CHROMAFFIN CELL GRAFTS TO THE CEREBRAL CORTEX OF NUCLEUS BASALIS MAGNOCELLULARIS-LESIONED RATS: TISSUE SPECIFICITY, DURATION OF PLACEMENT AND ESTABLISHMENT OF IMMUNOCYTOCHEMICAL PROTOCOLS

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Chromaffin Cell Grafts to the Cerebral Cortex of NBM-Lesioned Rats

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ABSTRACT

Cell transplantation to the brain and spinal cord is a well-established tool for studies on cellular and molecular mechanisms involved in development and plasticity. Experimental data from animals has suggested that restoration or preservation of function through cell transplantation has potential as a useful therapeutic approach in neurodegenerative disorders. Experimental evidence of graft-induced behavioral ameliorations has raised the question of the applicability of this technique to the cognitive neurodegenerative disorder, Alzheimer's disease (AD).

This present study is based on previous work from our lab that has indicated a potential for cortical grafts of chromaffin cells of the adrenal medulla to alleviate nucleus basalis magnocellularis (nbm) lesion-induced cognitive and neurochemical deficits; such lesions produce deficits which mimic certain of the deficits seen in AD. This study has found that chromaffin cell grafts ameliorate behavioral deficits and that a neurochemical recovery occurs in the cortex consequential to it. This particular behavioral improvement was evidenced 6 weeks but not 5 days post-graft, indicating a gradual effect by the graft. Additionally, in this behavioral test, control grafts of kidney cells were found to have no ameliorating effects. A fina objective to establish immunocytochemical techniques for detection of graft constituents was also actualized.

RÉSUME

Un outil bien établi dans l'étude des mecanismes moléculaires et cellulaire lors du developpement et de la plasticité neuronale est la transplantation cellulaire au cerveau et à la moelle épinière. Des donnees expérimentales ontenues chez l'animal suggèreut que la transplantation cellulaire pouvrait être utilisee à des fins therapeutiques pour restaurer ou préserver des fonctions dans des desordres neurodégénératifs. Des améliorations compartementales induites par ces greffes soulèvent la possibilite d'utiliser cette techniques dans le traîtement de la maladie d'Alzheimer (MA).

Cette étude baseé sur des travaux anterieurs de notre laboratoire qui ont indiqué une amélioration des deficits neurochimiques et cognitifs grâce aux greffes corticales de cellules chromaffines de la glande médullo-surrenale. Ces déficits induits par des lésions au noyau basal magnocellulaire (nbm) ressemblent à certains deficits observés dans la MA. Cette etude a permis de voir que des greffes de cellules chromaffines améliorent des déficiences comportementales en plus d'un retablessement neurochimique au cortex. Cette amélioration comportementale est observee apres la greffe, indiquant une amelioration graduelle suite à cette greffe. Dans ces mêmes epreuves comportementales, les greffes contrôles de cellules rénales n'ont aucun effet. L'établissement des techniques immunocytochimique pour la direction des composantes de greffes a aussi été réalisé dans le cadre de ce travail.

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On Giving - "You give but little when you give of your possessions.
It is when you give of yourself that you truly give."
On Friendship - "Your friend is your needs answered."
Kahlil Gibran - The Prophet

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1 INTRODUCTION

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disorder of the elderly. This disorder is characterized by diverse behavioral and biochemical symptoms and their neuropathological correlates. Due to the complex nature of the behavioral deficits and the wide variety of pathological changes, both neuroantomically and biochemically, determining the specific clinical symptoms due to a specific pathology is a Herculean task.

1.1.1 Behaviorial Pathology

The disease presents a highly complex symptomology. In AD, a characteristic cluster of behavioral symptoms is evident including language impairment, visuospatial deficits, anhedonia and the hallmark behavioral symptom, memory deficits. Secondary to these are the possible development of physical symptoms such as myoclonus, decreased sensory function and muscular rigidity resembling parkinsonism, all of which are associated with a greater severity of the disorder (DSM-IIIR, 1987).

1.1.2 Physiological Pathology

A diverse array of physiological changes are also presented in AD, the research of which is confounded by the fact that all these phenomena are characteristics found in varying degrees in normal aging brains as well. The histological hallmark of the disorder is senile or amyloid plaques and neurofibrillary tangles (NFT)(Perry et al., 1978).

However, other neuropathological signs are presented, as well, including neuronal loss or shrinkage in the neocortex, reduction in brain weight, ventricular enlargement, the presence of filamentous clusters called Hirano Bodies (Tomlinson, 1977) and granulovacuolar degeneration (Tomlinson and Kitchener, 1972). Deficiencies in neurotransmitter systems have been evidenced, the most consistent of which is a reduction in cholinergic markers in pathways projecting from the basal forebrain cholinergic nucleii, which includes the nucleus basalis of Meynert (nbM) and septal nuclei, to the neocortex and hippocampus (Bowen et al., 1976; Davies and Maloney, 1976; Whitehouse et al., 1982).

1.1.3 Cholinergic Hypothesis of Mnemonic Dysfunction in Humans

The loss of cholinergic markers in the brains of patients with AD (Davies and Maloney, 1976; Bartus et al., 1982) and evidence that anticholinergic agents such as scopolamine induce temporary deficits of memory (Drachman, 1976; Kopelman, 1986) led to the proposition that damage to a cholinergic system may be responsible for the behavioral or cognitive deficits seen in AD.

This cholinergic hypothesis of memory dysfunction has focused particular attention on the nbM and its neocortical innervations since the time that loss or atrophy of these neurons in AD was observed (Davies and Maloney, 1976; Bartus et al., 1982) and a decline in cholinergic markers such as the enzyme responsible for the synthesis of acetylcholine (ACh),

choline acetyltransferase (ChAT)(Bird et al., 1983), and the enzyme which degrades ACh, acetylcholinesterase (AChE)(Perry et al., 1978) in the cortex of patients were found to be correlated with the degree of dementia. A corollary of the cholinergic hypothesis was the proposal that lesions of these basal forebrain cholinergic neurons may provide an animal model of AD.

1.2 Animals Models

The creation of an ideal animal model that can mimic the pathology seen in AD is an essential step in developing therapeutic approaches to the disorder. A good animal model is useful for the development of effective therapies or for providing a better understanding of the biological mechanisms that underlie symptoms of the disease. It is important to remember that any element associated with the disease that one intends to model may only be secondary to the disease progression and not involved in the pathogenesis *per se*.

1.2.1 Pharmacological Studies in Animals in Support of Cholinergic Involvement in Cognitive Function

Initial studies in animals of the cholinergic hypothesis of cognitive dysfunction supported evidence in AD. Cholinergic antagonists such as scopolamine (Drachman and Leavitt, 1974; Saghal et al., 1990; Callahan et al., 1993) and hemicholinium (Caulfield et al., 1981; Muir et al., 1992) in experimental animals, were disruptive to various cognitive tasks. Furthermore, infusion of cholinergic antagonists directly into

the basal forebrain of rats also produced profound cognitive deficits(Muir et al., 1992).

1.2.2 Lesions to the Cholinergic Neurotransmitter System

In developing an animal model of AD, experimenters have focused on the basal forebrain cholinergic system. This is based on the aforementioned neuropathological changes that occur to this system in AD. It was hoped that manipulating the cholinergic transmission of these nucleii would produce a model mimicking at least some of the behavioral deficits that are evident in AD.

This system may be divided into two major subunits. One of these is the septo-hippocampal system, the cell bodies of which are located in the Medial Septal Area (MSA), and project to the hippocampal formation (Fibiger, 1982). The other subunit is the innominate-cortical system, the cell bodies of which are located in the nucleus basalis magnocellularis (nbm), a group of cholinesterase reactive neurons in the ventromedial corner of the globus pallidus, which projects cholinergic neurons to the frontal, temporal, and parietal neocortex (Wenk et al., 1984; Semba et al.,1987). The nbm is believed to be homologous to the nbM in humans and non-human primates (Fibiger, 1982) but is smaller and more diffusely organized (Wenk et al., 1984). While lesioning of both these systems have been used to study dysfunction and recovery of memory and related cognitive processes, this present work has used the nbm lesioning model.

