and Fr3, that are surrounded by the cingulate cortex medially and the insular and orbital cortices laterally and ventrally (242). The lack of a clearly visible inner granule cell layer (layer IV) and the contrasting presence of a predominant inner pyramidal layer with large and densely packed pyramidal cells (layer V) distinguishes the frontal areas from the laterally and caudally adjacent parietal cortex. Microstimulation and connectivity studies have added further to the characterization of areas Fr1 and Fr3 as the primary motor cortex. In this context, it is important to mention that cortical motor functions are not restricted to the frontal cortex areas in the rat. Several investigators have described a motor-sensory overlap for various somatotopic areas (58, 111, 154, 183).

Afferent projections to the frontal cortex arise from several ipsilateral thalamic subnuclei such as the ventrolateral, mediodorsal, ventromedial, magnocellular medial geniculate, posterior and parafascicular intralaminar, reuniens and rhomboid nuclei. All neocortical areas send afferent input to the frontal cortex. These afferent projections show relatively restricted projection patterns and are termed point-to-point fiber systems. This is in marked contrast to the widespread diverging distribution patterns seen for global system fibers. Global system afferents, i.e. projections arising from relatively small groups of cells and projecting widely to their destinations in the
forebrain, include cholinergic, dopaminergic, noradrenergic and serotonergic fiber systems. These global system fibers arise in the basal forebrain nuclei, substantia nigra, locus coeruleus and midbrain raphe nuclei, respectively. [For review see (243)]. Efferent connections from the frontal areas Fr1 and Fr3 terminate in several brainstem areas including the nucleus ruber, the cranial nerve motor nuclei and the pontine gray as well as the spinal cord (159).

**Parietal cortex**

The parietal cortex consists of four areas: the forelimb (FL), hindlimb (HL) and parietal areas 1 and 2 (Par1 and Par2). Par 1, FL and HL correspond to the primary somatosensory area (SI) and Par2 to the secondary somatosensory area (SII) (242).

Sensory information to the parietal cortex is derived mainly from contralateral cutaneous mechanoreceptors. This information is relayed via specific sensory thalamic nuclei such as the ventroposterolateral (VPL), the ventroposteromedial (VPM), intralaminar and posterior nuclei. Non-thalamic point-to-point system afferents arise from the ipsilateral primary motor cortex, contralateral parietal cortex and claustrum [See (243) for review]. The level setting global system fibers arising from basal forebrain (cholinergic), locus coeruleus (noradrenergic) and raphe nuclei (serotonergic) send extensive projections to the parietal cortex (See(243) for review). .

Efferent projections connect the parietal cortex to several thalamic nuclei such as VPL, VPM, intralaminar and reticular thalamic nuclei and also to zona incerta, frontal motor cortex, contralateral parietal cortex, claustrum, caudate-putamen, mesencephalon, pons, superior colliculus, medulla oblongata and spinal cord [See (243) for review].
Cell types and neurotransmitters present in the neocortex

The frontal and parietal cortices are complex structures with numerous putative transmitters and a large number of morphologically different neuronal cell types. The pyramidal cell is the predominant cell type (71-97%) (171, 172, 238). Other cell types include spiny stellate cells, multipolar cells, basket cells and bipolar cells. The internal granular layer (layer IV), which is predominant in the primary sensory areas, contains small perikarya cells that receive specific thalamocortical inputs. This layer is especially well developed in the parietal cortex, which contains most of the somatosensory representations. The internal pyramidal cell layer (layer V) contains large pyramidal cells that project to subcortical layers and is well developed in the motor areas.

Acetylcholine. The cholinergic innervation to the frontoparietal cortices originates in specific subsets of neurons located in the magnocellular basal forebrain nucleus (186). Cholinergic axons in the neocortex show a laminar termination pattern with the highest density in layers V and IV (72, 186). In addition to these extrinsic inputs, a quantitatively less important intrinsic network of axons from choline acetyltransferase (ChAT) positive perikarya has been described (71, 72).

Serotonin (5-hydroxytryptamine, 5-HT). The serotonergic innervation of the rat neocortex originates from the brainstem midline raphe nuclei. Axons travel via the medial forebrain bundle to the frontal cortical area and via the dorsal raphe cortical tract to the parietal cortex (4, 230). Measurements of cortical 5-HT content reveal regional differences with the prefrontal areas of the neocortex having the highest 5-HT content (177). 5-HT containing axons are localized throughout the thickness of the neocortex with the highest density in the superficial layers. The orientation of the serotonergic fibers varies within the different cortical regions and layers.
Noradrenaline (NA). Noradrenaline containing axons of the rat neocortex originate from the brainstem locus coeruleus which projects via the medial forebrain bundle, anterior septal region and internal and external capsules (73). Measurements of NA in the frontal, parietal, temporal and occipital areas (165) show decreasing concentrations along the rostrocaudal axis. NA containing axons show a layer-specific orientation with horizontal fibers in layer I, radially oriented fibers in layer II and III, short oblique axon segments in layers IV and V and strictly rostrocaudally oriented fibers in layer VI. The density of NA containing fibers decreases from layer I to layer VI.

Other major neurotransmitters such as glutamate, \( \gamma \)-amino-butyric-acid (GABA), dopamine and neuropeptides such as vasoactive intestinal polypeptide, somatostatin, cholecystokinin and opioid peptides are also present in neocortical neurons, where they often are found to be colocalized with other neurotransmitters or neuropeptides (242).

Functional role of cholinergic and monoaminergic systems

Although the cholinergic and monoaminergic systems have been studied extensively, their specific functions with regard to the neocortex are not as well characterized as other pathways, such as the thalamocortical projections. Their involvement in behavioral attention, brain plasticity and certain disease processes as well as in neuronal activity-level setting is suggested (66, 110, 133). All of the global systems, i.e., the basal forebrain derived cholinergic system, the coerulear noradrenergic system, the mesencephalic dopamine system, and the midbrain serotonergic system, are thought to be engaged in parallel in the performance of several types of attentional tasks. Particularly relevant to this dissertation, the noradrenergic
system has been implicated in the organization of adaptive responses to stress (88). An increase in firing rate of coerulear neurons and an increase in terminal release of noradrenaline is observed following exposure of an experimental animal to a stressful situation (100).

In addition to their contributions to behavioral responses, global systems are also hypothesized to be involved in CNS plasticity. For example, the removal of cholinergic inputs to the neocortex is reported to enhance neocortical morphological plasticity (109). The monoaminergic systems also are involved in neocortical plasticity (9, 37, 133, 164). Finally, global system pathways have been implicated in the pathogenesis of certain neuropsychiatric disorders as well as in neurodegenerative disorders such as Parkinson's disease and dementia of Alzheimer's type.

**Excitotoxin-induced lesions of the neocortex**

In the experiments described in this dissertation, fetal neocortical tissue was transplanted into excitotoxin induced lesions in the sensorimotor cortex of adult rats.

**Excitotoxins**

Glutamate is the most commonly occurring excitatory neurotransmitter in the central nervous system with receptors found on almost all types of neurons as well as on some glial cells. Although glutamate is normally present within the CNS both as part of a metabolic pool and as a neurotransmitter, abnormally high concentrations of this transmitter are lethal to neurons in vitro and in vivo. Since the initial report on the neurotoxic effect of this excitatory amino acid (138), several exogenous and
endogenous agents, showing both excitatory amino acid receptor affinity and the ability to cause neuronal degeneration, have been found [for review, see (179)]. In 1974 Olney introduced the term excitotoxins for agents that can kill neurons via binding to excitatory amino acid receptors. In addition to glutamate, the first described excitotoxin, other agonists with potent excitotoxic effects have been identified. These include agents such as N-methyl-D-aspartic acid (NMDA), kainic acid (KA), ibotenic acid, AMPA, quisqualic acid, and quinilinic acid. These and several other agonists bind to two different types of postsynaptic receptors. Receptors that directly control ion channel opening are termed ionotropic receptors and include NMDA, AMPA and KA receptors. Receptors that are coupled to G-proteins are termed metabotropic receptors and include several subtypes as well (6, 135, 234, 241).

In vitro experiments have revealed that neuronal death after acute exposure to excitotoxic amino acids can be divided into two phases. The initial phase is characterized by neuronal swelling and is dependent on the presence of extracellular Na$^+$ and Cl$^-$, and the second phase is characterized by a more delayed neuronal disintegration and is dependent on extracellular Ca$^{++}$. Both the initial and delayed phase of neuronal degeneration can alone produce irreversible neuronal injury, but the Ca$^{++}$ dependent process appear to be a universal feature of excitotoxic cell death [see (6, 145, 148) for reviews]. Excessive activation of the NMDA subtype of glutamate receptors has been implicated in the pathogenesis of several acute neurological disorders such as hypoxia-anoxia, stroke, epilepsy, hypoglycemia and more chronic neurodegenerative diseases such as Huntington's chorea, Alzheimer's disease and amyotrophic lateral sclerosis (for reviews, see (145, 148)). Patients with these disorders may therefore be especially vulnerable to the excitotoxic effects of
endogenous and/or exogenous agents or have been exposed to supranormal levels of excitotoxic agents.

Increased neuronal vulnerability to excitatory amino acids may occur following an impairment of ion pumps, Ca++ buffering mechanisms or free radical scavenging resources. Hypoxic/ischemic insults lead to impaired oxidative phosphorylation resulting in a decreased supply of ATP. Na+/K+ and Ca++ pumps are driven by ATP dependent mechanisms and a lack thereof results in impairment of these ion pumps. Failure of the Na+/K+ pump function decreases cellular membrane potential, which may overcome the voltage dependent Mg++ blockade of the NMDA receptor channel, resulting in overactivation of the NMDA receptor complex. Failure of Ca++ pump function results in impaired compensation for the rapid Ca++ entry through the NMDA receptor channel also resulting in an increase in NMDA receptor mediated cellular reactions. Excitatory amino acids are reported to stimulate the formation of the free radical nitric oxide, which has neurotoxic characteristics (55, 85). Impaired free radical scavenging mechanisms may therefore result in increased neuronal injury.

Exposure to supranormal levels of glutamate or other excitatory amino acids can be derived from intrinsic as well as extrinsic sources. Endogenous glutamate is found in abnormally high synaptic concentrations in situations where the presynaptic high affinity glutamate uptake system is overloaded or suppressed. For example, transient cerebral ischemia shows an elevated extracellular glutamate concentration when monitored with microdialysis (10). With regard to extrinsic sources, several of the glutamate receptor agonists are present in our diet, either occurring naturally or added by the food industry. Although neuropathological damage resulting from the consumption of the neurotransmitters themselves (found in relative high contents in some foods) has not been described in humans, several animal studies showing their
neurotoxic effect suggest that some naturally occurring or food additive excitatory amino acid receptor agonists can cause CNS damage. Reports of Domoate/blue mussel poisoning and BOAA/chick pea induced neurolathrysm provide support for this possibility (147).

*Intracortical excitotoxin injections*

Focal administration of excitotoxins is a commonly used lesion model for studies of CNS repair. In contrast to other lesion methods such as aspiration or excision methods, intracerebral injections of excitatory amino acids predominantly affect neuronal cell bodies and thus spare afferent axons and terminals coursing into or through the injection site (8, 46, 74).

Histological examination of the neocortex one week after application of KA or NMDA receptor agonists revealed a disruption of normal cytoarchitecture. Neuronal density decreased significantly while glial cell density increased (8, 113). Neurochemical markers, such as glutamate and GABA content/glutamic acid decarboxylase activity, representing neurons intrinsic to the cortex, were significantly reduced following excitotoxin application. In contrast, markers for afferent systems to the cerebral cortex such as ChAT activity, NA, dopamine and 5-HT content did not differ significantly from unablated controls (8, 113). These observations are in agreement with the axon sparing nature of excitotoxic lesions (46). Relative, but not absolute sparing of NADPH-diaphorase and neuropeptide positive neurons was observed following NMDA intracortical injections (8).

The pattern of neuronal degeneration within the cerebral cortex due to ischemia or Huntington's chorea shows several similarities to the cytopathological changes observed after injections of the excitotoxin NMDA. The resemblance in pathology
between the NMDA induced neuronal degeneration and the degeneration seen after cerebral ischemia and Huntington's disease and the ability to produce lesions with a constant size and localization renders this a useful model for the study of these disease states.

Neuronal pathway tracing

Several techniques are available for the tracing of neuronal connections (105, 106, 208). These can be divided into different subgroups according to their specific neuronal properties or functions. Four different methods are used in this dissertation to examine neuronal connectivity.

Retrograde axonal tracing

This technique is based on the neuronal property of retrograde axonal transport. Intracerebral injections of a fluorescent dye are taken up by axon terminals in the area of the injection and subsequently transported retrogradely towards the cell body. Once accumulated in the perikarya, certain tracers can be visualized up to several months later. The fluorescent tracers Fast Blue (FB) and Diamidino Yellow (DY) were used in the present study. Injections of two different tracers facilitate the analysis of connections to each injection site and also permit the determination of axon collaterals to both sites. This analysis is afforded because of differences in emission wavelengths (FB, 460 nm and DY, 510 nm) and subcellular distribution (FB, cytoplasmic granules and DY, nucleus).
Anterograde axonal tracing

Based on anterograde axonal transport, certain injected compounds are taken up by neurons located within the area of the injection site and transported away from the cell body (anterogradely). In contrast to retrograde tracing methods, which identify inputs into an injection site, this method identifies axons projecting away from the site of injection. In these studies, the anterograde tracer phaseolus vulgaris leucoagglutinin (PHA-L) was used. This particular tracer which is visualized by immunocytochemical methods results in small and well defined injection site after iontophoretic injections (86). This is especially useful for tracing transplant projections.

Direct visualization of transmitter specific pathways

This subgroup of tracing techniques is based on the direct visualization of products contained within a certain type of neuronal system. Enzyme or immunohistochemical methods are used in the detection of transmitter markers, i.e., enzymes involved in the production or degradation of a particular neurotransmitter, or of the actual neurotransmitter itself. In the anatomical examination of cholinergic, catecholaminergic and serotonergic fiber innervation of neocortical transplants the histochemical detection of AChE positive fibers (104) was used as a marker for cholinergic fibers, while tyrosine hydroxylase (TH) immunocytochemistry was used to detect catecholaminergic fibers and serotonin immunocytochemistry was employed to visualize serotonergic fibers.

Detection of functionally connected pathways

Several techniques are available for mapping of functionally connected parts of the nervous system. These methods include electrical stimulation with subsequent
recording of evoked neuronal activity, metabolic activation of specific pathways which is detected by the uptake distribution of exogenously administered 2-deoxy-glucose, and the stimulus evoked activation of immediate early genes. The latter method was used in this dissertation.

Trans-synaptic signals cause a rapid neuronal response ranging from milliseconds to seconds and even minutes. The slower responses are dependent on the induction of gene expression and can be modulated by neurotransmitters, membrane depolarization and neurotrophic growth factors. Trans-synaptic stimulation responsive genes are divided into two classes: (i) cellular immediate early genes (IEG) whose transcription is activated rapidly and transiently within minutes of stimulation (7, 96, 151), and (ii) late response genes, whose expression is induced over a time frame of hours (7, 35, 162). IEG’s are thought to encode regulatory proteins that control the expression of late response genes whose products then are thought to serve more specific effector functions in the neuronal response (7, 35, 162).

IEGs may be induced in the nervous system in several ways: convulsant drug-induced general seizures (see (152) for review), following direct stimulation of sensorimotor cortex (60, 182, 239), surgical lesions and nerve transections (197), NMDA injections (206), cerebral ischemia (119), light stimulation (3, 181), peripheral stimulation (13, 29, 59, 132, 140, 233) and psychological stressors such as introduction into an open field (101).