1.2.2a Neuroanatomical and Biochemical Effects of Nucleus Basalis Magnocellularis Lesions in Rats

The use of nbm lesions in rats causes degeneration of cholinergic projections to the neocortex and a consequent drop in measures of cholinerginicity in the cortex. This is intended to mimic a primary pathological feature of AD, a decrease in cholinergic markers in the cortex, as well as the mnemonic deficits (see sections 1.1.1 and 1.1.2, above). Thus, loss of nbm cells is correlated with a decrease in the level of biochemical markers for cortical cholinergic neurons ie., ChAT activity, AChE activity, ACh release and high affinity choline uptake (HACU)(LoConte et al., 1982; Arendash et al., 1987); this suggests that the nbm is the major source of extrinsic cholinergic input to the neocortex (Shute and Lewis, 1967; Lehmann et al.,1980).

Lesions resulting in cell loss in the nbm may be induced by either electrolytic techniques or excitatory amino acid (EAAs) neurotoxins. Electrolytic lesions destroy both cell bodies and fibres of passage. As such, it is difficult to distinguish whether any deficits seen are due to damage to cell bodies or damage to fibres passing through the area (Hepler et al., 1985). EAAs are preferred for nbm lesioning as they act only on cell bodies that possess a specific EAA receptor, depending on the neurotoxin. Therefore, they act on neurons originating around the site of injection while sparing passage fibres. While ibotenic and kainic acid were once the

most widely used neurotoxins, quisqualic acid and, subsequently, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), have been found to induce greater cholinergic depletion, with fewer harmful side effects. This appears to be because ibotenate or kainate affect other neurotransmitter systems in and around the nbm. Thus, they will induce lesions that are of sufficient size to decrease more than the cholinergic markers alone in the neocortex, which may often lead to quite severe debilitations to the general health of the animal. This certainly would affect the interpretation of results. Quisqualate, however, being more selective for cholinergic neurons appears to cause less damage to subcortical structures and thus has fewer undesired side effects (Dunnett et al., 1987).

A distinctive cluster of neuroanatomical and biochemical deficits are evident upon lesioning the nbm. The extent of damage seems to vary as a function of toxin type as well as concentration and placement within the nbm (Dunnett et al., 1987). A consistent decline in the markers of cholinerginicity, such as HACU (Wenk and Olton, 1984; Bartus et al., 1985), ChAT and AChE activity (Salamone et al., 1984; Wenk et al., 1984; Hepler et al., 1985; Hohmann and Coyle, 1988) are evident; the decreases in ChAT and AChE levels have been found in both unilaterally and bilaterally lesioned animals (Casamenti et al., 1988). Arendash and coworkers (1987) have reported that, in addition to the depletion of

cholinergic markers, the formation of neuritic plaque-like structures, neurofibrillary changes and cerebral atrophy in the cortex, hippocampus and entorhinal cortex were also seen in bilaterally lesioned animals using quisqualate. It is possible that this additional damage is due to the concentration or amount of neurotoxin used which led to damage of the neighbouring medial septal area and its projections to the hippocampus. However, in total, the model seems to parallel the fundamental neuroantomical and biochemical deficits seen in the human condition.

1.2.2b Behavioral Effects of Nucleus Basalis Magnocellularis Lesions in Rats

Behavioral deficits following nbm lesioning are clearly evident as measured by a variety of behavioral measures. These include the one trial passive avoidance paradigm (Dunnett et al., 1987), the T-maze reinforced alternation paradigm (Salamone et al., 1984) and the radial arm maze paradigm (Turner et al., 1992). This last paradigm has proven useful in that it is particularly well-suited to differentiate between deficits in reference memory and working memory. Reference memory refers to information concerning the task that is invariant from trial to trial and can be used on all trials. Working memory requires the use of recently presented information that is not useful beyond that particular trial (Beninger et al., 1989). Possible confounds such as deficits in spatial abilities or motivation and arousal can be avoided

using all these tests with the possible exception of one-trial passive avoidance learring.

Behavioral consequences of nbm lesioning seem to confirm the link between the biochemical symptoms and the behavioral symptoms of AD and confirm the validity of the model. A distinctive pattern of cognitive deficits caused by the various lesioning methods is evidenced by the behavioral paradigms. Lesioned animals all show deficits in the acquisition of new responses and long term potentiation, demonstrated in appetitive and aversively motivated tasks and in tasks of spatial localization (Salamone et al., 1984; Hepler et al., 1985; Wozniak et al., 1989; Imaizumi et al., 1991). Further, deficits of order memory, manifested in rats with nbm lesions are seen to mimic the same deficits of order memory seen in AD patients when administered a similar task (Kesner et al., 1986; Kesner et al., 1987).

As mentioned earlier, the most commonly presenting cognitive deficit of AD patients and often the first sign of the disorder is disruption of memory, specifically, memory for recent events and the ability to learn new information (DSM-IIIR, 1987). This seems to be the same pattern of behavioral loss evidenced in nbm-lesioned rats (Kesner et al.,1987). Thus, animals appear to mimic the mnemonic deterioration seen in AD, presumably by the reproduction of a subset of neuroantomical and biochemical pathology via lesioning of the nbm; for the past decade, this model has been widely used as

an animal model for AD.

1.2.2c Possible Dissociations Between the Cholinergic System and Memory Function

The traditional interpretation of the link between the cholinergic basal forebrain system and memory, per se, has recently been challenged (Dunnett et al., 1991; Fibiger, 1991). While these deficits on various behavioral measures may require intact memory, the conclusion that the disruptions are indeed reflective of mnemonic disruption rests on two assumptions. The first assumption is that the memory deficits are not due to damage incurred in other cognitive or non-cognitive systems. Secondly, the evident deficits are due to the destruction of cholinergic projections from the nbm and not some other system of neurons from the nbm. As we shall discuss later (section 5, GENERAL DISCUSSION) neither of these assumptions may be warranted. That nbm-lesions lead to profound cognitive deficits, there can be no doubt, but the functional interpretation of these deficits is not clear.

1.3 Grafting as a Therapy for Neurodegenerative Disorders

Cell transplantation to the brain and spinal cord is a well-established tool for studies on cellular and molecular mechanisms involved in development and plasticity. Despite this, its use as a therapeutic tool in treating neural damage in both the peripheral and the central nervous system is still in its infancy. However, an abundance of experimental data from animals has suggested that restoration or preservation of

function through cell transplantation has potential for a useful therapeutic approach. This work was ushered by demonstrations that feotal neural tissue transplanted into adult rat brain survives and even forms synaptic connections with endogenous tissue (Lund and Hauschka, 1976).

Functional recovery studies lent creedance to the initial neurophysiological observations. Functional compensations were evident using many experimental paradigms. Motor deficits in experimentally-induced Parkisonism were ameliorated using grafts of feotal dopaminergic neurons (Bjorklund, Segal and Stenevi, 1979; Perlow et al., 1979). Endocrine deficits were compensated for by intraventricular transplantation of both pituitary tissue and feotal hypothalamic tissue (Halasz et al., 1963; Keiger et al., 1982).

Evidence of recovery in experimental paradigms raised hopes that neural transplants would provide a method of treating human CNS degenerative disorders. Prompted by a lack of adequate medical therapy and severity of symptoms, cell transplantation has been proposed as a possible treatment for neurodegenerative diseases. Based on the early evidence of the grafts considerable capacity to become integrated into host neural pathways, the positive results obtained in the studies of function recovery led to the assumption that functional effects were due to the reassembly of compromised host neural connections or regeneration. However, more recent experiments have provided support for a different interpretation of graft

mechanism of action.

1.3.1 The Graft as a Neurotransmitter Pump

The 'neurotransmitter pump' hypothesis was based on studies of a dopaminergic nucleus, the substantia nigra and its projections to areas of the brain essential in motoric control. Destruction of this nucleus would render the animal unable to conduct movement. This could be restored by systemic administration of the dopaminergic precursor, L-dihydroxyphenylalanine (L-DOPA)(Carlsson et al., 1957) or dopamine (DA) agonists injected directly into the substantia nigra target, the striatum (Andén and Johnels, 1977). These measures of pharmacologically-induced motor behavior led to the proposal that transplants of cells into the area of neurotransmitter depletion that actively secrete the deficient neurotransmitter might compensate for the deficiency.

Thus far, the neurodegenerative disease that has seen the most clinical use of transplantation is Parkinson's Disease. This is most probably because the neuropathology is relatively well understood, involving the degeneration of a specific neurotransmitter system, the dopaminergic substantia nigra and its projections to the motor cortex (Hornykiewicz, 1989). Dopaminergic cells of feotal origin, from the ventral mesencephalic region and catecholaminergic chromaffin cells obtained from the patients own adrenal gland as an autograft have been attempted (Olson, 1990). Both of these techniques have provided mixed results with modest improvements being

observed in some of the symptomology (Backlund et al., 1985; Lindvall et al., 1987; Madrazo et al., 1990; Lindvall, 1991).

This has raised the question of the applicability of grafting to treat the neurodegenerative disorder AD; some researchers were more optimistic (Gash et al., 1985), others were less so (Lindvall, 1991). With its global cognitive and neurological impairment, prospects of the therapeutic relevance of grafting seemed low. Firstly, unlike PD, many neurotransmitter systems are deficient in AD; primarily cholinergic systems, but also noradrenergic and serotonergic systems, as well. Moreover, unlike motor performance where sensory and effector pathways are relatively well-mapped out, neither the processes, nor the neural bases for memory and cognition are sufficiently well understood. This is evidenced in the multitude of conflicting animal models proposed for AD. And further, there is no definitive evidence for a relationship between brain area and particular cognitive function. However, despite these disparaging issues, attempts to ameliorate cognitive deficits were initiated with encouraging findings, and continue to show promise.

1.3.2 Cholinergic Embryonic Tissue Grafts

Initial experimental research an CNS grafts using this model of AD supported both the cholinergic hypotheis of memory dysfunction and the possible applications of transplantation to AD. Based on Parkisonian models and substantia nigral transplantation work which hypothesized that graft mechanism

of action was via release of the deficient neurotransmitter, initial studies used grafts of feotal cholinergic neurons to the cerebrum of cholinergically lesioned animals.

Cognitive recoveries were evidenced in a variety of models employing different behavioral tasks. Cholinergic enriched grafts have been evidenced to reverse behavioral deficits in T-maze (Welner et al., 1988; Welner et al., 1990), radial arm maze tasks (Hodges et al., 1990), swim maze (Dunnett et al., 1985) and retention of passive avoidance tasks (Dunnett et al., 1985; Fine et al., 1985). These behavioral ameliorations have been evidenced to occur consequent to the reinduction of cholinerginicity in the host cortex (Welner et al., 1988; Hodges et al., 1990) and do not occur in animals receiving grafts of non-cholinergic brain tissue implying that the cholinergic systems in the cortex are involved in the behavioral amelioration. Furthermore, cholinergic-enriched grafts have been evidenced to produce cholinergic reinnervation to dennervated cortex (Fine et al., 1985).

1.3.3 Chromaffin Cell Grafts

While this work with cholinergic rich grafts supports the graft as a biological minipump, recent work from our lab using catecholaminergic chromaffin cell (CC) grafts from the adrenal medulla, has supported another interpretation of graft action. Using CC grafts, research from our lab has shown that increases in lesion-induced deficits of cholinergic markers in

the host cortex can be produced by placement of these grafts in the cerebral cortex of nbm-lesioned rats. These neurochemical ameliorations are coincident with improvements in T-maze spatial alternation behavior tested 3 months post-grafting (Welner et al., 1990). In addition to the betterment of spatial alternation behavior in the T-maze tested at three months, we have recently witnessed that certain lasting deficits on a battery of simple sensorimotor and attentional measures that were produced by nbm lesioning could be significantly ameliorated following the implantation of the CC grafts tested at 1.5 months; these improvements were not seen 5 days post-grafting (Welner and Koty, 1993).

These results were strong evidence against the original rationale underlying graft mechanism of action, that these grafts work via the diffusion of a behaviorally active substance. Firstly, that the tissue grafted into the cholinergic cortex was itself not cholinergic in nature, implies that the amelioration is not arising via the immediate secretion of a behaviorally active substance (at least not acetylcholine). Further, no immediately behaviorally active substance is implicated in that the cognitive amelioration is not immediate. If the graft did exert its effects via supplying the deficient neurotransmitter than its effects should be immediately evident. Rather the profile of the recovery is suggestive of a neurophysiological instead of neurochemical action of the graft.

Investigating possible mechanisms of CC graft action is complex. Many possible factors make it an ideal candidate as tissue for therapeutic grafting in the central nervous system and to investigate effects on a lesioned cholinergic system. Both CC and cells of the central nervous system are derived from embryonic neurological tissue. Further, chromaffin cells can change their morphology depending on extracellular influences. When exposed to nerve growth factor or when released from the effects of glucocorticoids that medullary cells are normally exposed to via adrenal cortical release, in culture, chromaffin cells change from a rounded to an elongated form, sending out processes, exhibiting neuron-like morphology (Ogawa et al., 1984). This is accompanied by a reduction in catecholaminergic expression and a consequential increase in cholinergic characteristics. Neonatal rat chromaffin cells in culture have been shown to produce functional cholinergic synapses. Cultured adult bovine chromaffin cells develop the ability to synthesize [^{3}H] acetylcholine from [^{3}H] choline provided extracellularly (Carmichael, 1986). Finally, chromaffin cells contain a variety of neuroactive substances such as dynorphins (Dumont and Lemaire, 1985), enkephalins (Lewis and Stern, 1983) and basic fibroblast growth factor (Gospodarowicz et al., 1986; Schweigerer et al., 1987; Baird et al., 1991), any of which may influence cellular activity.

1.3.3a Basic Fibroblast Growth Factor

The presence of bFGF, especially, in native chromaffin cells suggests an alternative mechanism by which a graft may exert a therapeutic effect. bFGF is a member of structurally related growth factors, chracterized by their high affinity to heparin and proteoglycans (Lobb et al., 1986). It is a multifunctional molecule which, in addition to its embryogenic, mitogenic and angiogenic effects on peripheral tissue, will effect all major cell types comprising the CNS. Thus bFGF has been observed to stimulate the proliferation of astrocytes and oligodendrites and to support the survival of post-mitotic neurons (Baird et al., 1986). It is neuroprotectant (Cheng and Mattson, 1991) and is a potent neurotrophe (Garcia-Rill et al., 1991), suggesting a role in regenerative events and wound healing.

Its nearly ubiquitous presence in the nervous system is highly suggestive of a role in wound repair in the nervous system. This is supported by evidence of increased levels of bFGF in and around sites of tissue damage in the CNS as measured by *in situ* hybridization (Logan et al., 1992), Western-blot analysis (Eckenstein et al., 1991; Frautschy et al., 1991; Takami et al., 1992) and immunocytochemistry (ICC) (Finklestein et al., 1988; Fraustchy et al., 1991; Kiyota et al., 1991; Takami et al., 1992; Gomez-Pinilla et al., 1992).