These studies all suggest that c-fos m-RNA or c-Fos protein detection [most antibodies directed against the c-fos protein product recognize Fos as well as other Fos related antigens] can be used as a technique for mapping the pattern of postsynaptic stimulation/activation. In contrast to the commonly used 2-deoxyglucose uptake activity mapping technique, this technique allows for activity mapping at the level of individual
cells. Studies using simultaneous Fos immunostaining and 2-deoxyglucose uptake techniques after electrical stimulation of the sensorimotor cortex suggest a close correlation between c-fos expression and neuronal activity (182, 197), although there are exceptions to the rule (119, 182).

The use of c-fos protein immunocytochemistry as a metabolic marker for tracing neuroanatomical connections, seizure pathways and sites of action of neuroactive drugs has been reviewed recently (59). While Fos immunocytochemistry can be a very useful methodological technique for tracing functioning active pathways a number of potential problems are recognized. For example, certain brain regions show a mismatch between Fos immunoreactivity and 2-deoxyglucose uptake, and some neurons do not show Fos elevation regardless of the stimulus. Accordingly, negative results do not necessarily imply a lack of activation. Secondly, mature neurons show a low but detectable constitutive expression of c-fos m-RNA. Parameters such as anesthetics and prestimulatory stress can influence c-fos m-RNA and protein expression to a degree that can interfere with correct interpretation of the results. It is therefore important to minimize the stressful stimuli presented to the animals and of course to include proper control groups when designing experiments. Lastly, Fos induction in neurons apparently requires strong activation, especially in anesthetized animals where neuronal bursting is required.

**Intracerebral neocortical transplantation**

The first attempts to graft tissue into the mammalian central nervous system were made one hundred years ago when Thompson transplanted neocortical tissue
between adult cats and dogs. These and other early studies generally showed poor transplant survival, and evidence for long-term neuronal survival was not convincingly demonstrated. In 1907, Del Conte who was the first investigator to transplant embryonic tissue to the CNS, concluded that the brain was an unfavorable site for implantation of most types of embryonic tissue. The first clear-cut evidence for successful grafting of neuronal tissue to the brain was reported by Tello (1911) who implanted predegenerated portions of sciatic nerve into cortex of dogs and by Dunn who grafted neocortical tissue from 10 day old rats into cortical cavities of littermates. The first successful report on the use of fetal nervous tissue as donor tissue appeared in 1940, when Le Gros Clark described good survival and differentiation of embryonic cortex grafted to the lateral ventricle of 6 week old rabbits. (See (51) for historical review).

The breakthrough for modern neural transplantation studies came in the early 1970's when three research groups independently reported successful and reliable survival of grafts to the brain (16, 52) and to the anterior chamber of the eye (163). These studies led to a more systematic characterization of the necessary conditions for obtaining reliable graft survival: (i) use of tissue taken from donors at embryonic or neonatal age when neurons are near their final cell division, and (ii) selection of transplant placement and procedure to optimize vascularization (217). Neuroanatomical connections between grafted tissue and the recipient mammalian CNS was first demonstrated in 1976 (20, 139), and was followed in 1979 by the first reports on functional effects of intracerebral transplants (18, 169).
Functional effects of intracerebral grafting

Transplanted tissue can influence the functional/behavioral capacity of host animals in several ways, as reviewed recently (15). These mechanisms include (i) nonspecific or negative consequences of the implantation surgery, (ii) trophic action on the host brain, e.g., via secretion of growth factors and/or the migration of glial cells into the host tissue, (iii) diffuse but constant release of neuroactive chemicals such as neurotransmitters and hormones, and (iv) anatomical integration with the host brain.

Scar formation, cyst formation and space occupying lesions are all examples of possible consequences of transplant surgery that do not allow for axonal growth and therefore may impede successful integration with the host brain.

With regard to trophic actions, experiments have demonstrated that transplants may provide trophic support for intrinsic host neurons that have lost their normal targets. For example, fetal transplants are reported to reduce lesion induced secondary neuronal atrophy, an effect that may be attributed by transplant production of target derived neurotrophic factors (102, 196, 204, 211).

The functional effect of transplant produced neuroactive chemicals is well demonstrated by studies involving the grafting of gonadotrophin releasing hormone (GnRH) rich tissue into GnRH deficient, hypogonadal mice (45). In this work, the grafts were found to innervate the median eminence and release the hormone into the portal vessels and thus improve GnRH related functions. In other work, chromaffin rich adrenal medulla grafts or fetal nigral grafts have been implanted into the denervated striatum in order to replace ablated dopamine inputs which normally arise from the substantia nigra. The functional integration of these grafts was demonstrated by recovery from abnormal circling behaviors (18, 220).
The implantation of retinal tissue after unilateral enucleation in newborn and in adult rats provides an excellent example concerning the establishment of transplant-host interconnections (124, 125). In this work, the functional reconstruction of visual pathways was demonstrated by the reestablished pupillary light reflex.

**Transplantation and lesion models**

Fetal neocortical tissue can be transplanted either as a single block of embryonic tissue or as a suspension of dissociated embryonic tissue. Neocortical transplantation has been performed using a variety of brain lesion models such as aspiration induced cavity lesions in newborn or adult hosts as well as excitotoxic- or ischemia- induced lesions in adult hosts. The reported enhanced transplant survival observed in grafts placed at one week after lesion placement is attributed to the production of injury induced trophic factors (142, 157). Similar results were observed when grafting into the thalamus (158), the hippocampus (229) and the striatum (131). Moreover, these latter studies reported an increased exchange of transplant-host connections when using the delayed grafting paradigm.

**Internal organization of neocortical grafts**

The organization of both block grafts and dissociated suspension grafts into clusters or whorls of cells generally makes them easily distinguished from the normal six layered structure of the unablated host cortex (41, 81, 153). Golgi staining studies of block transplants demonstrated the presence of both pyramidal and non-pyramidal cells that were comparable to cell types found in the normal intact neocortex (81). Immunocytochemical staining for ChAT, AChE, GABA, vasoactive intestinal peptide, neuropeptide Y, somatostatin and NADPH-diaphorase further showed that transplants
resembled normal intact neocortex (26, 69, 90, 141), and immunostaining for substance P, alpha-melanocyte or corticotrophin stimulating hormones, β endorphin or arginine-vasopressin showed the absence of labeled cells in the neocortical grafts as well as in intact neocortex (69). In contrast to these findings, other work found that the rate limiting enzyme in catecholamine synthesis, tyrosine hydroxylase, which normally is only transiently present during normal cortex development, had persisted for several months in cortical transplants (108, 167).

Intrinsic connections of fetal neocortical block transplants demonstrated patterns of interconnections that resembled normal cortex in that cells labeled from superficial injections of fluorescent retrograde transported tracers formed clusters and bands of both pyramidal cells and non pyramidal cells (84).

**Interconnections between neocortical transplants and host tissue**

Neocortical grafts implanted into the cortex of newborn recipient rats were found in several studies to receive extensive innervation from the host brain. Retrograde tracer injections into the transplants resulted in labeled host cells within the ipsilateral cortex, thalamus, basal forebrain, raphe nuclei, locus coeruleus and contralateral cortex (38, 40, 43, 44), all areas that normally innervate the somatosensory cortex. The efferent projections of transplants placed into newborn hosts have been examined using two different approaches. Retrograde fluorescent tracers were injected into different known target areas of normal cortical projections and anterograde tracers were injected into the transplants. Such studies showed that fetal neocortical tissue implanted into newborn hosts was able to send fibers to the ipsilateral cortex, striatum, thalamus, pons, pyramidal decussation and to the contralateral cortex and cervical spinal cord (39, 41, 43, 44, 70, 81, 209, 213).
Transplantation of fetal neocortical suspension or block grafts into adult recipients has been performed using the aspiration-, excitotoxin- or ischemia-induced neocortical lesion methods. Cell suspensions of fetal neocortical tissue transplanted into the ischemic or excitotoxic ablated cortex received afferent innervation from host neurons located in ipsilateral cerebral cortex, basal nucleus of Meynert, locus coeruleus, raphe nuclei, thalamus and the contralateral cerebral cortex (93, 113, 114, 203). Neurochemical markers for cholinergic (ChAT activity) and noradrenergic (NA) cortical afferents were found within neocortical suspension grafts (113). This latter study showed significantly lower levels of these neurochemical markers in transplants when compared to levels in normal cortex. Reports using blocks of fetal neocortical tissue grafted into cortical aspiration lesions showed a paucity of inputs from the ipsilateral thalamus (75, 84, 109, 210). The use of different transplantation methods as well as different lesion procedures in these studies makes it difficult to draw conclusions about possible differences between graft or lesion methods. Studies using retrograde and anterograde/retrograde tracing techniques on cortical grafts placed into the adult recipients failed to demonstrate significant efferent projections into the host CNS (91, 92, 113, 175, 195)

Functional effects of neocortical grafts

Electrophysiological techniques have been used to study both the intrinsic organization and functional connectivity of neuronal grafts. Transplantation of fetal neocortical tissue into aspiration-induced barrelfield cortical lesions of adult rats has revealed that the grafted neurons show electrophysiological properties similar to intact cortical neurons and that such grafts can respond to peripheral vibrissae stimulation [See (65) for review]. Electrophysiological studies of neocortical transplants implanted
into both newborn and adult hosts revealed similar results in demonstrating that such grafts can receive sensory input from the ipsilateral thalamus and contralateral forepaw (155, 156). Metabolic activation of transplant neocortical neurons by peripheral stimulation provided further evidence of anatomical and functional integration (94, 134).

Neocortical grafts also have been shown to ameliorate learning deficits resulting from cortical damage. For example, they facilitated the recovery of complex spatial and visual learning in adult rats with lesions of the neocortex (214). The removal of transplanted frontal cortex of adult rats that sustained lesion/transplantation as neonates resulted in a deterioration in motor performance which indicated that the transplants were functional within the host brain (184). A lack of transplant effect on cortical lesion induced impairments has also been described, i.e., fetal neocortical grafts placed into aspiration lesions of neonatal rats were not able to reverse deficits in beam walking (226). Importantly, these grafts did not exacerbate the impairment although graft-induced behavioral impairment has been reported (67).

The majority of studies involving functional recovery mediated by neocortical transplants indicate that the formation of transplant-host connections are of major importance. In this regard, graft induced functional recovery of a motor task did not occur until nine weeks post transplantation (175), and this corresponded with the re-establishment of host-transplant interconnections. In contrast to these findings, other studies showed a rapid recovery from lesion induced behavioral deficits suggesting that the recovery was mediated by transplant produced neurotrophic substances rather than by the formation of host transplant anatomical interconnections (67, 122, 214).
Trophic effects of neocortical transplants

Neocortical lesions in newborn rats result in the atrophy of cortical projecting thalamic nuclei. However, this atrophy can be significantly attenuated by transplanting fetal neocortex into cortical lesions (102, 196, 211). While this protection may be permanent (196, 211) or temporary (102), these studies suggest that the target derived trophic interaction necessary for the survival of developing neurons can be reestablished by graft replacement of lost target neurons. In similar work, cortical suspension transplants protected nucleus basalis cholinergic neurons from atrophy induced by cortical lesions made by excitotoxin injections (204).
CHAPTER III

CONNECTIVITY OF FETAL NEOCORTICAL BLOCK TRANSPLANTS
PLACED IN EXCITOTOXIN-INDUCED NEOCORTICAL LESIONS OF
ADULT RATS

Summary

Fetal neocortical block grafts placed into newborn recipients are able to exchange axonal connections with the host central nervous system as shown in several previous experiments. The present study examined the connectivity of fetal neocortical block transplants placed into the excitotoxically ablated cortex of adult rats. Young adult rats received injections of the excitotoxic amino acid NMDA into the sensorimotor cortex area one week prior to receiving a fetal (E14-15) neocortical transplant. At 3-6 months post transplantation, afferent and efferent connections of these grafts were examined by injecting the transplants with the fluorescent retrograde tracers FB and DY or with the anterograde tracer PHA-L.

Retrogradely labeled neurons were observed within several host brain regions including the ipsilateral neocortex, several thalamic nuclei, subcortical areas such as claustrum and lateral hypothalamus, nucleus basalis, dorsal raphe nuclei and locus coeruleus. Fibers labeled with PHA-L were found extending throughout the transplants but with rare exceptions fibers were not observed within the host brain.
The experiments showed that neocortical block grafts placed into the excitotoxically ablated neocortex receive afferent input from areas in the host brain that normally innervate the sensori-motor cortex. The extensive PHA-L positive axonal labeling found within the grafts demonstrated the ability of the grafted neurons to establish extensive intrinsic graft connections. Their failure to project out of the grafts suggests that the mature host brain does not provide a permissive environment for neurite extension.

Introduction

Intracerebral grafting is an excellent experimental tool for studying the neuronal development and plasticity of the cerebral cortex (38, 39, 40, 41, 90, 91, 93, 113, 114, 161, 185, 204, 210, 211). These studies showed that tissue blocks grafted into the cerebral cortex of newborn rats as well as cell suspensions grafted into the cerebral cortex of adult rats received afferents from host brain areas that normally project to the cerebral cortex. These inputs arose from several areas including the ipsilateral and contralateral cerebral cortex, basal nucleus of Meynert, locus coeruleus, raphe nuclei and thalamus (38, 41, 43, 44, 93, 113, 114, 203). In contrast, related work showed fewer connections when small blocks of neural tissue were transplanted into lesion cavities made in the cerebral cortex of adult recipients (75, 84, 109, 210). However, the placement of grafts into excitotoxin induced lesions led to an apparently better host regenerative response than found with other commonly used transplantation paradigms involving the placement of the grafts into aspiration lesion cavities or transplantation without prior ablation (131, 170, 229).
The reconstruction of damaged neural circuitry is considered a particularly important aspect concerning the ability of neuronal grafts to influence the host nervous system. Considering that block neocortical transplants are reported to demonstrate several features that are reminiscent of normal cortical structure and intrinsic connectivity (39, 44, 81, 84), this method of grafting would seem to enable transplants to become more functionally integrated into the host nervous system. Accordingly, the present experiment was designed to examine the host-transplant connectivity of block grafts placed into excitotoxic cortical lesions made in adult rats.

In addition to providing anatomical information concerning the integration of transplants with the host nervous system, examination of the afferent and efferent connections of neocortical transplants also provides information of the regenerative or growth properties of specific neuronal populations under the given conditions. Our findings of host projections into the transplants demonstrate that the immature grafts provide a suitable environment for the growth of mature axons. In contrast, the observed absence of transplant axonal extension into the host nervous system indicates that the adult central nervous system is non-permissive for axonal growth, an effect which has been attributed to insufficient levels of extracellular or cellular substrates for growth and growth factors as well as to the presence of neurite growth inhibiting factors (121, 191, 216).
Materials and methods

Excitotoxic NMDA-lesions

Eight-week old male Long-Evans black-hooded rats (n=20) were anesthetized with sodium pentobarbital (42 mg/kg) and placed in a stereotaxic apparatus. The skull was opened at the level of the coronal suture by use of a trephine, and five 0.4 µl injections of an NMDA solution (total of 500 nmol) were stereotaxically placed into the neocortex at a depth of 1.5 mm. The coordinates for the five injections were made in relation to bregma: 1) 2.5 mm lateral and 1.5 mm anterior, 2) 3.5 mm lateral and 2.5 mm anterior, 3) 3.5 mm lateral and 0.5 mm anterior, 4) 1.5 mm lateral and 2.5 mm anterior and 5) 1.5 mm lateral and 0.5 mm anterior. Each injection were made over a 5 min time interval followed by an additional 5 min diffusion pause before the cannula was withdrawn. After covering the injection site with a bone flap, the incision was closed with wound clips. The animals received neocortical grafts one week later.

Transplantation

Donor tissue was obtained from Long-Evans black-hooded fetuses removed at 14-15 days of gestation from sodium pentobarbital anesthetized (42 mg/kg) pregnant dams. The skull and meninges overlying the telencephalic part of the fetal brain were removed, and a 2-3 mm² block of presumptive cortical tissue was dissected out and placed in sterile Ringer's solution. While the transplants were being prepared, the adult recipients that previously sustained NMDA-induced lesions were anesthetized (sodium pentobarbital, 42 mg/kg) and the wound clips and bone flap from previous surgery were removed. The donor tissue was now aspirated into a glass cannula mounted on a
Hamilton syringe and then slowly injected into the lesion area. Again the bone flap was lightly placed on top of the fetal donor tissue / lesion area and the skin sutured.

**Retrograde and anterograde tracers**

At 3-6 months post-transplantation, the animals were again anesthetized with sodium pentobarbital and placed in a stereotaxic instrument. The bone flap was carefully removed and the area underneath was examined for the presence of a graft. Transplants were usually identified by a slightly lighter color than the host brain, by superficial blood vessels surrounding the graft and by a slight protrusion in relation to the surrounding host tissue. In ten animals, one rostral injection of DY (0.2 µl of a 2% solution) and one caudal injection of FB (0.3 µl of a 2% solution) were placed within each graft at a depth of 1.5 mm using a Hamilton syringe. In the remaining ten animals, the anterograde tracer PHA-L was iontophoretically injected into the transplants. A glass micropipette containing a 2.5% PHA-L solution in 0.02 M sodium phosphate buffered saline (pH 7.4) was lowered 1.5 mm into the center of the transplants, and a positive current of 5.5 mA pulsed in 7 sec. cycles for 20 minutes was driven through the micropipette by means of a Midgard ionotophoretic instrument in order to expel the PHA-L.

**Histological procedure**

At eight days after injection of retrograde fluorescent tracers DY and FB and at two weeks after injection of anterograde tracer, the animals received an overdose of pentobarbital (84 mg/kg) and were perfused transcardially with a brief rinse of a 8% sucrose, 4% glucose, 8% saline solution followed by 500 ml of fixative (4% formaldehyde, 3.5% sucrose and 2.5% MgSO4). The brains were removed from the
skull immediately, immersed overnight in 30% sucrose, frozen on dry CO2 and stored at -70°C until sectioned frontally at 30 µm in 3 parallel series.

In the retrograde tracing study, one histological series was left unstained and coverslipped with Entellan, whereas the remaining two adjacent series were stained with toluidine blue or for AChE (104). The unstained sections were examined for fluorescent labeling using a Leitz fluorescent microscope. The distribution of fluorescent cells observed in representative sections throughout the brain was plotted using a X-Y digitizing system (Minnesota Datametrics). The localization of labeled neurons was then determined from comparison of the X-Y plot, the AChE-stained sections and a stereotaxic atlas (168).

The brains injected with the anterograde tracer PHA-L were stained with toluidine blue or for AChE. One free floating series was reacted immunohistochemically for PHA-L according to the protocol from Vector laboratories. The reacted brain sections were mounted on gelatine covered slides, coverslipped and analyzed by means of both brightfield and darkfield light-microscopy.

Schematic representation of the experimental design
Results

Transplant survival and histology

Transplants were histologically identified in 19 of 20 animals. They were usually confined to the host neocortex extending ventrally to the corpus callosum. The transplants generally appeared to be well incorporated into the host brain with no obvious barrier at the graft-host interface (Fig 1). Remnants of the lesion cavity however were occasionally observed at the interface. The normal cytoarchitectural lamination were in all cases replaced by characteristic patterns of whorls and bands of cells (39, 41) The histological examination of dye injected grafts showed that in nine animals at least one of the two dyes was confined to the transplant (Fig 2). The injection sites were usually located halfway between the surface and the corpus callosum. Six of the PHA-L injected rats had successful injections confined to the grafts with no indication of passive spread to the host (Fig 3).

AChE histochemistry revealed that AChE-positive fibers were present within all the grafts examined (Fig 4). In transplants with good structural integrity with the host brain, such fibers were seen to reach all parts of the grafts, but in general the fiber density was lower than normal and highest near the graft-host interface.

Retrograde fluorescent labeling

The observed distribution of retrogradely labeled neurons within the host brain is demonstrated in Figure 5. Retrograde neuronal labeling was found in several ipsilateral host thalamic nuclei in six out of nine animals with injections confined to the transplants. The labeling was most consistent in the venterolateral (Fig 6) and posterior thalamic nuclei. Less consistent but prominent labeling was observed in the
venteromedial thalamic nucleus, while labeling in medial dorsal, reuniens, rhomboid and central lateral and central medial thalamic nuclei were observed in a few animals only. No apparent topographic distribution of thalamic labeling was recognized with respect to the rostral-caudal distribution of the injections within the grafts.

In two cases, labeling of ipsilateral neocortical cells was observed caudal to the graft within the somatosensory regions SI and SII. These retrogradely labeled neurons were mainly located within the deep and superficial layers of the cerebral cortex. No labeling was observed in the contralateral cortex.

A few labeled neurons were found ipsilaterally in several other subcortical areas including the claustrum and lateral hypothalamus. The nucleus basalis, dorsal raphe nuclei and locus coeruleus, areas giving rise to global cholinergic, serotonergic and noradrenergic axons, showed varying degrees of labeling with the most intense and consistent labeling being found in nucleus basalis.

_Anterograde PHA-L labeling_

PHA-L immunoreactive fibers were found extending throughout the transplants (Fig 7). A few fibers crossed the graft-host interface, but they were only traced to host areas immediately adjacent to the transplant. Careful analysis of sections throughout the host cortex, thalamus, striatum, red nucleus, internal capsule and pontine gray revealed no PHA-L positive fibers.


Discussion

The present study showed that fetal neocortical tissue, transplanted in block form, can survive and establish connections with the host brain when grafted into the excitotoxically lesioned neocortex of adult rats. In this context, our results using fluorescent tracers demonstrated that host projections to the transplants originated from sources that normally innervate sensori-motor cortex. Fluorescent labeled thalamic neurons were found in six out of nine cases analyzed, but variations in both distribution and density were seen. Typically, frequent and relatively dense labeling was found within the host venterolateral, venteromedial and posterior thalamic nuclei corresponding to normal thalamic projections to the fronto-parietal cortices, where the transplants were placed. These findings also corresponded to previous work demonstrating host projections to transplants placed into aspiration cavities made in the cerebral cortex of newborn hosts. Also similar to previous work with newborn recipients, less frequent and more sparse labeling was found in thalamic nuclei such as the medial dorsal nucleus, central lateral nucleus, central medial nucleus and the rhomboid nucleus, which also normally project to fronto-parietal cortices.

Host cortical labeling was found primarily in the deeper layers of the ipsilateral primary and secondary somatosensory cortex and in the claustrum in accordance with the presence of normal afferent connections from these areas to the rat sensori-motor cortex. In contrast to previous studies, we found no evidence of projections from the contralateral cortex.

In addition to innervation from areas characterized by having rather precise projections, e.g., the thalamus and the cortex, the transplants also received inputs from areas with more global projections, like the basal nucleus of Meynert, locus coeruleus.
and midline raphe. The topographic distribution of retrogradely labeled cells within the nucleus basalis and the substantia innominata is in accordance with several studies on cholinergic fronto-parietal cortical input from the basal forebrain nuclei in normal animals (149, 186, 237). Unlike previous work using newborn hosts (38), retrograde labeling was not found within the horizontal limb of the diagonal band. The consistent retrograde labeling found in the basal nucleus/substantia innominata of all animals receiving retrograde fluorescent tracers corresponds to the presence of AChE-positive fibers within all the grafts. The distribution of fluorescent labeled neurons in the locus coeruleus and the dorsal midline raphe concurs with labeling studies performed on normal animals (76, 136) and on animals receiving neocortical transplants at birth (38).

Placement of fluorescent dye injections into the center of the grafts was attempted in order to reduce tracer spread to the host brain. This however only retrogradely labeled host cells projecting to the central parts of the grafts, thereby excluding cells which project only to the periphery of the transplants. Earlier reports have shown that host callosal input to neocortical grafts placed in newborn hosts are limited to the ventral part of the graft (44) and that thalamic and cortical afferents to striatal grafts mainly innervate the peripheral part of the grafts (176, 240). Our results correspond to these observations in that animals with superficial tracer injections generally exhibited the least amount of host labeling.

Thalamic innervation of neocortical grafts placed into the adult neocortex has previously been reported to occur only when the host animals also sustained lesions of the basal forebrain cholinergic projection system or in case of cell suspension grafts (68, 109). The present study demonstrated that block transplants placed into excitotoxic lesions also can receive host thalamic inputs, thereby extending previous work demonstrating host thalamic inputs to suspension grafts placed into the excitotoxically...
ablated cortex of adult rats (113). In related work, the grafting of fetal neocortical block transplants into adult hosts with aspiration lesions, that axotomized cortical afferents, failed to demonstrate the regeneration of 'thalamo-transplant' connections (210). In contrast, the observed innervation of block grafts placed into excitotoxic lesions may be attributed to the remaining structural network of afferent fibers that survive after excitotoxin-induced lesions (46, 74). It is alternatively possible that the observed thalamic labeling is due to the sprouting of fibers innervating the adjacent non ablated cortex. Related studies transplanting immature tissue into excitotoxin induced lesions of the hippocampus, thalamus and striatum have reported similar results regarding point to point as well as global system fiber innervation (131, 170, 229).

Neocortical transplants placed in newborn hosts have in several recent studies been shown to project to several areas of the host CNS such as the ipsi- and contralateral cortex, striatum, thalamus as well as the pontine gray, the caudal medulla and spinal cord (39, 41, 43, 44, 81, 209, 213). In the present study using adult recipients, transplant injections with PHA-L led to extensive axonal labeling within the grafts but only sparse labeling within host brain areas immediately adjacent to the transplants. Previous studies, using retrograde and retrograde/anterograde tracing techniques, also failed to demonstrate significant projections from neocortical grafts placed into adult recipients (91, 113).

The abrupt termination of transplant axons at the graft-host interface, as observed in our animals, suggests that barriers or factors within the host adult CNS may have inhibited transplant axonal outgrowth. Phenomena such as the presence of physical glial scar barriers (see (178) Reier and Houle, 1987 for review), inhibitors associated with myelin producing oligodendrocytes (191) or astrocytes (146) and lack of trophic factors (201) have all been suggested to explain how the adult nervous
system inhibits neurite outgrowth. The apparent inability of transplant axons to penetrate the host subcortical white matter at the graft-host interface suggests that dense bundles of host myelinated axons may have presented a physical barrier to axonal outgrowth. However, recent work demonstrating neurite growth inhibitory activity by two membrane proteins localized in oligodendrocytes and CNS myelin (32, 34, 192) indicates the presence of a biochemical inhibition to transplant axonal outgrowth rather than merely a passive physical barrier imposed by myelin. The presence of neurite growth inhibitory factors within the host CNS would also explain the absence of graft axons growing laterally into the host brain where no physical subcortical white matter barrier is present. The observed regeneration of severed axons in the presence of an antibody directed against these neurite growth inhibitory proteins further demonstrated the presence of biochemical inhibitory factors within the adult CNS (30, 187).

Extensive CNS tissue degeneration typically result in the formation of astroglial-mesenchymal scars that traditionally have been thought to constitute a major obstacle to axonal regeneration (178). In vitro studies of axonal growth on glial scar associated cellular and extracellular components have suggested that the growth inhibiting character of glial scars is correlated with the expression of certain extracellular matrix molecules (146). Previous studies of neocortical transplantation have showed variable degrees of gliosis at the graft-host border ranging from complete absence to complete encapsulation of the grafts (81, 130, 153). Indirect evidence that the adult CNS represents a non-permissive environment for axonal growth was provided by studies demonstrating extensive axonal elongation by CNS neurons allowed to grow into grafted peripheral nerves (11, 54). Presumably, this paradigm enabled the growing axons to avoid the inhibitory effects exerted by the CNS. Similarly, cortical transplant neurons might also be able to extend axons into the host
CNS if neurite inhibitory factors could be avoided or neutralized. That fetal neocortical grafts can send axons into the newborn's recipient host brain (209) demonstrates that such grafts indeed have the capacity for axonal growth in the proper environment.
FIGURE 1

Neocortical transplant with characteristic whorls and bands of cells. Toluidine blue.
Scale bar = 500 µm.

FIGURE 2

Fast Blue injection site within a transplant. Dense labeling of adjacent transplant cells helps demarcate the graft-host interface. Scale bar = 500 µm.
FIGURE 3
PHA-L injection site and transplant-host interface (dashed line). No labeled axons are seen to cross the interface. Scale bar = 150 µm.

FIGURE 4
AChE positive fibers on both sides of transplant-host interface, but with less density on the transplant side (upper left) as compared to the adjacent host tissue (lower right). Scale bar = 50 µm.
FIGURE 5
Camera Lucida drawings illustrating the distribution of retrogradely labeled neurons within the host brain. Labeling within the transplant and more caudal brain stem sections is not shown. FR, frontal cortex; SI, primary somatosensory cortex; SII, secondary somatosensory cortex; CPU, caudate putamen; VHC, ventral hippocampal commissure; LV, lateral ventricle; VL, ventrolateral thalamic nucleus; IC, internal capsule; PO, posterior thalamic nuclei; LH, lateral hypothalamus; 3V, third ventricle; VM, ventromedial thalamic nucleus; C, crus cerebri; SN, substantia nigra.
FIGURE 6
Fluorescent retrogradely labeled cells within the host ventrolateral thalamic nucleus after Fast Blue injection into transplant. Scale bar = 100 µm.

FIGURE 7
Camera lucida drawing showing the PHA-L injection site and PHA-L labeled fibers extending throughout the transplant. Very few labeled fibers were observed within the host tissue.
CHAPTER IV

BIOCHEMICAL AND ANATOMICAL ANALYSIS OF CHOLINERGIC, NORADRENERGIC AND SEROTONERGIC INNERVATION OF FETAL NEOCORTICAL TRANSPLANTS PLACED IN EXCITOTOXIN-INDUCED NEOCORTICAL LESIONS OF ADULT RATS

Summary

Fetal neocortical block transplants were implanted into the excitotoxically ablated sensorimotor cortex of adult rats in order to examine the density of innervation and distribution of presumptive host derived afferent fibers within these transplants. Cholinergic fiber innervation was examined at three months post grafting by measuring acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) enzyme activities within the grafts and within the corresponding host cortex by radiochemical enzyme assays as well as by AChE histochemistry for the visualization of AChE positive fibers. Noradrenergic and serotonergic inputs were examined by high performance liquid chromatography (HPLC) measurements of noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT) concentrations as well as by tyrosine hydroxylase (TH) and 5-HT immunocytochemistry for the visualization of monoaminergic fiber distribution. Our
results demonstrated that the grafts contained significantly lower levels of neurotransmitter markers when compared to normal unablated cortex. The anatomical analysis showed an unequal fiber distribution within the transplants. Areas adjacent to the host tissue revealed a relatively dense fiber innervation when compared to the density observed within the more central parts of the transplants, and the anatomical data therefore supported the biochemical data in suggesting an overall lower cholinergic and monoaminergic innervation of fetal neocortical transplants placed into the lesioned adult cortex when compared to normal cortex.