1.3.3b Trophic Activity of Basic Fibroblast Growth Factor

That bFGF is present in a high concentration in CC is

suggestive of many different possible mechanisms of action to account for the ameliorating effects of the grafts. Perhaps the graft may act via the release of bFGF that stimulates growth and whose effects on host neurons is not immediately evident. Instead of actively promoting regeneration, the graft release of bFGF may act as a neuroprotectant that may be responsible for the amelioration, rescuing pathways from further degeneration. This assumes that the neurotoxic effects on the nbm and cholinergic projections are still in progress two weeks following the graft placement allowing protection via a graft secretagogue. Alternatively, the graft may exert a neurophysiological recovery on the cortex indirectly via its influence on an intermediary entity. This option includes a possible effect on support cells in and around the graft. Gliosis produced by the grafting procedure may result in the release of a neurotrophic factor that acts directly to support the cholinerginc innervation (Yoshida and Gage, 1991). Infiltrating macrophages, commonly observed in and around implant sites, may promote neuronal survival by releasing a growth factor (Frautschy, Walicke and Baird, 1991). Therefore, grafts may mediate neural plasticity by the release of growth factors or stimulation of trophic factors that supercede any resoration of function directly by the grafted cells themselves, ie. neurotransmitter release.

2 OBJECT OF PRESENT EXPERIMENTS

The main goal of the present work was threefold. Firstly, in order to further the observation that CC grafts are able to improve simple behaviors at 6 weeks post-graft, but not 5 days post-graft (Welner and Koty, 1993), we wished to test whether lesion deficits in the more complicated T-maze task would also be bettered at 6 weeks post-graft in comparison to a short 5-day post-graft interval. Secondly, since grafts of control tissues are able to better behavioral deficits in certain cases (Pezzoli et al., 1988; Przedborski et al., 1991), we wanted to test here whether a non-specific peripheral tissue would produce any effects on T-maze behavior, also at the two time points post-graft indicated above. Thirdly, we wished to establish immunocytochemical techniques suitable for the detection of chromogranin A, to identify endocrine cells, tyrosine hydroxylase, to mark catecholaminergic cells, glial fibrillary acidic protein, to visualize astrocytes and for basic fibroblast growth factor. Results are presented here for control adrenal tissue and for normal and lesioned brain; future studies will map these substances in CCs grafted to brain.

The first two objectives are addressed in Chapter 3 as a manuscript submitted for publication. The third objective, detailing the establishment of immunocytochemical techniques for the markers listed above, is elaborated in Chapter 4 of this thesis.

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Chromaffin cell grafts to the cerebral cortex of nucleus basalis magnocellularis-lesioned rats ameliorate a lesion-induced behavioral deficit: Graft specificity and duration of placement

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Summary: Grafts of adrenal chromaffin cells, but not control grafts of kidney cells, to the cerebral cortex of nucleus basalis magnocellularis (nbm)-lesioned rats are able to ameliorate lesion-induced behavioral deficits in performance of a T-maze alternation task evaluated after long-term (1.5 month) but not short-term (5 day) post-grafting intervals. An increase in lesion-induced deficits in cortical acetylcholinesterase staining is also evident in the chromaffin cell grafted group, but not the kidney cell grafted group, at 1.5 months post-grafting.

One of the neurological hallmarks of Alzheimer Disease (AD) is degeneration of the nucleus basalis of Meynert (nbM) and its corresponding projections to the cortex[25]. This is accompanied by a reduction in cortical cholinergic activity[2]. Traditionally, this neural pathology has been linked to the memory deficits characersitic of AD. Consequently, lesioning of the analogous structure in the rat, the nucleus basalis magnocellularis (nbm), has been considered to model certain aspects of AD pathology and its behavioral consequences[8,15,21,22].

Much recent testing has studied the ability of transplanted tissues to reverse certain of these behavioral and anatomical deficits. Cholinergic enriched grafts have been evidenced to produce cholinergic reinnervation to denervated cortex[9,21] and have been shown to reverse behavioral deficits in T-maze[21,23], radial arm maze tasks[13], swim maze[6] and retention of passive avoidance tasks[6,9]. Furthermore, these behavioral ameliorations have been evidenced to occur consequent to the reinduction of cholinergicity in the host cortex; neither the beneficial behavioral effects nor the enhancement of cortical cholinergicity occur in animals receiving grafts of non-cholinergic brain tissue implying that the cholinergic systems in the cortex are involved in the behavioral amelioration[13,21].

Research from our lab has shown that similar increases in lesion-induced deficits of cholinergic markers in the host cortex can be produced by placement of chromaffin cell (CC)

grafts in the cerebral cortex of nbm-lesioned rats; these neurochemical ameliorations are coincident with improvements in T-maze spatial alternation behavior tested 3 months post-grafting[23]. The rationale for using grafts of CC's, which are normally catecholaminergic in nature, to investigate effects on functions following a lesion of a cholinergic pathway is based on the ability of adrenal CC's to express neuronal and cholinergic phenotypes under certain conditions, such as when removed from the high levels of circulating glucocorticoids secreted by the adrenal cortex and especially in the presence of nerve growth factor (see 23). Further, CC's contain a variety of neuroactive substances such as dynorphins[4], enkephalins[16] and basic fibroblast growth factor[1,11,20] which, in of themselves, may have neuroprotective effects on the cortical tissue of the host lesioned rat. The mechanism by which these grafts are having their behavioral effects is presently under investigation.

In addition to the betterment in spatial alternation behavior in the T-maze tested at three months[23], we have recently witnessed that certain lasting deficits on a battery of simple sensorimotor and attentional measures that were produced by nbm lesioning could be significantly ameliorated following the implantation of the CC grafts tested at 1.5 months; these improvements were not seen 5 days post-grafting[22]. The duration of graft placement therefore becomes an important question which can give us some information as to

possible mechanisms of action of these grafts (see discussion). Therefore, the first objective of the present study was to determine whether effects in the cognitive task using the T-maze would follow the same time course, that is, would be evidenced at 1.5 months but not after short-term (5 day) placement. In addition, previous work had shown that grafts of non-cholinergic, control brain tissue did not produce betterment of lesion-induced deficits in spatial alternation behavior; a non-specific peripheral tissue has so far not been employed in our studies investigating the effects of CC grafts[22,23]. A second objective of this study is , therefore, to employ a sham graft of peripheral tissue to study the question of tissue specificty in these grafting studies; as the choice of cells for a sham graft, we use suspensions of kidney cells (KC).

In the present experiments, male rats (Sprague-Dawley, 175-200g; Charles River Laboratories, Montreal) were trained on a paired-trial alternating choice task in the T-maze. Prior to training, rats were fed a restricted diet of approximately 5% of their normal body weight until they reached 80% of their free-feeding weight. Thereafter, they were fed approximately 6.5% of their normal body weight which served to maintain them at 80%.

A total of seventy-seven rats were pretrained on a paired-trial alternating choice task in the T-maze. Rats were introduced to the maze by allowing them free access for 3

minute periods six times per day for three days. Following this rats were administered paired trials six times/day for four days/week until they reached a criterion of 80% correct responding. Rats were placed at the start of the long arm of a T-shaped maze which had one short arm blocked off; the rat was therefore forced to run into the unblocked arm to receive a food reward. On the next trial of the pair, the barrier was removed and the rat was free to make a choice of entering the previously blocked arm to get another food pellet and score a correct response or to enter the previously unblocked arm, receive no reward, and score an incorrect response. The choice was randomnly varied between left and right.