Introduction

Numerous studies have examined the potential of using intracerebral neuronal transplants in the reconstruction of damaged neural circuitry. In this regard, fetal neocortical block grafts demonstrate features that are reminiscent of both normal cortical structure and intrinsic connectivity (39, 44, 81, 84). Such transplants are therefore considered good candidates for restoring functional connectivity in the damaged brain, a possibility which also is supported by studies of host-transplant connectivity (38, 40, 43, 44, 189, 210). Using retrograde neuronal tracing methods, these studies demonstrated that neocortical block transplants placed into newborn or adult recipient rats receive inputs from several areas of the host brain. While these afferents qualitatively resembled normal connectivity, quantitative analysis of transplant afferents was considered imprecise primarily because of difficulties in controlling placement of injections within the transplants. Quantification based on retrograde
labeling methods is further complicated by studies indicating that certain axonal projections are not distributed uniformly within cortical transplants (44, 81).

The present study was initiated to provide a quantitative measure of host derived afferents within fetal neocortical block transplants placed into the excitotoxically ablated cortex of adult rats. Transplant levels of ChAT and AChE activities were measured by radiochemical enzyme assays and used as an index of cholinergic innervation. Noradrenaline and serotonin transplant levels, as determined by HPLC, provided indices of noradrenergic and serotonergic innervation. Histochemical detection of AChE positive fibers as well as immunohistochemical detection of catecholaminergic and serotonergic fibers within the transplants provided morphological data regarding innervation patterns.

Our histological results confirmed previous anatomical tracing studies by showing transplant innervation by catecholaminergic, serotonergic and AChE positive fibers. The presence and density of these 'global' projections, which were consistently less than those found in normal host cortex, corresponded to biochemical measures of neurotransmitter levels.

Materials and methods

Eight-week old male, Long-Evans, black-hooded rats (n=24) were anesthetized with sodium pentobarbital (42 mg/kg) and placed in a stereotaxic apparatus. The skull was carefully opened at the level of the right forelimb sensorimotor cortex using a trephine. As described previously in more detail (189), five injections (0.4 µl) of an
NMDA solution (total of 500 nmol) were injected into the right forelimb motor representation area of the neocortex at a depth of 1.5 mm. After covering the injection/lesion site with a bone flap, the incision was closed with wound clips. The animals received transplants seven days later.

Donor tissue was obtained from Long-Evans, black-hooded fetuses removed at 14-15 days of gestation from sodium pentobarbital anesthetized (42 mg/kg) pregnant dams. During the preparation of transplant donor tissue, the animals that received the excitotoxic lesions one week earlier were anesthetized with sodium pentobarbital and their wound clips and bone flaps removed. The skull and meninges overlying the telencephalic part of the fetal donor brain were removed, and a 2-3 mm² block of presumptive cortical tissue was dissected into a sterile Ringer's solution. The donor tissue was gently aspirated into a glass cannula mounted on a Hamilton syringe and subsequently injected as a single block into the cortical lesion area. The bone flap was again placed lightly on top of the fetal donor tissue/lesion area and the skin sutured. At the conclusion of transplant surgery, the pregnant dams were killed by an overdose of sodium pentobarbital (84 mg/kg).

Tissue sample preparation

At three months post grafting, the animals used for biochemical analyses were decapitated and their brains quickly removed and placed on an ice cooled copper plate. Only animals with easily identified transplants were used for biochemical analyses (n=16). Two coronal slices of the brain were made at levels immediately rostral and caudal to the graft. The transplant was then dissected under a surgical microscope, placed in a micro centrifuge tube, and immediately frozen on dry ice. Control tissue,
i.e., frontal cortical tissue in a position exactly contralateral to the transplant, was also
dissected. The tissue samples were stored at $-70^\circ$ C.

**Radiochemical enzyme assay for ChAT and AChE**

Choline acetyltransferase enzyme activity was determined according to the
method of Fonnum (83). Briefly, tissue was homogenized in a 75 mM sodium
phosphate buffer at pH 7.4. The homogenate was then incubated in a buffer solution
containing $[^3H]$-labeled acetyl Co-A, one of the substrates for ChAT. By adding a 3-
heptanone/TPB scintillation solution mixture to the incubation medium, synthesized
acetylcholine (ACh) can be efficiently determined in the hydrophobic phase in contrast
to the labeled Acetyl Co-A. The amount of labeled ACh was determined with a
scintillation spectrometer. ChAT activity was expressed as nanomol $[^3H]$ACh/mg
protein/hour.

AChE activity was estimated by a radiochemical enzyme assay using $[^3H]$AChI
in the buffer solution. Because the substrate acetate/$[^3H]$acetate is hydrophilic, the
samples were frozen to discard the top organic layer containing the unconverted
$[^3H]$AChI. Scintillation counting on the aqueous layer was then performed. AChE
activity was expressed as nanomol $[^3H]$acetate/mg protein/hr. Protein content was
determined in triplicates according to standard techniques (137).

**High Performance Liquid Chromatography**

Transplant and control cortex samples were individually sonicated (KONTES
Micro ultrasonic cell disrupter) in 500 µl 0.1M phosphate buffer pH 7.0. They were
then centrifuged for 3.5 min. at 15,000 g, and the supernatant filtered through a 0.45
micron Acro LC3A filter. 5-HT was detected using an HPLC system, which consisted
of an Altex 110A metering pump, a Rheodyne 7125 inject port with 50 µl sample loop and a C18 reverse phase column (10 cm x 4.6 cm, 3 mm particle size, Rainin Instr., Woburn, MA). Samples were detected by electrochemical detection using a Bioanalytical Systems LC-4B electrochemical controller with a LC-17 transducer consisting of a glassy carbon working electrode and Ag/AgCl reference electrode. The potential of the working electrode was set at +0.7V relative to the reference electrode. NA levels were detected using a Rainin rabbit HP/HPX drive module pump and a Rheodyne 7125 injection port with a 100 µl sample loop were used. The column and the transducer were the same as for 5-HT detection. The electrochemical controller was a LC-4A Bioanalytical Systems. The potential of the working electrode was +0.8V relative to the reference electrode.

The mobile phase for detection of 5-HT consisted of 0.1 M monobasic anhydrous sodium phosphate containing 8% (v/v) HPLC grade methanol (Burdick and Jackson Labs, Muskegon, MI). For detection of NA the mobil phase consisted of 0.1 M sodium phosphate buffer containing 3 mM 1-octane sulfonic acid (Sigma Chemicals) and 4% (v/v) HPLC grade methanol. The buffers were filtered through 0.45 µm pores (Nylon-66 filters, Rainin), degassed under vacuum and pumped through the column at a flow rate of 1.0 ml/min. Identification of respective monoamines was based on retention times of authentic standards (Sigma Chemicals). Monoamine concentrations within the samples tested were calculated from comparisons of sample peak heights to peak heights of the authentic standards (1ng/ml). NA and 5-HT levels were expressed as ng/mg protein. Protein content was determined in duplicates (137).
Statistical analysis

Transplant values for each transmitter marker were compared to control tissue values using the paired Student t-test.

Histology

At three months post grafting, six animals were sacrificed with an overdose of sodium pentobarbital (84 mg/kg) and perfused transcardially with a 4% paraformaldehyde solution. The brains were immediately removed from the skull and allowed to sink over night in a 30% sucrose solution before they were frozen. The brains were sectioned frontally in four series at 30 µm. One series was stained with toluidine blue for routine histological examination. The remaining series were reacted histochemically for AChE (104) or immunocytochemically for TH (the rate limiting enzyme in catecholamine synthesis) or 5-HT.

For immunocytochemical visualization of TH and 5-HT, the sections were reacted free-floating. Initially the sections were incubated in 10% normal goat serum (NGS) and 0.3% Triton X-100 in phosphate buffered saline (PBS) for 30 min and then for 48 hours in the primary antiserum directed against TH (Pelfreeze, diluted to 1:100) or 5-HT (Incastar, diluted to 1:4000) in 1% NGS, 1% fetal calf serum in PBS. One-hour incubations in a secondary biotinylated anti rabbit antibody (1:200) and streptavidin (1:250) with 3 X 10 min rinses (PBS with 0.3% Triton X-100) in between was used for intensification and detection of the primary antibodies. Following incubation in the secondary antibody, the sections were blocked (30 min) for endogenous peroxidase in a 100% methanol/0.1% H₂O₂ solution. Finally, the sections were reacted with 0.05% 3,3'-diaminobenzidine and 0.01% H₂O₂ for 6 min, rinsed in tris buffer and mounted onto gelatin-coated slides before they were osmium intensified.
The histological analysis made no attempt on formal quantification, and the descriptions are therefore based on direct observations of the histological material.

*Schematic representation of the experimental design*

![Diagram showing experimental design]

**Results**

At sacrifice 22 of 24 recipients showed obvious transplant survival and growth. All the transplants that were analyzed histologically \( n=6 \) were confined within the excitotoxically ablated cortex and appeared to be well integrated into the host brain with no remnants of damaged tissue or a lesion cavity at the transplant-host interface (Fig 1). Histologically, the grafts characteristically showed whorls and bands of cells instead of the laminar organization of normal neocortex. The cell density appeared comparable to the unablated sensorimotor cortex of the opposite hemisphere.

Tissue samples from seven animals were used for the radiochemical enzyme assays and tissue from nine animals was used for HPLC analysis. The transplants
showed statistically significantly (p<0.01) lower levels of ChAT and AChE activities and also of NA and 5-HT content as compared to unablated control tissue (Fig 2).

AChE histochemistry revealed positive fibers located throughout the extent of all the grafts analyzed histologically. The fibers were generally observed to cross the graft-host interface without any apparent hindrance. In one transplant the AChE fiber density approached that of the normal unablated cortex, but in the other grafts the density appeared near normal only in the ventral regions of the grafts and near the graft-host interface (Fig 3). A few AChE positive cell bodies were observed within the transplants, and a marked increase of AChE positive cells were observed in the host tissue immediately adjacent to the graft border. One transplant also revealed a marked increase in AChE positive cell bodies in the tissue adjacent to the host parenchyma (Fig 4). Adjacent sections immunostained for the acetylcholine synthesizing enzyme ChAT revealed no positive cell bodies in the above described areas. The large round/oval AChE positive cells near the graft-host interface had few neurites and were clearly distinct from that of the small fusiforme, bipolar, ChAT positive cortical neurons found in unablated cortex. Morphologically, the described AChE positive cells were distinctly different from the large polymorphic, multipolar, ChAT positive basal forebrain neurons. Furthermore, the size of these AChE positive neurons was much larger than that of similar stained neurons located within the contralateral unablated neocortex.

TH immunoreactive fibers were seen crossing the graft-host interface, and a few fibers appeared to enter from the pial surface. These fibers were mostly confined to the areas adjacent to the graft-host interface although some were also present in the center and most dorsal parts of the grafts (Fig 5). The overall density of TH positive fibers within the transplants was clearly subnormal in all cases. A few TH positive cell bodies were observed close to the graft-host border in two transplants.
5-HT immunocytochemistry showed positive fibers predominantly located close to the graft-host border. Reactive fibers however were also observed within the center of the transplants. No apparent orientation of the fibers was observed (Fig 6). As for TH positive fibers, the total density of 5-HT positive fibers was clearly less than in normal sensorimotor cortex.

Discussion

The results of this experiment demonstrate that cerebral neocortical transplants placed into the excitotoxically ablated neocortex of adult rats contain the neurotransmitters ACh, NA and 5-HT, although at lower levels than in the opposite cortical hemisphere, as determined using biochemical methods. Histochemical and immunocytochemical data supported these findings and further demonstrated an apparently diffuse but unequal fiber distribution within the transplants. These findings combined with previous anatomical tracing analysis [55] demonstrate the innervation of cortical block transplants by cholinergic, noradrenergic and serotonergic inputs, and other studies suggest that such global fiber systems reinnervate fetal neuronal transplant in a less constrained manner than point to point fiber systems (212, 229, 246). Moreover, the morphological findings of the present study support similarities between global fiber systems with regard to graft innervation in that comparable innervation patterns were noted for AChE positive, catecholaminergic and serotonergic fibers.

In agreement with previous work (39, 41, 189, 210), transplants were characterized by whorls or clusters of cells interposed by bands of white matter rather
than the laminar patterns found in normal cortex. Routine Nissl staining further revealed that transplant cell density was comparable to the density of cells within normal cortical tissue. Therefore, measurements of neurotransmitter levels based on protein content were probably not complicated by histological differences.

The presence of AChE positive as well as TH- and 5-HT immunoreactive fibers crossing the graft-host interface provided evidence of transplant integration with the host brain. This integration was demonstrated previously by retrograde labeling of neurons in the host basal forebrain, locus coeruleus and the raphe nuclei after injections of fluorescent tracers into neocortical grafts placed into adult rats (93, 113, 189, 210). However, the occurrence of AChE-positive neurons within cortical grafts and also within the adjacent host cortex suggests that the AChE positive fibers found within the grafts may have arisen to some extent from intrinsic graft neurons and/or from AChE positive host cortex neurons and therefore not solely from the cholinergic basal forebrain neurons. This possibility is supported by related work involving the grafting of fetal neocortical cell suspensions into adult recipients, which indicated that 25% of the total AChE fiber density within the grafts was intrinsically derived (114). These previous findings correspond to observations that basal forebrain innervation normally constitutes approximately 67% of the ChAT staining in the parietal cortex, with cortical interneurons providing the remaining innervation (72). Similarly, the reported presence of tyrosine hydroxylase positive perikarya in neocortical transplants (108, 167) might provide an intrinsic source of transplant catecholaminergic innervation. However, these neurons are not believed to be catecholamine producing, and only an occasional TH positive cell body was observed within our grafts.

The large AChE positive cells observed in close proximity to the graft-host border most likely represents non-cholinergic cells. Immunocytochemical methods
revealed no ChAT-positive cells in these areas, and morphologically the AChE positive cells were clearly distinct from ChAT positive neocortical and basal forebrain neurons. The fusiforme and bipolar shaped ChAT positive neurons of the cerebral cortex were found previously to contain vasoactive intestinal polypeptide [21], and therefore immunocytochemical detection of this peptide may further help to rule out the cholinergic nature of these AChE positive cells which are apparently ChAT negative. Transient expression of the acetyl cholinesterase enzyme in neocortical pyramidal neurons, and in neurons of the ventrobasal complex of the thalamus as well as in thalamocortical fibers [29, 44, 53], suggests that AChE is a developmentally active molecule involved in cellular maturation, axonal guidance and/or synaptogenesis. That AChE expression at the graft-host interface may reflect events related to growth and development corresponding to the observed increase of growth factors found both within embryonic/neonatal transplant donor tissue [35, 47, 67] and in association with neuronal lesions [50, 63].