Sixty three of the pretrained rats were lesioned; the remaining comprised the unoperated control group. Rats were anesthetized with Nembutal (50mg/kg, i.p.) and placed in a stereotaxic apparatus. Lesioning consisted of bilaterally injecting 0.12M quisqualic acid dissolved in 0.1M phosphate buffer (pH 7.4), in two sites of each nbm. Each injection was delivered over a period of four minutes to the following coordinates: A: +0.2 mm (bregma), L: ±3.4 mm (midline), V: - 7.0 mm (dura) and A: +1.0 mm, L: ±2.6 mm, V: - 7.3mm with the incisor bar set at 5.0 mm above the interaural line. Twenty-two percent, or 14 of 63 rats, died during the lesioning procedure; of these, three died preoperatively due to the anesthetic and the remaining eleven died from effects of the lesion. Ten days following lesioning, 17 of the lesioned rats

received transplants of the control graft, consisting of a suspension of KC (about 2,000 cells/μl). Eighteen received a suspension of CC (about 2,000 cells/μl) derived from the adrenal glands of an equal number of adult donors. While under Nembutal anesthesia as described above, animals received two μl aliquots of one of the cell suspensions into six sites extending over the frontal and parietal cortex. The coordinates were: A: +2.7 mm (bregma), L: ±4.0 mm (midline), V: -3.5 mm (dura); A: +0.2 mm, L: ±5.0 mm, V: -2.0 mm; A: -2.3 mm, L: ±5.0 mm, V: -2.3 mm with the incisor bar set at zero. Chromaffin cells were isolated as referenced by Welner et al., 1990[23]. The remaining 14 of the lesioned animals comprised the lesion alone group.

To form the five day time point group, following the grafting procedure, six rats were taken from the 14 total control rats, eight rats were taken from the 14 lesioned animals, seven rats were taken from the 17 KC grafted animals, and nine animals were taken from the 18 CC grafted animals for retesting on the T-maze. Retesting consisted of 24 trials, 6 per day, with the number of correct responses being recorded. Six weeks later, the remaining rats were retested, as above, however, an initial day of maze 'familiarization' was included wherein the rats were administered six unscored trials. Results for each time point, from the behavioral testing and from the rating of the neurochemical marker, below, were analyzed by one way analysis of variance (ANOVA)

followed by the Fisher's Least Significant Difference post-hoc test.

Table 1: Difference between pre- and post- scores on T-maze performance

	5 day	(N)	1.5 month	(N)
Control	-3.7 ± 4.1	(8)	8.3 ± 3.3	(8)
Lesion	7.6 ± 4.7	(6)	18.7 ± 3.8 §	(6)
Lesion + KC	13.7 ± 4.4 §	(7)	14.4 ± 2.9	(10)
Lesion + CC	11.9 ± 3.9 §	(9)	4.2 ± 3.1 *	(9)

§ $p<0.05$ compared to control group for the respective time point

* $p<0.05$ compared to lesion group for the respective time point

The behavioral effects from both the 5 day and the 1.5 month time point post-grafting experiments are listed in Table 1 as the difference betweeen pre- and post-surgery scores of performance in the T-maze for control, lesion alone, lesion + KC and lesion + CC groups of animals. It can be seen that, at the 5 day time point, whereas the animals in the control group improve their performance, indicated by the negative pre minus post score, all other groups performed less well (ANOVA: $F_{(3,26)} = 3.54$; $p<0.028$). The lesion alone group scores, while not significantly different from control values, show a trend

towards being reduced; this coincides with the partial depletion of cortical AChE observed at this time point (Figs. 1, 3, 5). The groups receiving either the KC grafts or the CC grafts are significantly different from controls but not from the lesioned rats, thus clearly, neither of the grafts seem to have any beneficial effect at this short-term time point (ANOVA: $F(3,29) = 3.71$, $p<0.022$).

Analysis of variance of the 1.5 month behavioral data produced an F value of 3.71, $p<0.022$. Post-hoc testing indicated that a significant difference was produced by the nbm lesion compared to control, as expected. Moreover, animals receiving the KC graft were not significantly different from the lesion group. In contrast, the performance of animals receiving the CC graft was significantly ameliorated compared to that of the lesion group and the lesion plus KC group while it was not significantly different from the control group. Thus, at this time point, CC grafts appear to ameliorate the behavioral deficits induced in the lesioned alone animals; KC grafts have no effect.

To verify the lesion in the nbm and to investigate potential cholinergic changes in the cortex, staining for AChE, a marker for cortical cholinergic neurons, was carried out histochemically in tissue from a subgroup of animals randomnly chosen. Thus, following the behavioral retesting, animals were anaesthetized and perfused intracardially with 100-200 ml of 0.1M phosphate buffered saline (PBS), pH 7.3,

followed by 250-350 ml of 4% paraformaldehyde solution in PBS. Brains were removed and postfixed for one hour and sunk in 15% sucrose in 0.1M PBS, pH 7.4, containing 0.1M sodium azide for 24 hours. Brains were then sliced on a cryostat to a thickness of 20 μm. AChE staining was performed according to the method of Karnovsky and Roots[14]. Staining in the nbm and cortex was assessed by first rating the control group to determine the range of staining that control samples spanned. The three groups of lesioned, lesion with KC graft and lesion with CC graft at both time points were then assessed by a rater unaware to which group the samples belonged. Staining in the regions of the cortex, from +3.2 to -2.8 mm from bregma, and at the level of the nbm was assessed according to the method described by Welner et al.[24]. The rating scale used was 3 = very intense staining, 2 = intense, 1 = poor and 0 = absent. The left and right hemispheres were judged separately.

Analysis of the AChE staining at the 5 day time point is reflective of the behavioral data. As illustrated by Fig. 1, analysis of AChE staining in the nbm confirmed the lesion with the lesioned alone, lesion + KC and lesion + CC showing a loss of AChE-positive magnocellular cells in the nbm, in comparison to the control group. Analysis of variance of this data produced an F value of 8.61, $p<0.001$ with post-hoc testing indicating a significant reduction of AChE in the nbm in both the right and left hemispheres for all groups compared to

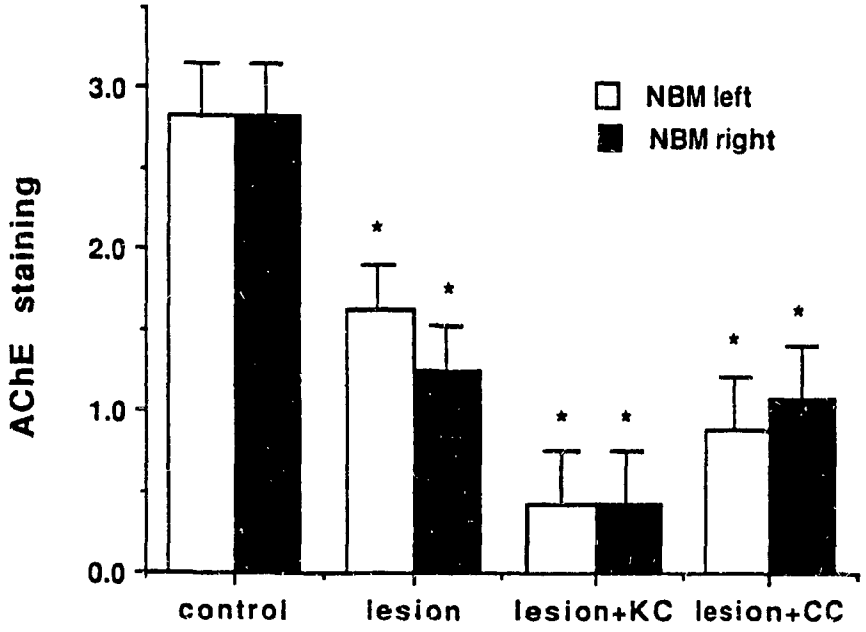

Fig. 1 Rating of AChE staining in the nbm from respresentative animals from the 5 day time point confirms the effectiveness of the lesion. N= 3, 4, 3, 3 for control, lesion, lesion + KC and lesion + CC groups, respectively.