While noradrenergic projections to the cerebral cortex normally arise from the locus coeruleus (232), peripheral nervous system sympathetic fibers may enter the CNS via blood vessels as observed following cholinergic denervation of several brain regions, including the cerebral cortex (17, 48, 50). Accordingly such peripheral inputs may also innervate cortical transplants, but previous work demonstrated that transplant noradrenergic fibers arise at least partly from coerulear neurons (40, 93, 113, 189). Similarly, the observed serotonergic innervation of cortical grafts is believed to arise from the brainstem mesencephalic dorsal and median raphe nuclei as demonstrated in previous studies of cortical transplants (40, 93, 113, 189). No intrinsic source of serotonin has to our knowledge been reported within the cerebral cortex.
The ChAT and AChE enzyme activities found in our block transplants correspond to recent findings showing significantly lower than normal levels of ChAT enzyme activity in fetal suspension grafts (113). The below normal and non-uniform distribution of AChE positive fibers within the block grafts of the present study also supports previous work involving the placement of suspension grafts into the ischemic or excitotoxically ablated cortex (93, 113) as well as the implantation of block transplants into the cortical aspiration lesions (210). Similar to our findings for cholinergic markers, transplant NA and 5-HT levels were significantly lower than in control tissue. These biochemical findings correspond to our immunocytochemical observations of catecholamine and serotonin fiber distribution, which in turn correspond to anatomical analyses of noradrenergic and serotonergic fiber distribution within suspension grafts placed into the ischemic or kainic acid ablated cortex of adult rats (93, 113). Host-derived transplant innervation therefore does not seem to depend on the nature of the transplant, i.e. suspension versus block transplants.

The growth of cholinergic and monoaminergic fibers into fetal neocortical transplants placed into the ablated cortex of adult rats suggests the sprouting of axons from the adjacent nonablated cortex or perhaps the regrowth of axons that were previously deprived of their neuronal targets. Although studies have suggested that these global fiber systems are able to regrow following CNS lesions (19, 77, 173), the growth of mature CNS axons is generally considered to be quite limited, and this impaired regrowth has been attributed to the presence of glial associated growth inhibitory factors (5, 24, 146, 180, 192, 193, 202). However, that mature CNS axons do in fact maintain the capacity for extensive axonal growth has been shown in several experiments demonstrating the importance of suitable cellular and extracellular factors including trophic factors in supporting neurite outgrowth in the mature CNS.
Presumably, fetal neocortical transplants are likely to provide such a suitable cellular milieu, i.e., the lack of neurite growth inhibitory factors and the presence of growth promoting substances, as evidenced by studies of transplant afferents originating from several areas of the adult host brain (38, 40, 44, 63, 64, 81, 91, 93, 113, 189, 210). However, the observed subnormal innervation of adult recipient grafts suggests a less than optimal balance between the availability of growth promoting and growth inhibiting factors.

The significance of cholinergic and monoaminergic innervation of neuronal grafts is unknown. Numerous studies, however, suggest that these systems can influence brain development and plasticity (9, 22, 37, 56, 79, 89, 109, 120, 126, 133, 143) as well as trophic factor production (82, 194). In this context, the presence of cholinergic and monoaminergic fibers within transplants may be similarly important to the growth and functional integration of transplants with the host nervous system. Accordingly, the observed levels of these transmitters within transplants may be insufficient to promote complete transplant-host integration, and indeed studies using neuronal transplants to repair or replace damaged neural circuits generally report a transplant-host connectivity that is considerably below normal innervation densities. Although we are unable to explain why cholinergic and monoaminergic fibers were more dense along the transplant periphery, these findings correspond to reports indicating that other inputs are also more dense along the transplant margins [14, 24]. While host-derived neural projections in general may be unable to invade deeper into neuronal transplants because of unknown mechanisms, the possibility is raised that their ingrowth may be influenced by the corresponding location of cholinergic and monoaminergic fibers.
FIGURE 8
Neocortical transplant at three months after grafting. Instead of normal cortical lamination, transplanted cells are arranged into whorls or clusters with interposed bands of white matter. Scale bar = 300 µm
FIGURE 9
Transplant ChAT and AChE activities and NA and 5-HT levels are presented in comparison to control tissue values. Values are expressed as the mean + S.E.M. * = p < 0.01.
FIGURE 10

Bright field photomicrograph showing AChE positive fibers within neocortical transplants.

A. Transplant fiber density (right of dashed line) close to the graft-host border appears to be comparable in density to the adjacent host cortex.

B. AChE fiber density near the caudal margin of a transplant.

C. AChE fiber density within the center of the same transplant shown in panel B.

Scale bar = 50 µm.
FIGURE 11

A. AChE–positive fibers and cell bodies (arrows) within the host cortex contralateral to the transplant.
B. AChE reactivity within the transplant (left of dashed line) and adjacent host cortex. AChE–positive cells (arrows) at transplant borders were more numerous than observed in the contralateral host cortex. Same magnification as panel A.
C. Higher magnification of AChE–positive cell bodies within the host cortex adjacent to the transplant-host interface.
Scale bars: A = 200 µm, C = 50 µm.
FIGURE 12

Photomicrographs illustrating TH immunoreactivity within neocortical transplants.

A. Immunoreactive fibers are seen crossing the interface between host (left of dashed line) and transplant.

B. Numerous immunoreactive fibers observed near the caudal part of a transplant.

C. Density of immunoreactive fibers within the center of the same transplant shown in panel B.

D. TH-positive cell body (arrow) found within a graft. Although some fibers are observed within this transplant, several host fibers appear to terminate at the border.

A, B and C are shown in dark-field and correspond to the 50 µm scale bar shown in B. Scale bar in D = 50 µm.
FIGURE 13
Dark field photomicrographs showing 5-HT-immunoreactive fibers within transplants.
A. Immunoreactive fibers seen at the graft-host border. Host tissue is to the left of the dashed line.
B. Immunoreactive fibers seen within the caudal part of a transplant.
C. 5-HT positive fiber density within the center of the same transplant shown in panel B.
Scale bar = 50 µm; same enlargement in A, B and C.
CHAPTER V

EXPRESSION OF C-FOS mRNA AND C-FOS PROTEIN IN NEOCORTICAL TRANSPLANTS PLACED INTO EXCITOTOXIN-INDUCED LESIONS OF ADULT RATS.

Summary

Using anatomical and electrophysiological methods, previous studies from this laboratory demonstrated that fetal neocortical block transplants placed into the excitotoxically ablated cortex of adult rats can establish functional connections with the host central nervous system. In order to further examine functional integration at the level of genomic activation/molecular signaling systems, transplants were analyzed for the presence of c-fos mRNA and Fos protein labeled cells following a 20 min exposure of the animal to a novel open-field environment. This behavioral testing paradigm, which previously was shown to induce expression of c-fos mRNA in several brain regions including the sensorimotor cortex, was administered at three months after grafting. The transplants were found to express c-fos mRNA as well as c-fos protein following novelty exposure. These labeled cells were distributed primarily along the peripheral margin of the grafts. This study therefore suggests that at least some neurons within the transplants became integrated with host pathways at the level of molecular signal transduction.
Introduction

The survival and differentiation of fetal neocortical tissue transplanted into newborn or adult hosts and the establishment of normal patterns of connections between the grafts and the host central nervous system suggest that neuronal grafts can become functionally integrated with host brain circuitries (36, 37, 38, 39, 40, 41, 43, 44, 90, 91, 93, 113, 114, 189, 203, 209, 210, 211). In this regard, neocortical grafts have been reported to improve lesion-induced behavioral deficits (174, 214) and to rescue and form connections with neurons that are injured or threatened by lesions (196, 204, 211). The increased electrical and metabolic activity detected within grafts following peripheral stimulation (27, 28, 92, 134, 155, 156) provides further evidence of functional integration.

Other issues regarding the integration of neocortical transplants concern whether they are able to transduce extracellular signals at the molecular level in the same way as normal cortical neurons. Recent studies have described cellular mechanisms useful for addressing these questions by demonstrating that the immediate early gene c-fos and its protein product can be rapidly induced in the central nervous system following a variety of external stimuli (152). These findings indicate that the detection of c-fos mRNA or the protein product Fos in neurons can be used as an activity mapping technique for demonstrating pathways involved in transmitting information regarding specific stimuli (59, 152).

In further study of transplant functional integration at the level of molecular signal transduction, the present study examined the expression of c-fos mRNA as well as Fos/Fos related antigen (FOS) protein in animals that received fetal neocortical grafts placed into excitotoxin-induced cortical lesions. C-fos mRNA and FOS protein
expression, which was induced by placing the animals in a novel open-field environment according to previous work (101), was observed within several areas of the unablated brain as well as within the neocortical grafts.

**Materials and methods**

*Excitotoxic lesions*

The excitotoxic method presently used to induce cortical lesions has been described recently (189). Briefly, during sodium pentobarbital anesthesia (42 mg/kg), eight-week old Long-Evans black-hooded male rats (n=10) were secured in a stereotaxic instrument and received five injections of NMDA (500 nmol) into the right frontal forelimb sensorimotor area of the neocortex (154). The injections were made stereotaxically using a Hamilton micro-syringe attached to an electrode carrier. The coordinates for the five 1.5 mm deep injections were made in relation to bregma: (i) 2.5 mm lateral and 1.5 mm anterior, (ii) 3.5 mm lateral and 2.5 mm anterior, (iii) 3.5 mm lateral and 0.5 mm anterior, (iv) 1.5 mm lateral and 2.5 mm anterior and (v) 1.5 mm lateral and 0.5 mm anterior. Each injection was made over a five min time interval followed by an additional five min diffusion pause before the canula was withdrawn. The animals received neocortical transplants one week after the excitotoxin injections.

*Transplantation*

Donor tissue was obtained from Long-Evans black-hooded fetuses (embryonic day 14-15) that were surgically removed from sodium pentobarbital anesthetized (42 mg/kg) dams. The overlying skull and meninges were removed, and 2-3 mm² plates of
presumptive neocortical tissue were dissected from the telencephalic vesicle. The blocks of cortical tissue were placed into a sterile Ringer's solution, drawn into a glass canula attached to a 50 µl Hamilton syringe and transferred to the excitotoxically ablated area of the recipient neocortex. Transplants were kept in place by a bone flap that was created at the time of lesion placement. The donor dams were killed immediately after removal of the last fetus by intracardiac injection of an overdose of sodium pentobarbital (84 mg/kg).

Open-field environment

Three months after transplantation the rats were prepared for open-field testing. One week prior to this test, the animals were handled daily for five minutes in order to reduce handling stress at the time of testing. On the day of testing eight animals were placed, one at a time, in the center of a well lit open-field box (40' x 40' x 10'). The floor of the open-field box was divided by black lines into 25 squares. Four equidistant holes were located towards the center of the box floor. The animals were left within this open-field environment for 20 min and transferred back to the home cage where they were left for an additional 30 minutes before being sacrificed by decapitation. Two animals served as base-line controls and were not placed within the open-field box but were sacrificed immediately following removal from their home cage. Behavioral activities of the animals were videotaped during the 20 min. period in the open-field chamber and subsequently analyzed with regard to, (i) number of wall and center squares entered, (ii) number of hind limb rearings and (iii) number of nose pokes into one of the four holes.
Histology

After sacrifice the brains were removed, immediately frozen in \(-30^\circ C\) isopentane and placed in a \(-70^\circ C\) freezer until sectioned into six parallel series of 18 \(\mu m\) sections using a Leitz cryostat. One series was stained with toluidine blue for routine histological examination, a second series was used for in situ hybridization detection of c-fos mRNA, and a third series was reacted immunocytochemically for Fos and Fos related antigens.

In situ hybridization. The tissue slides were fixed for five min in a 4\% paraformaldehyde solution. Following fixation the slides were rinsed in PBS, Triethanolamine-HCL (TEA), acetylated with 0.25\% acetic anhydride in TEA and rinsed in 2 X saline sodium citrate buffer (SSC). They were then dehydrated in increasing ethanol concentrations, delipidated in chloroform and air dried. A 259 base pair c-fos cRNA probe (complementary to nucleotides 1838 to 2116 of rat c-fos cDNA) was transcribed in the presence of \(^{35}\text{S}-\text{UTP}\) (Amersham, Lake Forest, IL) yielding a specific activity of \(1\text{-}5 \times 10^8\ \text{cpm/}\mu\text{g}\). The transcribed probe was added to the hybridization solution \([0.6 \text{ M NaCl}, 10 \text{ mM Tris}, 2 \times \text{ Denharts, 1 mM EDTA, 10\% dextran sulfate, 50\% formamide, 0.1 mg/ml salmon sperm DNA, 0.5 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 0.1\% sodium thiosulfate, 100 mM dethiothreitol (DTT) and 0.1\% sodium dodecyl sulfate (SDS)]\) immediately prior to use. After adding the probe solution \(20 \times 10^6 \text{ cpm/ml}\), the slides were kept in a humidified incubator at \(60^\circ C\) for 20 hours. Following hybridization the slides were rinsed in 2 \times SSC, the non hybridized RNA digested with RNase A \(20 \mu\text{g/ml; 30\%C}\) and rinsed in RNase buffer. Finally, the slides were washed with increasing stringency of SSC to a final stringency of \(0.1 \times \text{SSC/60\%C}\).
The hybridized slides were apposed to Hyperfilm Bmax (Amersham Inc. Lake Forest, IL) for 12 days. For photographic emulsion coating the slides were dipped in Kodak NTB-3 nuclear tract emulsion and exposed for 3-4 weeks. The emulsion coated slides were counter stained with cresyl violet.

**Immunocytochemistry.** Tissue sections immediately adjacent to sections hybridized for c-fos mRNA detection were removed from the freezer and allowed to reach room temperature before they were fixed for 5 min in a 4% paraformaldehyde solution. The sections were then reacted immunocytochemically to detect FOS proteins. The protocol provided by the manufacturer of the primary antibody (OA-11-823, Cambridge Research Biochemicals) was followed exactly with exception of the incubation time and temperature which was changed from the recommended 72 hours at 4°C to 48 hours at room temperature. The primary sheep anti FOS antibody was used in a 1:1000 concentration which resulted in only the occasional staining of neurons within the sensorimotor cortex of the normal non-stimulated rat.

**Data evaluation**

The precise delineation of graft-host borders was determined from emulsion coated slides that were counter stained with cresyl violet as well as from toluidine blue stained sections. The emulsion coated slides were analyzed using both bright- and dark-field light microscopy. The distribution of c-fos mRNA labeled cells within representative sections was illustrated using a camera lucida microscope attachment.

Five sections from each of the brains of the novelty exposed animals and from the home cage control animals were chosen randomly and the total number of c-fos mRNA and FOS protein labeled cells within the cross-sections of the transplants and contralateral host cortices were counted. The number of labeled cells per area was then
determined using NIH's image software for cross-sectional area measurements. Using Dunn Bonferroni's t-test for multiple comparisons, and Student's unpaired t-test, comparisons were made between the following groups: (i) c-fos mRNA in transplants versus contralateral host cortex of open field exposed rats, (ii) FOS protein in transplants versus contralateral host cortex of open field exposed rats, (iii) c-fos mRNA in transplants/contralateral host cortex versus FOS protein in transplants/contralateral host cortex of open field exposed rats (iv) c-fos mRNA in transplants/contralateral host cortex of open field exposed rats versus c-fos mRNA in transplants/contralateral host cortex of home cage controls and (v) FOS protein mRNA in transplants/contralateral host cortex of open field exposed rats versus FOS protein in transplants/contralateral host cortex of home cage control rats.

The density of c-fos mRNA and FOS protein labeled cells within grafts was compared to the behavioral data for each animal using Spearman's correlation test.

*Schematic representation of the experimental design*
Results

Histological analysis revealed the presence of surviving transplants within six of the eight animals that were exposed to the novel open field environment and within the two home cage base-line control animals. Animals that did not have surviving transplants were excluded from further analysis. The grafts were localized mainly in the sensorimotor neocortex forelimb area. They did not demonstrate normal laminar patterns but were organized into whorls and bands of cells with white matter interposed. A thin 'band' of non-neuronal cells usually demarcated the graft-host border (Fig 14).

The behavioral activity patterns of the six animals that showed viable transplants upon histological analysis are listed in Table I.