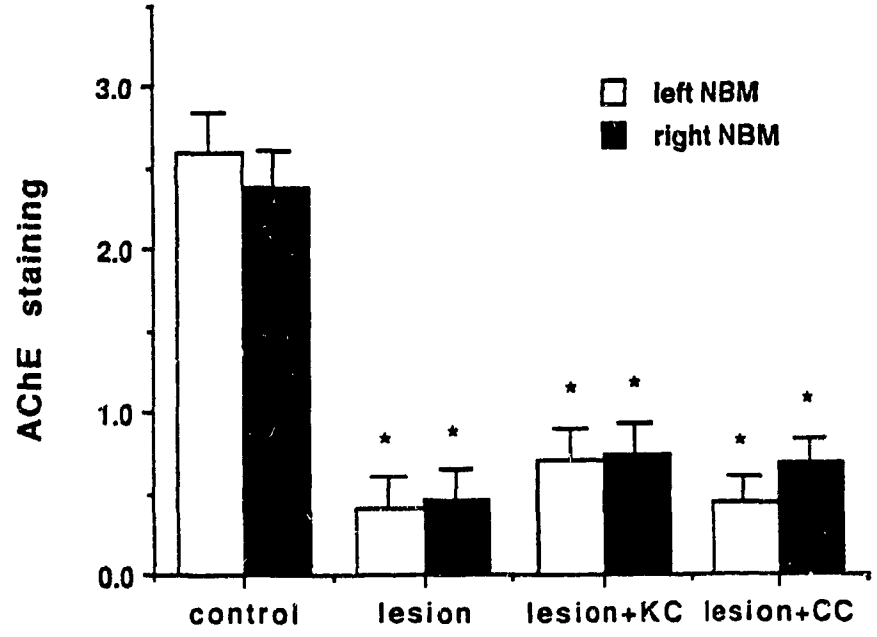

Fig. 2 Rating of AChE staining in the nbm from respresentative animals from 1.5 month time point confirms the effectiveness of the lesion. N= 4, 6, 6, 9 for control, lesion, lesion + KC and lesion + CC groups, respectively.

control. Analysis of the AChE staining in the cortex (Fig. 2) indicates a clear cholinergic deficit in the cortex as induced by the lesion. Analysis of variance of this data produced an F value of 3.98, $p<0.005$ with post-hoc testing indicating significant differences in both hemispheres between the control group and all other groups.

Analysis of the AChE staining in the nbm at the 1.5 month time point confirmed the effectiveness of the lesion (Fig. 3). The lesioned alone, lesion + KC and lesion + CC show a pronounced loss of AChE-positive magnocellular cells in the nbm, in comparison to the control group. Analysis of variance of this data produced an F value of 15.37, $p<0.001$. Post-hoc testing indicated a significant reduction of AChE in the nbm in both the right and left hemispheres. Furthermore, these deficits are greater than at the 5 day time point. This is noteworthy in that this may reflect the degeneration of cells within the nbm over time. Analysis of the AChE staining in the cortex indicates a clear cholinergic deficit in the cortex as induced by the lesion (Fig. 4). Analysis of variance of this data produced an F value of 12.76, $p<0.001$ and post-hoc testing indicated significant differences in both hemispheres between the control group and all other groups. However, the group receiving the CC graft shows an amelioration of AChE levels in both hemispheres; this group is significantly different from both the lesion alone and KC grafted groups. This, however, is a partial amelioration as a complete return

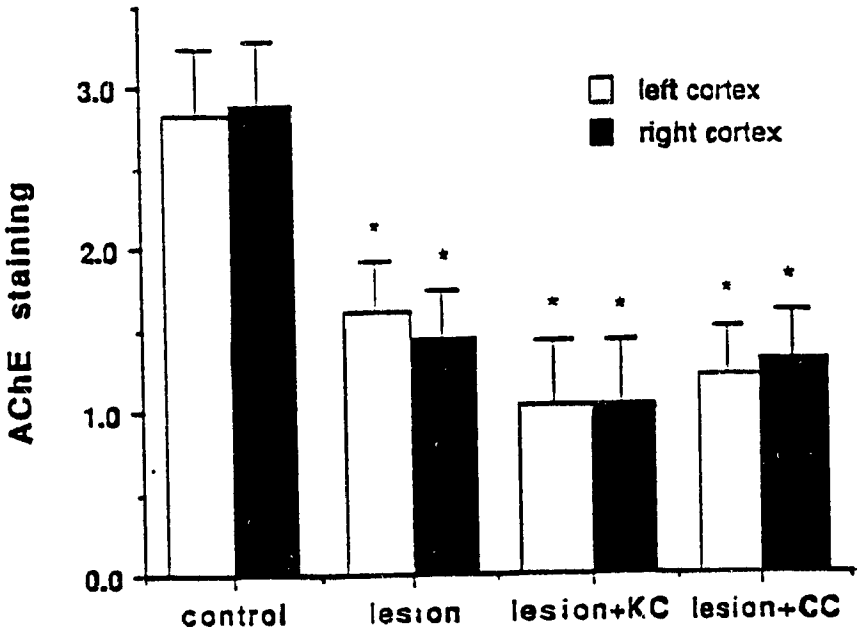

Fig. 3 Rating of AChE staining in the cortex at the 5 day time point. Note the significant difference between the control group and the remaining three, indicating the absence of cholinergic amelioration at this time point. N= 3, 4, 3, 3 for control, lesion, lesion + KC and lesion + CC groups, respectively.

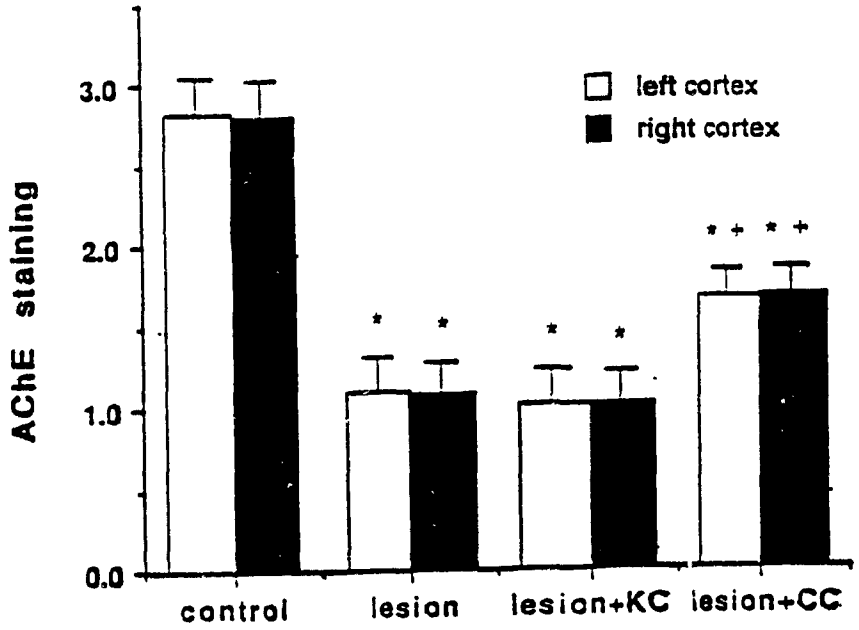

Fig. 4 Rating of AChE staining in the cortex at the 1.5 month time point. Although there is a significant difference between the control group and the remaining three groups, as in the previous figure, only the group receiving the CC grafts shows a significant recovery of AChE staining compared to either the lesion or lesion plus KC graft group. N= 5, 6, 6, 9 for control, lesion, lesion + KC and lesion + CC groups, respectively.

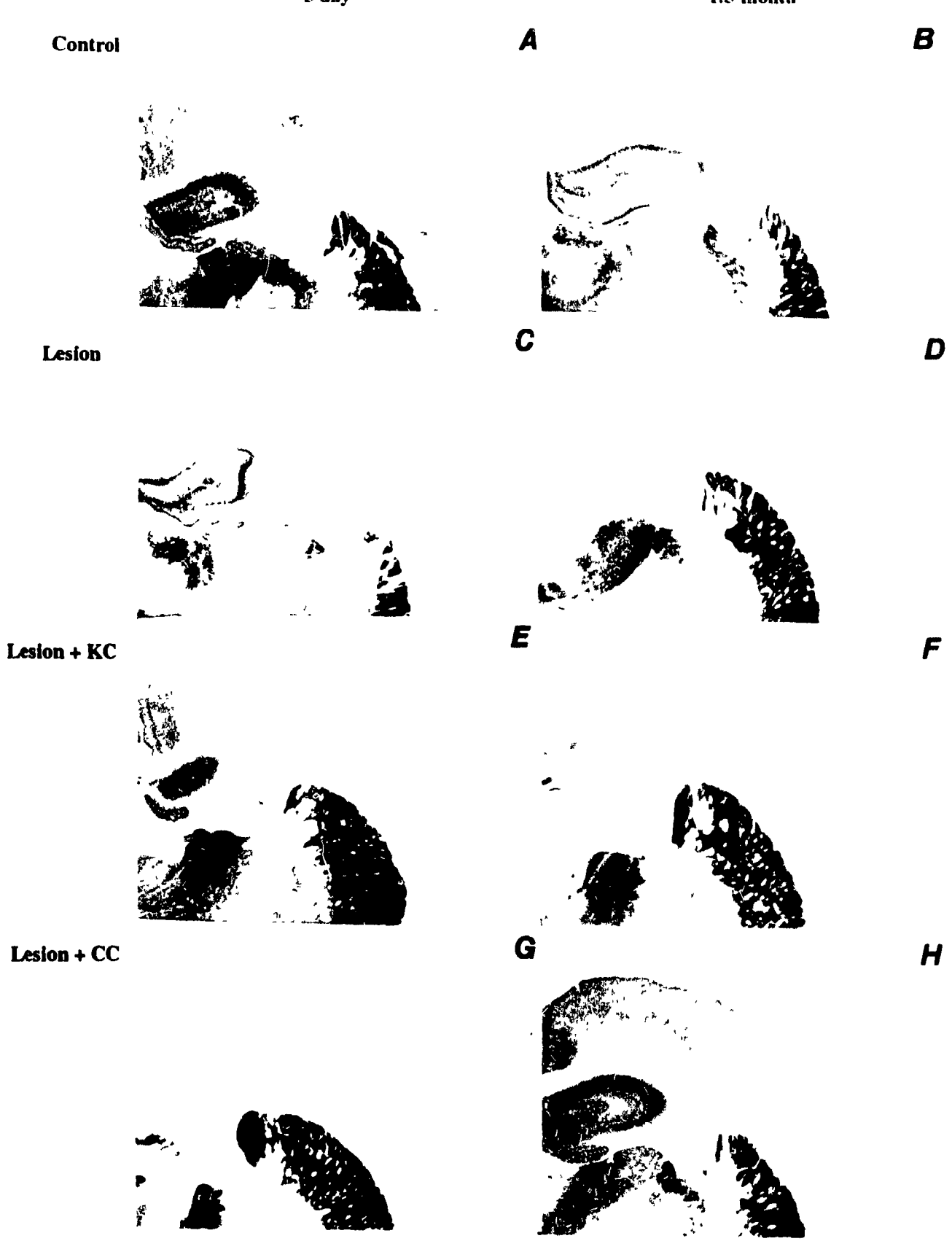

Fig. 5 Photomicrographs of acetylcholinesterase staining in representative cerebral cortices from the control (A,B), lesion (C,D), lesion + KC graft (E,F) and lesion + CC graft (G,H) groups at both the 5 day (A,C,E,G) and 1.5 month (B,D,F,H) time points. Note the prominent loss of AChE positive neurons in the lesioned animal and the lesioned plus KC grafted animal at both time points. Moreover, note the presence of enhanced AChE staining in the cortex of a lesioned plus CC grafted animal only at the 1.5 month time point. Magnification x 5.3.

to control levels is not evidenced; the values for the CC group remain significantly different from those of the control group.

Fig. 5 shows photomicrographs of the staining pattern seen in the cerebral cortex of representative control (A,B), lesioned alone (C,D), lesion + KC (E,F) and lesion + CC (G,H) animals at 5 days and 1.5 months, respectively. The photomicrographs indicate that a partial cholinergic amelioration accompanies the behavioral amelioration in nbm lesions following long-term placement of CC but not KC.

Thus, CC grafts to the cerebral cortex of lesioned animals are able to reverse lesion-induced deficits in performance of a task involving spatial memory at 1.5 months post-grafting. Further, this effect was not evidenced in the KC control grafted group and, as such, suggests that it is due to the type of cell grafted and not a result of the grafting procedure itself. This finding is further supported by the increased cortical staining in host brain which is evident in the CC grafted but not the lesion alone or KC grafted animals.

These results confirm previous reports of the potentially therapeutic effects of CC grafting to the cortex. As our lab has evidenced an amelioration of T-maze performance at the 3 month time point and a further amelioration of certain deficits on simple sensorimotor or attentional measures at 1.5 month post-grafting, it is interesting to note from the present study that an amelioration of the T-maze performance

can also be seen at 1.5 months post-grafting.

The present results are strong evidence against the notion that CC grafts work directly via the diffusion of a behaviorally active substance. Firstly, no behavioral amelioration is immediately evident; if the graft did exert its effects via supplying a behaviorally active substance, such as the deficient neurotransmitter, then its effects should be immediately apparent. Rather the profile of the recovery is suggestive of a delayed action of the graft. Perhaps the graft takes time to associate with host tissue or may act via release of a neuroprotectant or neurotrophe whose effects on host neurons is not immediately evident. This is especially significant in light of preliminary findings from our lab that CC grafts contain basic fibroblast growth factor (bFGF)[26]. As mentioned earlier, native chromaffin cells are known to contain bFGF[24], a multifunctional molecule which, in addition to its embryogenic, mitogenic and angiogenic effects on peripheral tissue, will effect all major cell types comprising the CNS. It is neuroprotective[3] and is a potent neurotrophe[10], suggesting a role in regenerative events and wound healing. Therefore, the behavioral and physiological effects observed, post-grafting, may possibly be mediated through bFGF action.

Recent evidence has suggested that allocortical cholinergic systems that do not lay within the nbm's primary cortical projection may mediate many cognitive behaviors[8,18,19].

Lesions to cholinergic innervations of the hippocampus[12,17] and the medial prefrontal cortex[5,7], both of which originate from basal forebrain nucleii rostral to the nbm, have been evidenced to produce mnemonic deficits[19]. Our results strongly indicate that, at least for the T-maze task, cortical systems are heavily implicated. Due to the specific placement of the grafts in the frontal and parietal cortex, and the localized increase in cholinerginicity to these regions, the seat of this behavioral task appears to be cortical in nature.

In summary, transplants of adult chromaffin cells, but not kidney cells, to the cerebral cortex of lesioned animals are able to ameliorate the deficits in performance of T-maze behavior and partially ameliorate the cholinergic deficits caused by nbm lesioning as early as 1.5 months following grafting. These results seem to indicate that the graft-induced amelioration, both behaviorally and neurochemically, is cell type specific and not a procedural phenomenon.

Practically, the use of chromaffin cells offers a source of neuroactive tissue that has certain advantages inherent to its use. Firstly, since chromaffin cell tissue can be 'autografted', problems with graft rejection may be minimized and the use of immunosuppressants unnecessary; in the same vein, however, it should be acknowledged that problems associated with dual surgery, that is, retrieval of CC tissue and then cortical transplantation, may exist. Secondly, this tissue offers a more ethically acceptable source than

embryonic or fetal tissue for transplant therapy. Further, this area of research provides leads as to the possible factors responsible for the observed effects of the graft and, if indeed a neurotrophic secretagogue such as bFGF is found responsible for the observed behavioral and physiological effects, the therapeutic use of this tissue would not be limited to any specific neurochemical system.