In situ hybridization for c-fos mRNA of brain sections taken from animals that were exposed to the novel open-field environment revealed a very intense hybridization signal in the cingulate and medial frontal cortex as well as in the pyriform cortex. Intense hybridization was also observed within the lateral septum and dorsal part of the striatum. In the unoperated hemisphere, the sensorimotor cortical area corresponding to the location of the grafts on the operated side showed intense hybridization labeling in the superficial and deep cortical layers with a more sparse signal in between (Fig 15). Immunocytochemical analysis showed a close correlation of FOS labeled cells with those areas identified by in situ hybridization (Fig 16 and Table II). In contrast to the labeling seen after open-field exposure, little base-line hybridization or FOS immunoreactivity was found within the host brain regions or within the grafts when animals were sacrificed immediately following removal from their home cages. Cell densities of c-fos mRNA or FOS protein labeled cells within transplants and
contralateral host cortices of home cage animals were significantly lower than densities within corresponding brain areas of open field exposed animals; \( p < 0.004 \).

Brain sections from open-field exposed animals showed c-fos mRNA and FOS protein labeled cells within all the transplants analyzed (Fig 15 and Fig 16). Although the densities of mRNA as well as protein labeled cells were significantly higher than the densities observed within transplants of home cage animals, the densities of labeled cells within the transplants were significantly lower than normal cortex (Table II). Furthermore, labeled cells were not distributed evenly throughout the transplants but were localized primarily near the graft-host interface or at the dorsal surface of the grafts (Fig 17). The density of c-fos mRNA labeled cells within host cortex as well as within transplants was not significantly different from the density of FOS protein labeled cells within corresponding brain regions of open field exposed rats.

No significant correlation was found between animal activity patterns and the densities of labeled cells within the transplants or contralateral host cortex of animals exposed to the novel open field environment.

Discussion

In situ hybridization and immunohistochemical techniques were employed to examine c-fos mRNA and FOS protein induction within neocortical grafts. After placing the animals in a novel open-field environment for 20 min, c-fos mRNA and FOS protein labeled cells were consistently observed within transplants that had been grafted into excitotoxin-induced cortical lesions. In this regard, the expression of c-fos mRNA and FOS protein within the grafts or the contralateral host cortex of
environmentally stimulated animals was invariably higher than the low to absent expression within transplants of adult recipients that were not placed within the novel environment. However, the density of mRNA or protein positive cells was significantly lower than observed in normal host sensorimotor cortex. Furthermore, the distribution of labeled cells near the margins of the transplant differed from the patterns observed in the host cortex.

Although the transplanted cortex demonstrated abnormal labeling patterns, the presence of these cells suggested that they became functionally integrated with host neuronal pathways. The similarity between the c-fos mRNA and FOS protein densities within the grafts of stimulated animals further indicates that the grafted neurons were capable of inducing transcription as well as translation of the c-fos gene in a fashion similar to that of normal cortical neurons.

Several previous studies have indicated that c-fos mRNA/protein expression may serve as a marker for mapping the pattern of postsynaptic stimulation within the CNS (29, 47, 59, 140, 152, 233). This genomic response, however, can be induced following a wide variety of stimuli including physiological and psychological stress (13, 14, 29, 42, 47, 101, 132, 140, 152, 233). Therefore, in order to reduce c-fos expression induced by non-specific stimuli such as stress from handling prior to placement in the open-field box, the experimental animals were handled daily in the week prior to the experiment. Home cage animals, which were handled daily but not placed in the open-field box, revealed only sparse baseline c-fos mRNA and FOS protein expression within the brain including the transplants, indicating that the observed c-fos mRNA/protein expression in our experimental animals is due largely to novelty related stimuli.
In addition to providing a marker for neuronal pathway activation, expression of the immediate early gene c-fos also may be associated with events such as cell growth and differentiation (1, 2, 199). Therefore, the developmental state of the neocortical transplants may be important to the interpretation of these data. Several studies have reported the presence of c-fos mRNA/protein within the developing cortex during late embryogenesis and early postnatal development suggesting an involvement in events like neurite outgrowth, programmed cell death or myelination. Tritiated thymidine autoradiography of embryonic cortical tissue implanted into various areas of the newborn rat brain showed histogenesis to take place between embryonic days 15 and 22 (53, 116). This timing corresponds to the normal birth dates for cortical neurons, suggesting that neurogenesis and differentiation of transplanted cortical neurons follow intrinsic cues, and this interpretation is supported by recent work that examined the postnatal development of estrogen receptors in neocortical grafts (160). However, other work revealed apparently immature anatomical as well as functional features of grafted tissue (80, 95, 112, 115, 158). Such results indicate a disruption of the intrinsic developmental time course or alternatively that extrinsically derived factors are important for correct anatomical and functional development of CNS neurons. Again, the absence of hybridization staining in the control home cage animals with three months old grafts supports the assumption that the staining observed in the grafts after open-field exposure is due to the novel open-field stimulus and not to delayed developmental events.

Induction of c-fos in neocortical transplants in a behavioral paradigm that results in similar genomic activation of normal unablated neocortex provides evidence for functional integration with host neuronal pathways at the level of molecular signaling systems. Although the functional significance of c-fos gene induction is not
clear in detail, it is known that transcription factors of the FOS family, after
dimerization with a transcription factor from the JUN family of immediate early genes,
can regulate the expression of late response/target genes leading to the production of
NGF, preproenkephalin and preprodynorphin (61, 107, 150, 207, 239).

Although early response genes are known to be induced by extracellular stimuli
and their subsequent activation of multiple intracellular second messengers, the present
study does not elucidate the mechanisms by which the immediate early genes are
activated in response to a novel open-field exposure. It is known, however, that
physiological and psychological stress can activate the central noradrenergic system,
and that such activation can induce c-fos mRNA as well as Fos protein in neurons
within the cerebral cortex (12, 13, 14, 88, 97, 100, 198, 200, 218, 219). Because
exposure to a novel open-field is a psychological stressor where at least some of the
novelty related responses are regulated by the central noradrenergic system (57), the
immediate early gene response may be mediated in part via stress induced activation of
the noradrenergic system.

The possible role of the noradrenergic system in activating immediate early
genes within neocortical block transplants is supported by work demonstrating the
noradrenergic innervation of fetal neocortical block transplants placed into the
excitotoxically ablated cortex of adult rats (190). In this recent study from our
laboratory, biochemical analysis of transmitter markers for
noradrenergic/catecholaminergic as well as cholinergic and serotonergic inputs to
neocortical transplants showed significantly lower levels than normal sensorimotor
cortex. Additionally, histological evaluation revealed that the peripheral and dorsal most
parts of the grafts received the major part of the innervation. The topographic
distribution of catecholaminergic fibers (and cholinergic and serotonergic fibers)
accordingly corresponds to the distribution of c-fos mRNA/Fos positive neurons observed in the present study.

In *summary*, the observed expression of c-fos mRNA and c-fos protein in fetal neocortical transplants after novel open-field exposure indicates that transplant afferents from the adult recipient CNS can induce immediate early gene expression and accordingly mediate long-term functional effects. These findings support previous work demonstrating transplant functional integration at the electrophysiological and metabolic level after peripheral stimulation (26, 28, 94, 134, 155).
Table 1. Open field motor activity for six rats with neocortical transplants.

<table>
<thead>
<tr>
<th>ANIMALS</th>
<th>SQUARES ENTERED</th>
<th></th>
<th>REARS</th>
<th>NOSE POKES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wall</td>
<td>center</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>73</td>
<td>8</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>174</td>
<td>19</td>
<td>45</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>209</td>
<td>55</td>
<td>42</td>
<td>26</td>
</tr>
<tr>
<td>D</td>
<td>198</td>
<td>46</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>E</td>
<td>203</td>
<td>40</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td>F</td>
<td>214</td>
<td>57</td>
<td>47</td>
<td>27</td>
</tr>
</tbody>
</table>
Table 2. C-fos mRNA and FOS protein positive cells in transplant and host cortex of animals exposed to a novel open field environment.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Number of c-fos mRNA labeled cells/mm²</th>
<th>Number of FOS-protein labeled cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>transplant</td>
<td>host cortex</td>
</tr>
<tr>
<td>A</td>
<td>8.40±1.52*</td>
<td>40.41±2.99</td>
</tr>
<tr>
<td>B</td>
<td>7.26±0.92*</td>
<td>41.73±4.99</td>
</tr>
<tr>
<td>C</td>
<td>2.24±0.34*</td>
<td>35.56±6.51</td>
</tr>
<tr>
<td>D</td>
<td>1.10±0.26*</td>
<td>33.33±2.68</td>
</tr>
<tr>
<td>E</td>
<td>2.66±0.26*</td>
<td>35.10±2.41</td>
</tr>
<tr>
<td>F</td>
<td>3.81±0.76*</td>
<td>34.69±2.26</td>
</tr>
<tr>
<td>control</td>
<td>0.03±0.03</td>
<td>0.14±0.14</td>
</tr>
</tbody>
</table>

The density of c-fos mRNA and FOS-protein labeled cells within the transplants of animals exposed to an open field environment are significantly lower (* = p<0.05, Dunn Bonferroni t-test for multiple comparisons) than the c-fos mRNA/FOS-protein densities within host cortex of the same animals. The densities for both mRNA and protein labeled cells within transplants and host cortex of novelty exposed animals were significantly higher than the densities within similar tissues of homecage control animals (p < 0.004, Student t-test).
FIGURE 14

Coronal section showing the characteristic appearance of whorls and bands of cells within a three month old neocortical transplant (inside the dashed line). Toluidine blue.

Scale bar = 300 µm
Figure 15

C-fos mRNA labeled cells within contralateral host cortex and within neocortical transplants. Dark field (A, B and C) and bright field (D, E and F) photomicrographs showing c-fos mRNA labeled cells identified by in situ hybridization within the contralateral host sensorimotor cortex (A, B and D) and within neocortical transplants (C, E and F) from animals placed in a novel open field environment for 20 min. Panel A is a low magnification showing labeled cells throughout the depth of contralateral host sensorimotor cortex. Panel B-F are shown at the same magnifications. Arrowheads show examples of individually c-fos mRNA labeled cells. Scale bars: A = 100 µm; B and D = 25 µm.
FIGURE 16

C-fos immunoreactive cells observed within the contralateral host sensorimotor cortex (A) and within neocortical transplants (B and C) of animals exposed to a novel open field environment. Scale bar = 25 µm.
FIGURE 17
Camera lucida drawings showing the distribution of c-fos mRNA labeled cells within four different neocortical transplants from animals exposed to an open-field environment for 20 min. Each dot represents a c-fos mRNA labeled cell. T = transplant, cc = corpus callosum.
CHAPTER VI

FETAL NEOCORTICAL TRANSPLANTS GRAFTED INTO NEOCORTICAL LESION CAVITIES MADE IN NEWBORN RATS. AN ANALYSIS OF TRANSPLANT INTEGRATION WITH THE HOST BRAIN

Summary

Fetal neocortical transplants placed into frontal cortex aspiration lesion cavities in newborn rats have been shown to survive and exchange connections with the host brain. In order to further study the afferent innervation of such transplants, enzyme- and immunohistochemical techniques were employed to examine the distribution of cholinergic, catecholaminergic and serotonergic fibers within the transplants, and radiochemical enzyme assays and high performance liquid chromatography were used to determine the content of neurotransmitter markers for these same fiber systems. To examine functional integration of the transplanted neurons in terms of activation of molecular signaling systems, the graft recipient animals were exposed to a novel open field environment. This behavioral testing paradigm is known to induce c-fos mRNA and Fos protein within several areas of the normal brain, including the sensorimotor cortex. Subsequent detection of the induction of this particular immediate early gene (transcription as well as translation) in the grafts would accordingly indicate genomic
activation and therefore functional integration at the level of molecular signaling systems. Our results showed that these global fiber systems are distributed evenly throughout the extent of three month old neocortical grafts and that the content of transmitter-related markers for these systems does not differ significantly from control cortex. Open field exposure of the grafted animals resulted in c-fos mRNA and Fos protein expression of cells distributed throughout the transplants. We conclude that the 'global' fiber system innervation of neocortical transplants placed into newborn rats is similar to the innervation of normal cortex and that grafted neurons respond to host brain activation at the level of molecular signaling systems.

**Introduction**

Intracerebral neuronal grafting has been commonly used to study the reconstruction of the damaged central nervous system. For example, fetal neocortical tissue implanted into the aspiration ablated neocortex of newborn rats was found to develop extensive interconnections with the host CNS (38, 40, 43, 44, 81, 209). However, while these projections resembled connections innervating the normal unablated sensorimotor cortex, the utilized anatomical tracing techniques provided little information regarding the distribution and density of afferent fibers within the grafts.

In addition to providing a neuroanatomical basis for the integration of neocortical grafts with the host CNS, the importance of extrinsically derived inputs for the development of fetal neocortical transplants is suggested by experiments involving the placement of neocortical grafts into the anterior chamber of the eye (21, 166). When co-grafted with another piece of immature brain tissue, neocortical grafts displayed a
more 'normal' development than when transplanted singly. This effect of co-grafts on cortical graft development may find its basis in the extensive literature demonstrating the profound effects exerted by afferent fibers on cortex development (117, 164, 224).

With reference to the possible effect of transplant afferents on both graft development and functional integration with the host CNS, the present study of fetal neocortical grafts placed into newborn recipients was designed: (i) to examine the cholinergic and monoaminergic innervation of transplants by using histochemical and immunocytochemical methods, (ii) to relate these findings to biochemical assays of these neurotransmitter markers in order to provide a quantitative assessment of fiber innervation and (iii) to examine the functional integration of the transplanted neurons as indicated by the expression of the immediate early gene c-fos. Our results showed that 'global' system afferents, i.e., cholinergic, catecholaminergic and serotonergic fibers, were present throughout the grafts, that the content of neurotransmitter markers in the transplants was similar to the content within control cortex and that neurons located throughout the transplants became functionally integrated with the host CNS.

Materials and Methods

One-day old, Long-Evans, black-hooded, rats (n=28) were anesthetized by hypothermia, and the skulls were opened at the level of the right coronal suture. A unilateral cerebral cortical lesion, located 1-2 mm lateral to the midline and immediately rostral to the coronal suture, was made by gentle aspiration of 2-3 mm² of neocortical tissue. Cortical transplants obtained from Long-Evans fetuses, removed at 14-15 days of gestation from sodium pentobarbital anesthetized (42mg/kg) pregnant dams, were
immediately transferred into the lesion cavity. In obtaining the grafts, the skull and meninges overlying the telencephalic part of the fetal donor brain were removed, and a 2-3 mm² block of presumptive sensorimotor cortical tissue was dissected and placed in a sterile Ringer's solution. The donor tissue was then gently aspirated into a glass cannula mounted on a Hamilton syringe and subsequently injected as a single block into the cortical lesion cavities. The transplants were held in place by a bone flap, the skin incision was sutured, and the pups were returned to their mothers until weaning. At the conclusion of transplant surgery, the pregnant dams were killed by an overdose of sodium pentobarbital (84 mg/kg). All subsequent procedures were performed at three months post grafting.

**Histology**

Four graft recipients were sacrificed by an overdose of sodium pentobarbital (84 mg/kg) and perfused transcardially with a 4% paraformaldehyde solution. The brains were immediately removed from the skull and allowed to sink overnight in a 30% sucrose solution before they were frozen and sectioned frontally in four series of 30 µm. One series was stained with toluidine blue for routine histological examination, and the remaining series were reacted histochemically for AChE (104) or immunocytochemically for TH (the rate limiting enzyme in catecholamine synthesis) or serotonin/5-HT. The sections to be stained immunocytochemically were incubated free-floating for 48 hours in the primary antibodies [TH (Pelfreeze) diluted 1: 100; 5-HT (Incstar) diluted to 1:4000] before they were processed by the biotin-avidin-peroxidase technique using 3,3′-diaminobenzine as a chromogen.
At three months post transplantation, 17 animals were decapitated and their brains quickly removed and placed on an ice cooled copper plate. Only animals in which the transplants were easily identified were used in this portion of the study (n = 15). Two coronal sections were made rostral and caudal to the graft. Transplants were then dissected under a surgical microscope, placed in a micro centrifuge tube and immediately frozen on dry ice. Control tissue, i.e., frontal cortical tissue in a position exactly contralateral to the transplant, was also dissected for analysis. The tissue samples were stored at -70°C.

Choline acetyltransferase and acetylcholinesterase activities within graft and control tissue from seven experimental animals were determined using radiochemical enzyme assays. ChAT enzyme activity was determined according to the method of Fonnum (83). Briefly, tissue was homogenized in a 75mM sodium phosphate buffer at pH 7.4. The homogenate was then incubated in a buffer solution containing [3H]-labeled acetyl Co-A, one of the substrates for ChAT. By adding a 3-heptanone/TPB scintillation solution mixture to the incubation medium, synthesized ACh can be efficiently determined in the hydrophobic phase. The amount of labeled ACh was determined with a scintillation spectrometer. ChAT activity was expressed as nanomol [3H]ACh/mg protein/hour. AChE activity was also estimated by a radiochemical enzyme assay using [3H]AChI in the buffer solution. Because the product acetate/[3H]acetate is hydrophilic, the samples were frozen to discard the top organic layer containing the unconverted [3H]AChI. Scintillation counting on the aqueous layer was then performed. AChE activity was expressed as nanomol [3H]Acetate/mg protein/hr. Protein content was determined in triplicate (137).

High performance liquid chromatography was used to determine NA and 5-HT contents. Transplant and control cortex samples from eight experimental animals were
individually sonicated (KONTES Micro ultrasonic cell disrupter) in 500 µl 0.1M phosphate buffer pH 7.0. They were then centrifuged for 3.5 min. at 15,000 g, and the supernatant filtered through a 0.45 micron Acro LC3A filter. 5-HT was detected using an HPLC system, which consisted of an Altex 110A metering pump, a Rheodyne 7125 injection port with 50 µl sample loop and a C18 reverse phase column (10 cm x 4.6 cm, 3 mm particle size, Rainin Instr., Woburn, MA). Samples were detected by electrochemical detection using a Bioanalytical Systems LC-4B electrochemical controller with a LC-17 transducer consisting of a glassy carbon working electrode and Ag/AgCl reference electrode. The potential of the working electrode was set at +0.7V relative to the reference electrode. For detection of NA a Rainin rabbit HP/HPX drive module pump and a Rheodyne 7125 injection port with a 100 µl sample loop were used. The column and the transducer were the same as for 5-HT detection. The electrochemical controller was a LC-4A Bioanalytical Systems. The potential of the working electrode was + 0.8V relative to the reference electrode. The mobile phase for detection of 5-HT consisted of 0.1 M monobasic anhydrous sodium phosphate containing 8% (v/v) HPLC grade methanol (Burdick and Jackson Labs, Muskegon, MI). For detection of NA the Mobil phase consisted of 0.1 M sodium phosphate buffer containing 3 mM 1-octane sulfonic acid (Sigma Chemicals) and 4% (v/v) HPLC grade methanol. The buffers were filtered through 0.45 µm pores (Nylon-66 filters, Rainin), degassed under vacuum and pumped through the column at a flow rate of 1.0 ml/min.

Identification of respective monoamines was based on retention times of authentic standards (Sigma Chemicals). Sample concentrations were calculated from the peak height of these standards (1ng/ml). Protein content was determined in duplicate (137) and neurotransmitter content was expressed as ng/mg protein.
Transplant values were compared to control tissue values using the paired Student t-test.

*C-fos analysis.*

One week prior to open field testing, the animals (n=7) were handled daily for five minutes in order to reduce handling induced stress. On the day of testing, five animals were placed in the center of a well lit open field box (40' x 40' x 10'). The floor of the open field box was divided by black lines into 25 squares, and four equidistant holes were located towards the center of the box. At the time of testing the experimental animals were removed from their home cage and placed within the open field environment where they were left for 20 min. Subsequently, they were placed into their home cages for 30 minutes and then sacrificed by decapitation. Two animals that were not placed within the open field box but were sacrificed immediately following removal from their home cages served as controls.

After sacrifice, the brains were removed immediately, frozen in 30°C isopentane and placed in a -70°C freezer. The brains were sectioned through the area of the transplants into 6 parallel series of 18 µm sections using a Leitz cryostat. One series was stained with toluidine blue for routine histological examination, a second series was used for in situ hybridization detection of c-fos mRNA, and a third series was reacted immunocytochemically for FOS antigens.

*In situ hybridization.* The tissue slides were fixed for 5 min. in a 4% paraformaldehyde solution, rinsed in PBS, TEA, acetylated with 0.25% acetic anhydride in TEA and then rinsed in 2 x SSC. They were then dehydrated in increasing ethanol concentrations, delipidated in chloroform and air dried. A 259 bp c-fos cRNA probe (complementary to nucleotides 1838 to 2116 of rat c-fos cDNA) was transcribed in the presence of 35S-UTP (Amersham, Lake Forest, IL) yielding a specific activity of
1-5 x 10^8 cpm/µg. The transcribed probe was added to the hybridization solution (0.6 M NaCl, 10 mM Tris, 2 x Denharts, 1 mM EDTA, 10% dextran sulfate, 50% formamide, 0.1 mg/ml salmon sperm DNA, 0.5 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 0.1% sodium thiosulfate, 100 mM DTT and 0.1% SDS) immediately prior to use. After adding the probe solution (20 x 10^6 cpm/ml) the slides were kept in a humidified incubator at 60°C for 20 hours. Following hybridization the slides were rinsed in 2 x SSC, the non hybridized RNA digested with RNAse A (20 µg/ml; 30°C) and rinsed in RNAse buffer. Finally, the slides were washed with increasing stringency of SSC to a final stringency of 0.1 x SSC/60°C. The hybridized slides were apposed to Hyperfilm Bmax (Amersham Inc. Lake Forest, IL) for 12 days. For photographic emulsion coating, the slides were dipped in Kodak NTB-3 nuclear tract emulsion and exposed for 3-4 weeks. The emulsion coated slides were counter stained with cresyl violet.

Film autoradiograms were used to analyze c-fos mRNA density (35S/mg protein) within the transplant and control contralateral sensorimotor cortex. A 14C plastic standard curve was exposed alongside tissue sections, and subsequently compared to a brain mash standard with increasing amounts of 35S/mg protein (25). For each open-field exposed animal, the densities within the grafted tissue were compared to the densities within the contralateral control cortex using Student's paired t-test. The densities within transplants of open field exposed animals were furthermore compared to the densities within transplants of home cage control animals using Student's unpaired t-test.

**Immunocytochemistry.** Before the sections were reacted immunocytochemically for FOS antigens, they were fixed as described above for in situ hybridization. The
protocol provided by the manufacturer of the primary antibody (OA-11-823, Cambridge Research Biochemicals) was followed except that the incubation time was reduced from the recommended 72 hours at 4°C to 48 hours at room temperature. The primary sheep anti-FOS antibody was used in a 1:1000 concentration which resulted in only the occasional staining of neurons within the sensorimotor cortex of the normal non-stimulated rat.

The precise delineation of graft-host borders was determined from emulsion coated slides that were counterstained with cresyl violet and from toluidine blue stained sections. The emulsion coated slides were analyzed using both bright- and dark-field light microscopy. The distribution of c-fos mRNA labeled cells within representative sections was illustrated using a camera lucida microscope attachment.

_Schematic representation of the experimental design_
Results

At the time of sacrifice, 24 of 28 transplant recipients showed obvious transplant survival and growth.

Histological analysis

Three out of the four animals in this group had surviving transplants. The grafts were structurally well integrated with the host brain with no remnants of the original lesion at the graft-host interface (Fig. 18). Although they displayed whorls and bands of cells in contrast to the normal laminar organization of the sensorimotor cortex, the cell density within the grafts appeared similar to the density of cells within normal neocortex.

AChE positive fibers extended across the transplant-host interface and distributed evenly throughout the graft with a density that appeared comparable to the normal unablated sensorimotor cortex (Fig.19A,B). AChE positive cell bodies were occasionally found within the transplants and the adjacent host cortex. TH positive axons were also observed coursing throughout the transplant with no specific orientation or pattern (Fig.19C, D). Several fibers crossed the graft-host interface with a few entering from the pial surface. 5-HT immunocytochemistry revealed serotonergic fibers extending evenly throughout the graft (Fig.19E, F), and these fibers were also observed crossing the graft-host interface. No TH or 5-HT positive cell bodies were observed within the transplants.

The axons of all three systems entered the grafts in fairly equal densities along the entire host-transplant interface without any apparent particular points of entry.

Neurochemical analysis
Tissue samples dissected from the grafts and from the contralateral host cortex of 15 animals were examined for ChAT and AChE activity using radiochemical enzyme assays (n=7) or for NA and 5-HT content using HPLC analysis (n=8). Two rats that did not show a clearly definable graft-host border were excluded from the experiment. ChAT and AChE activities in grafted tissue were marginally lower than in control tissue (0.01<p<0.05), but no statistically significant differences were observed between graft and control tissue NA and 5-HT contents (p < 0.01) (Fig.20).

**Open field testing**

Four of the five animals exposed to the novel open field environment and the two control animals had surviving transplants. As described previously (101), rats exposed to an open field environment demonstrated c-fos mRNA labeled cells within several brain areas including the pyriform, the medial frontal, and the cingulate cortices, the dorsal-most part of the striatum, the lateral septum and most importantly for the present study, the sensorimotor cortex. C-fos mRNA hybridized cells were also distributed throughout the neocortical transplants (Fig. 21 and 22). Densitometric measurements of c-fos mRNA content within the transplants from animals exposed to the open-field environment were not significantly different from the contralateral sensorimotor host cortex (Table III). The distribution of FOS immunoreactive cells within the host brain and transplants from open-field exposed animals appeared comparable to the observed pattern of c-fos mRNA labeled cells (Fig.21).

In contrast to animals exposed to the open field environment, the homecage control animals showed very little or no expression of c-fos mRNA or FOS labeled cells within the host cortex or within the cortical grafts. Students's paired t-test showed significantly higher c-fos mRNA densities within transplants/control tissues of open
field exposed animals compared to densities within transplants/control tissues of home cage control animals.

**Discussion**

Fetal neocortical grafts placed in aspiration lesion cavities made in newborn rats received cholinergic, catecholaminergic and serotonergic afferents in normal amounts as examined by biochemical measures. These observations were supported anatomically using enzyme- and immunohistochemical methods. Additionally, the expression of c-fos mRNA and FOS protein within the transplants after open field stimulation provided evidence for transplant functional integration at the level of molecular signal transduction.

**Anatomical integration.** The presence of AChE, TH and 5-HT positive fibers entering and distributing within the grafts corresponds to several previous neuroanatomical studies demonstrating the neuroanatomical integration of neocortical transplants with the host nervous system (38, 40, 43, 44, 81). Our additional data on transmitter levels within the transplants reflect our histological observations indicating that the densities of cholinergic and monoaminergic afferents within the transplants were within normal levels. This is in contrast to other host-derived transplant afferents arising from the contralateral sensorimotor cortex and the ipsilateral thalamus that appeared mainly to innervate the peripheral parts of neocortical transplants (38, 44, 81).

Concerning the origin of graft afferents, some axons or neurotransmitter markers detected within the transplants may be derived from cells intrinsic to the grafts
rather than from axons entering from the host CNS. Indeed, AChE positive neurons were observed within our grafts, and in previous work AChE positive neurons within neocortical grafts placed into the excitotoxically ablated cortex of adult rats were found to contribute approximately 25% of the total AChE innervation (114). This observation corresponds to normal cortex where intrinsic neurons give rise to 33% of the total ChAT staining (72).

TH positive neurons have also been found within neocortical transplants, but they were not believed to be catecholamine producing (108, 167). In our animals, neither TH positive nor 5-HT positive neurons were found within the transplants excluding possible intrinsic sources of NA or 5-HT. Although the TH positive and presumably noradrenergic projections to the grafts are likely to arise from the host locus coeruleus, other extrinsic sources may exist. For example, peripheral sympathetic nerve fibers enter the CNS along blood vessels and may sprout into the CNS parenchyma following central cholinergic denervation (17, 48, 49). Such peripheral noradrenergic fibers may accordingly contribute both to the observed TH positive fibers and to the measured NA content within the neocortical grafts.

The observed extensive projections of host global system fibers to the grafts differ from the innervation of neocortical grafts placed into excitotoxic or ischemic lesions of the adult rat neocortex. In these transplantation paradigms, afferent fibers were distributed primarily to areas adjacent to the graft-host interface (93, 113, 190), and biochemical markers for host afferents demonstrated significantly lower levels within the grafts as compared to control tissue (190). The use of newborn recipients in our study is likely to account for the apparent increased innervation of the transplants. The importance of recipient age in relation to graft innervation is suggested when considering that previous work showing significantly less than normal graft innervation
involved the placement of transplants into adults that sustained excitotoxin–induced lesions (190). Such axon–sparing lesions would seem to better enable the spared host axons to innervate the graft as compared to the axotomizing cavity lesions used in our present work were it not for age differences (131, 170, 229). In addition to differences in age and lesion methods, the present experiment differs from previous work in that our animals received transplants immediately following lesion placement whereas studies involving adult hosts commonly introduce a 1-2 week delay between lesion and grafting. However, the apparent benefit of delayed grafting in promoting transplant growth and development (157) appear less important when using newborn recipients.

*Functional integration.* Induction of the immediate early gene c-fos in the nervous system can occur after a wide variety of stimuli that cause neuronal activation, and it is therefore suggested to be a valuable technique for mapping neuronal pathways (59, 152). Since, however, several forms of stress also can induce c-fos within the CNS (101, 198, 200, 218) this has to be acknowledged and possibly controlled when c-fos gene induction is used for functional mapping of neuronal pathways. In this regard, the absence of both c-fos mRNA expression and Fos protein staining within homecage control animals indicated that the genomic response observed after open field exposure was not due to handling stress but rather due largely to novelty related stimuli.

Although the relationship between the genomic response and open field behavioral stimulation indicates that the transplanted neurons were integrated functionally with host CNS neuronal pathways, the specific neuronal pathways involved are unknown. However, the central noradrenergic system is of particular interest because physiological as well as psychological stressors, including exposure to a novel open field can activate this system.
(88, 100) and also because activation of the noradrenergic system can induce the c-fos gene within the cerebral cortex (12, 13, 14, 97, 218, 219). Furthermore, the central noradrenergic system has been reported to be directly involved in the behavior of animals exposed to a novel open field environment (57). The involvement of the noradrenergic system in transplant c-fos gene induction after exposure to a novel open field environment is further supported by our observations of the distribution of catecholaminergic fibers throughout the grafts. This extensive distribution pattern in turn corresponds to the widespread distribution of transplant cells showing c-fos mRNA/FOS protein expression. This possible correlation becomes more plausible when our present findings are viewed in relation to previous related work involving the placement of neocortical grafts into adult recipients. Catecholaminergic fibers were distributed to the peripheral margins of the grafts (93, 113, 190) which also were the only areas displaying c-fos mRNA/Fos protein induction after open field stimulation (188).

Although the precise role of immediate early gene proteins within the CNS is not fully understood, several of these proteins are reported to function as transcription factors for 'downstream' genes and thereby as nuclear mediators that couple external stimuli to long-term changes in gene expression within a particular cell (152). The induction of the immediate early gene c-fos within transplanted neurons following an external stimulus therefore indicates that the host CNS can influence graft neurons in a more permanent way than suggested by previous reports demonstrating electrophysiological and metabolic integration with the host CNS (27, 28, 94, 134, 156).
Table 3. Average c-fos mRNA densities as measured from film autoradiograms.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Transplant density</th>
<th>control ctx. density</th>
<th>Graft density / control ctx. density</th>
</tr>
</thead>
<tbody>
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<td>1073A</td>
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<td>21315±1276</td>
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<td>1073D</td>
<td>16685±706</td>
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The transplant and contralateral sensorimotor cortex (control ctx.) c-fos mRNA densities were measured in 7 sections from each animal. Densities were determined from known brain mash standards and a C14 standard, that was exposed along with the brain sections. The densities were measured by the NIH image program, and expressed as $^{35}$S dpm/mg protein ± one standard error of the mean. Using a paired Student t-test, transplant densities were not significantly different from control tissue densities for any of the four open-field exposed animals.
FIGURE 18

Coronal section showing the cellular organization of a three month old neocortical transplant located within the black line. Nissl stain. Scale bar = 500 µm.
FIGURE 19

Darkfield photomicrographs showing AChE-positive fibers (A, B), TH immunoreactive fibers (C, D) and 5-HT immunoreactive fibers (E, F) within the host neocortex (A, C, E) and neocortical transplants (B, D, F). Scale bar = 50 µm.
FIGURE 20
Transplant ChAT and AChE activities and NA and 5-HT levels presented in comparison to control tissue values. Values are expressed as the mean ± S.E.M. * = 0.05 > p > 0.01.
transplant tissue
control tissue
FIGURE 21

C-fos mRNA and FOS positive cells within contralateral host cortex and within neocortical transplants. Brightfield photomicrographs showing c-fos mRNA positive cells, demonstrated by in situ hybridization (A, B) and Fos protein-like immunoreactive cells (C, D) within the host cortex (A, C) and neocortical grafts (B, D) of animals exposed to an open field environment. Arrows show examples of c-fos mRNA labeled cells. Scale bar = 25 µm.
FIGURE 22
Camera lucida drawing showing the distribution of c-fos mRNA labeled cells within a neocortical transplant and within the host cortex on either side of the encircled transplant from an animal exposed to the open field environment. The medial convexity of the cortical hemisphere is to the left. Each dot represents a c-fos mRNA labeled cell.
CHAPTER VII

GENERAL DISCUSSION

The functions of the adult mammalian CNS are critically dependent on the formation and maintenance of specific axonal connections. The neocortex in particular shows patterns of very complex but specific connectivity (127). Bearing this highly specific organization in mind, it is not surprising that injury to the neocortex or to neurons connecting with the neocortex can lead to functional impairments. Because mature neurons have lost their ability to proliferate and therefore to replace lost neurons and neuronal connections, basic research has focused intensely on mechanisms underlying development, plasticity and repair of the nervous system. In this regard, neuronal transplantation is a powerful experimental technique for the study of the development and re-establishment of lost neural connections. Neuronal grafting may also have clinical applications in the restoration of lost function.

Stroke and the use of neuronal transplants

Stroke is responsible for approximately one-third of the deaths occurring from cardiovascular disease, which in itself represents the third leading cause of death worldwide (23). Stroke is significantly more prevalent among the elderly, and because
the world population is gradually aging, stroke and its associated problems for survivors present an increasing burden to health care systems.

Stroke results in ischemic changes leading to neuronal death. The time course of neuronal degeneration depends on the degree of ischemia, i.e., the more severe the ischemia the less time it takes for neurons to become irreversible damaged. This time period from onset of the vascular insult to irreversible cell damaged is referred to as the window of opportunity or the therapeutic window. This window usually represents a period of only a few hours during which clinical interventions such as reperfusion or protective measures must be instituted in order to achieve optimal effects in reducing neuronal damage (23, 31). If therapeutic treatment is not initiated within this time frame, ischemic neurons will degenerate, which often causes an additional secondary degeneration of neurons projecting into the neuron depleted area. Also, secondary degeneration may occur among cell populations deprived of inputs originating from neurons located in the area of primary insult.

Currently, no useful therapeutic interventions exist that can reverse the functional deficits resulting from ischemia-induced neuronal death (118). Basic as well as clinical neuroscience research has focused therefore not only on mechanisms responsible for stroke-induced neuronal death and on the rescuing of reversibly damaged neurons but also on mechanisms that can improve lost functions following death of the ischemically injured neurons. In this regard, implantation of fetal neural tissue in animal models of cerebral ischemia may provide information about the regenerative mechanisms and potential of neurons that are affected secondarily by ischemia. Furthermore, transplantation of fetal neural tissue may present a possible therapeutic tool in the restoration of lost function.
Commonly used experimental stroke models include vascular occlusion models in which the blood supply to certain regions of the brain is transiently or permanently arrested, and chemical models in which application of glutamate or glutamate agonists results in neuropathological changes that resemble the changes that occur after ischemia.

The purpose of this dissertation was to examine the hypotheses that fetal neocortical transplants placed into ischemia-like, excitotoxin-induced neocortical lesions of adult rats could become functionally integrated with the host brain.

**Anatomical integration**

The use of fetal neural transplants to restore function in the damaged CNS is believed to involve one or more of the following mechanisms: (i) graft induced trophic actions on the host brain, (ii) diffuse transplant release of neurotransmitters or neurohormones (biological mini pump) and (iii) synaptic integration of graft and host neural pathways (15). For neocortical grafts, the establishment of host-transplant connectivity is especially important for achieving complete integration and amelioration of injury-induced impairment, although the recovery of certain functions is suggested to occur via different mechanisms as well (123, 215). Moreover, the study of host-graft interconnections may provide insight into the anatomical and functional interactions between different neural systems and the regenerative capacity of damaged neurons.

Regarding anatomical integration of fetal neocortical transplants, our experiments using blocks of fetal tissue (Chapters III and IV) combined with
experiments using fetal suspension grafts (93, 113), provided evidence of host derived afferent inputs from several areas of the host brain. These inputs arose from areas that normally send afferent projections to the sensorimotor cortex of the rat. The distribution and degree of innervation by cholinergic and monoaminergic fiber systems, however, significantly differed from normal cortex. Here it is important to re-emphasize, that the neuroanatomical integration of fetal suspension transplants did not appear more extensive than the integration of fetal block transplants. Considering that block transplants are reported to show features that are reminiscent of normal cortical intrinsic connectivity (84), this method of grafting would seem to enable block transplants to become better integrated into the host brain and therefore would be the preferred method for neocortical transplantation.

Differences in transplant-host interconnections were also found when comparing transplant innervation densities between adult and neonatal hosts (Chapters IV and VI). This apparent age-related difference in graft innervation by global system inputs corresponds to studies examining point to point projections to hippocampal transplants placed into newborn or adult hosts (223, 229, 244). This previous work also showed that the point-to-point type innervation was significantly enhanced with delayed grafting into CNS lesions. Similar findings have been demonstrated for global system fiber innervation of striatal grafts (131, 227). The placement of block grafts into excitotoxin-induced lesions in adult rats and aspiration lesion cavities in neonates, as done in our experiments, makes it difficult to directly compare transplant innervation densities in the two transplant models. However, since axon-sparing excitotoxin-induced lesions would seem to better promote transplant innervation, the increased density in our aspiration lesion model indicates that recipient age represents an overriding factor.
Transplant neurons must extend axons to host cortical and subcortical areas in order for complete neuroanatomical integration to take place. Studies using newborn recipients have in fact demonstrated transplant efferents to most areas that normally receive cortical projections (39, 70, 209), while adult recipient neocortical grafts demonstrated only very sparse efferents to the host brain (Chapter III) and (92, 113, 175, 195). However, intratransplant injections of the anterograde anatomical tracer PHA-L showed an extensive network of axons coursing throughout the transplant placed into adult hosts suggesting that the grafted neurons indeed did have the capacity for axonal growth, (Chapter III) and (92).

The regrowth of injured axons generally does not occur in the adult CNS, and this phenomena is generally attributed to a paucity of growth promoting factors and to the presence of growth inhibiting factors (146, 192, 201). In this regard, successful long distance axonal growth into peripheral nerve transplants is reported (54). Peripheral nerve grafts contain substrates and trophic factors that are favorable for axonal growth and do not contain oligodendrocyte-myelin associated neurite growth inhibitory factors. Therefore, by enhancing the growth promoting activities and by decreasing the growth inhibiting activities within the adult CNS, increased regrowth of axons may be achieved. In this regard, CNS injury is reported to induce a time-dependent increase in neurotrophic activity that favors the survival and growth of neural implants as well as the development of afferent innervation (131, 142, 157, 170, 227, 229). Improved graft survival and development was also reported after intracerebral injections of basic fibroblast growth factor (144).

Several factors that actively inhibit neuronal growth have been identified within the adult CNS. Examples include lesion induced glial scars and their production of two extracellular matrix molecules, chondroitin-6-sulfate proteoglycan and cytotactin that
posses growth inhibitory activities as well as myelin associated proteins that also exert growth inhibitory activities (32, 33, 146, 180, 191, 192). Presumably, these factors are able to overwhelm lesion-produced trophic factors and thus inhibit axonal regeneration after injury.

Transplanted fetal neural tissue provides a milieu that is permissive for axonal growth. Several studies demonstrate high concentrations of trophic factors within the developing CNS (103, 228, 235). Accordingly, host afferent fibers that grow into the transplants might be expected to grow throughout the grafts. However, the predominance of host brain afferents observed in our studies were distributed to the periphery of the transplants. This restriction in the distribution of transplant afferents may reflect a lack of available synaptic sites on neurons located centrally in the grafts. These sites are likely to be occupied by intratransplant connections. At the time of grafting, transplant interneurons are rapidly growing and differentiating, and their axons may therefore occupy the majority of available synaptic sites prior to the ingrowth of adult host fibers. This competitive interaction of transplant and host axons for available synaptic sites has previously been described for hippocampal transplants (222, 245).

**Functional transplant-host integration**

Functional integration of neocortical transplants with the host brain is closely related to the formation of host-transplant interconnections and can be examined by: (i) restoration of lesion-induced behavioral deficits, (ii) electrophysiological activation of
transplanted neurons, (iii) metabolic activation of transplanted neurons and (iv) genomic activation of transplanted neurons.

In the present studies, the functional integration of fetal neocortical grafts placed into the damaged host brain was examined at the level of molecular signaling systems. In this regard, the immediate early gene c-fos is a commonly used marker for the detection of functional neuronal pathway activation (59). Moreover, this technique allows for detection of individually activated cells.

A major focus of this dissertation work concerned transplant innervation by host derived cholinergic and monoaminergic projections. These global fiber systems originate from a limited set of distinctly located neurons that project widely to several different areas of the CNS. Such projections are thought to be important in setting the baseline activity levels of their target neurons. Stress induced activation of the locus coeruleus derived central noradrenergic system (88, 100) and subsequent induction of the immediate early gene c-fos (13, 14, 97, 101) may represent an example of neuronal activity level setting. In this regard, transplant neurons showed c-fos mRNA and FOS protein expression after the animals were exposed to a novel and therefore stressful open-field environment. These findings indicated that the host nervous system is capable of modifying the activity levels of transplanted neurons in response to a behavioral stimulus. Comparable findings were observed using both newborn and adult hosts. However, the distribution and density of c-fos mRNA labeled neurons throughout the 'newborn' grafts, which was similar to patterns found within normal cortex, differed from the uneven and less dense distribution found in 'adult' transplants (Chapter V and VI). This corresponds to several reports indicating that grafts are better integrated when using newborn recipients.
The precise function of cellular IEG's is not known. Their protein products however are thought to function as cellular transcription factors and therefore to mediate long-term functional effects. The expression of IEG's within neocortical transplants therefore extends previous reports that showed short-term metabolic and electrophysiological activation of transplanted neurons (27, 36, 87, 94, 134, 155, 156).

Concluding remarks

The results obtained from the experiments in this dissertation showed that fetal neocortical transplants become anatomically and functionally integrated with the neonatal and the adult host brain. For the adult recipients, however, the degree of innervation and functional integration was markedly different from that of normal neocortex, and therefore future experiments on neocortical transplants may focus on the enhancement of anatomical connections. In this regard, the regrowth of adult CNS neurons appears to be critically dependent on a balance between growth promoting and growth inhibiting factors present within the nervous system. Interventions that shift this balance toward growth promoting factors may therefore enhance the formation of transplant-host interconnections. For the neocortical transplant-lesion paradigm, such interventions may include: (i) attempts to find the optimal time for tissue implantation after CNS injury, (ii) application of specific growth factors (e.g., bFGF) in order to further promote the growth promoting activity in areas surrounding the graft and (iii) application of growth inhibitor neutralizing factors such as antibodies directed towards
the myelin associated growth inhibitory factors NI-35 and NI-250 and towards extracellular matrix components with growth inhibiting activities.

If basic research can provide sufficient evidence for enhanced connective interchange and for behavioral improvement exerted by neocortical transplants, clinical trials involving neuronal grafting for the repair of stroke and other neocortical insults can be envisioned. However, a very important problem regarding the clinical application of neuronal transplantation concerns the use of immature (fetal) donor material. Due to the ethical ramifications associated with the use of human fetal neural tissue, extensive searches for alternative donor sources may be necessary. Thus, basic research has focused on the use of genetically modified non-neuronal cells that are not obtained from fetuses (221). This approach is especially valuable for grafting paradigms that rely on the release of neurotransmitters, neurohormones or of neurotrophic factors for functional integration.

The use of genetically modified cells is likely not to be feasible in transplants of neocortex and other neural tissues where host-transplant integration depends upon the development of functional and appropriate neuroanatomical connections. Furthermore, studies of neocortical transplants used to reconstruct damaged connections demonstrated the importance of using donor issue derived from fetal brain areas that correspond to the host brain lesions (36, 70). Neuronal transplants also have been effective in rescuing neurons that are threatened but not immediately killed by neuronal lesions (102, 196, 211) Perhaps, the permanent rescue of these cells is dependent upon establishing synaptic contact with the grafted neurons. Alternatively, rescue may be mediated by graft produced trophic factors. In this light, the appropriate neurotrophic factors may be produced by genetically modified non-neuronal cells. Such methods can perhaps reduce the secondary neuronal degeneration induced by ischemic lesions.
Donor material obtained from non-human fetuses may also present a possible source of tissue for neuronal transplantation. Again, however, ethical concerns may arise. The grafting of non-species specific CNS tissue furthermore introduces questions about immunological rejection of the transplants and extensive investigations are currently undertaken in order to determine the necessity of immunosuppressive therapy in the xeno-grafting paradigm.

Currently, the clinical applicability of neocortical transplantation remains uncertain. However and perhaps more importantly, continued research in this field undoubtedly will provide additional important information on the anatomical and functional interactions between different neural systems and on the mechanisms underlying repair and reconstruction of the damaged nervous system.
APPENDIX

Schematic representation of the transplantation procedure.

See Materials and methods sections under Chapters III to VI for detailed description of the transplantation procedure.

Block grafts injected from microsyringe into NMDA induced lesions made in adult rat sensorimotor cortex.
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The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

[Signature]

Date: 4 April 95

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