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4 VISUALIZATION OF GRAFT CONSTITUENTS

After establishing the ameliorative properties of CC grafts both behaviorally and neurochemically, investigating the mechanism of action of the graft would be the next logical step. This may be accomplished by first assessing the presence of neuroactive compounds expressed in the graft over time. These substances would include, but not be limited to, the growth factor bFGF, a marker of catecholaminergic activity tyrosine hydroxylase (TOH), and a marker of cholinergic activity, choline acetyltransferase (ChAT). It would also be of interest to characterize the changes in the tissue surrounding the graft by measuring the levels of glial fibrillary acidic protein (GFAP), a marker of gliosis.

Immunocytochemistry (ICC) would be the prime candidate as a technique to detect the presence of these substances within the graft. As such, the next phase of the experiment involved establishing protocols for these techniques. Following this, these protocols would then be employed on grafted tissue directly.

4.1 Immunocytochemistry Overview

Immunocytochemistry is a technique which allows visualization of chemically distinct substances within tissue. The name is derived from the fact that the technique utilizes immunological reactions between molecules derived from the immune system, <u>in vitro</u>, to label the desired substances. The reason for this is that macromolecules, particularly proteins

and polysaccharides, that occur naturally in tissues can be extracted and used as antigens. Upon introduction to a host animal they give rise to the formation of antibodies which will attach to the antigen with a particular specificity and affinity.

These primary antibodies, upon extraction from the host, can attach to the antigen in tissue where that antigen is found. By labelling the antibody, the desired antigen can be visualized. It is this in vitro immunological attachment of a marker to the antigen that is the basis for ICC.

The two major types of ICC techniques can be distinguished on the basis of whether the marker compound used for identification of the site of the antigen-antibody reaction is attached directly to the antibody or indirectly to an anti-immunoglobulin intermediary. Directly labelling the primary antibody greatly reduces the reactivity of the antibodies. Furthermore, the direct antibody method is limited in that a differently labelled antibody preparation is needed for each antigen to be localized. For this reason, the more sensitive ICC labelling technique is the indirect method and is therefore the one primarily used.

Indirect labelling methods employ intermediary immunoglobulins that complex with both the antigen and the marker compound. Theoretically, the procedure is quite simple. A primary antisera that has been raised against the desired antigen is incubated with tissue, binding to molecules of the

antigen. Following this, a secondary antibody which recognizes the primary antibodies as an antigen is added. This secondary antiserum is prepared by injecting the IgG of the species used for the production of the primary antiserum, in an animal of a different species.

Visualization of the antigen consists of adding a substrate to which the secondary antibody will immunologically attach. This substrate may be labelled via fluoresence. More common is the use of a complex of substrate and an enzyme. The enzyme initiates the reduction of an electron-capturing marker molecule, which changes color and precipitates, labelling the antigen-antibody complex.

This is only a brief overview of some of the basic principles of ICC. Furthermore, many refinements have been applied to these basic principles, further improving the technique. (Please see section APPENDICES for more detailed explanations.)

4.2 Rationale

As mentioned above, the next logical step of the study employing grafts of CC would be to address the characterization of expression of neuroactive substances by the graft. As ICC techniques were new to our lab, establishing methodology to visualize the following compounds was a primary goal. First, since bFGF seems to be a promising neurotrophe, it was of prime focus. Also, the detection of a marker of catecholaminergicity, tyrosine hydroxylase, would serve to

resolve whether the graft changes its original phenotypic expression when in the surrounds of cortical tissue. Chromogranin A, a marker of endocrine cells, would serve to visualize whether CCs maintained endocrine morphology after grafting. Furthermore expression of GFAP in and around the graft site would further serve to characterize the nature of the graft effects.

4.3 Materials, Methods and Results

4.3.1 Tissue Preparation

In the present experiments, male rats (Sprague-Dawley, 350-400g, Charles River Laboratories, Montreal) were anaesthetized and perfused intracardially with 100-200 ml of 0.1M phosphate buffered saline (PBS), pH 7.3, followed by 250-350 ml of 4% paraformaldehyde solution in PBS. Brains were removed and postfixed for one hour and sunk in 15% sucrose in 0.1M PBS, pH 7.4, containing 0.1M sodium azide for 24 hours.

4.3.2 Slide Mounted Immunocytochemistry

Brains and adrenal tissue were sliced on a Leitz or Bright cryostat to a thickness of 20μm and slide mounted. Immunocytochemistry was carried out using the Avidin-Biotin complex(ABC) Vectastain Elite Kit(Vector Lab). Buffer used for all immunocytochemistry was 0.01M PBS, pH 7.3. All slices were first bathed in 0.01% hydrogen peroxidase/ methanol solution to eliminate endogenous peroxidase activity, followed by a 20 minute wash in buffer. Sections were then incubated for 30 minutes in PBS containing 1.67% normal goat serum. The

primary antibodies were incubated on slide mounted sections for 24 hours at 4°C. Polyclonal antibodies to bFGF for the primary antiserum were kindly provided by Dr. Andrew Baird of The Whittier Institute for Diabetes and Endocrinology, La Jolla, California. These rabbit antibodies are raised against a 1-24 synthetic fragment of bovine bFGF. Slices were washed for 20 minutes with buffer and then incubated with the secondary biotinylated antibody for 30 minutes. After a 20 minute washing, slides were incubated with avidin-PAP complex for 60 minutes and then developed in 0.25% 3,3 diaminobenzidine tetrahydrochloride(DAB) solution. Sections were then dehydrated in graded alcohols, cleaned with xylene and mounted with permount.

Three controls were used during the ICC experiment (see Table A). Firstly, as a negative control, some slices, during each attempt, were exposed to every step except incubation with the primary antisera. This eliminated the possibility of misidentifying nonspecific labelling due to nonspecific binding of the secondary antibody. Secondly, a positive control tissue in which the antigen is known to be present was stained to better elucidate specific staining in the experimental tissue. Thirdly, ICC on another set of slices is run alongside the experimental slices using a different antisera which is known to stain, when employing the same protocol as the primary antisera of interest. This control served to eliminate the possibility that a procedural error

Table A. Immunocytochemical controls

	Negative Tissue Control	Positive Tissue Control	Standard Procedural Control	Primary Antiserum Control
Procedure	Omission of primary antiserum	Use of known immunoreactive tissue	different antiserum, original protocol	Preincubation of primary antibody with antigen prior to use
Objective	Identification of non-specific immunoreactivity	Identification of specific immunoreactivity	Identification of procedural error	To ascertain specificity of antigen/antibody reaction

might have occurred which prevented a positive signal from being obtained. One final control must be done to ascertain that the primary antiserum was indeed responsible for specific staining. Before the ICC trial is performed, the primary antiserum should first be incubated with the antigen that it was raised against. This should serve to block any binding sites on the antibody and thus eliminate all specific binding. At this time, this last control was the only control not as yet performed.

Furthermore, a series of experiments were performed as methodological controls (see Table B). In separate trials, different steps of the ICC protocol were omitted or included. If any of the trials, other than the one that included all three steps produced positive staining, this would identify that antiserum preparation or combination of antisera as a source of nonspecific binding , producing a false positive signal.

Initial trials with the polyclonal antibody to bFGF on control tissue were succesful (See Figure A (low power), B (high power)). As evidenced in Figure A, cells of the adrenal medulla(am) are stained darker then the surrounding cortex. Also, almost all cells of the glomerulosa zone (gz), surrounding the cortex are immunoreactive. Few cells in the intermediary fasciculata zone (fz) and reticularis zone (rz) appear immunoreactive. Under higher magnification in Figure B, the specificity of staining is evident with cellular

Table B. Procedural Control								
Trial ID	1	2	3	4	5	6	7	8
Primary Antiserum	θ	+	θ	θ	+	θ	+	+
Secondary Antiserum	θ	θ	+	θ	+	+	θ	+
Tertiary Complex	θ	θ	θ	+	θ	+	θ	+
Staining Present?	no	no	no	no	no	no	no	yes

θ-omitted step +-included step
Identification of the source of potential non-specific binding

Figure A adrenal gland, polyclonal antibody to bFGF, magn. 84x

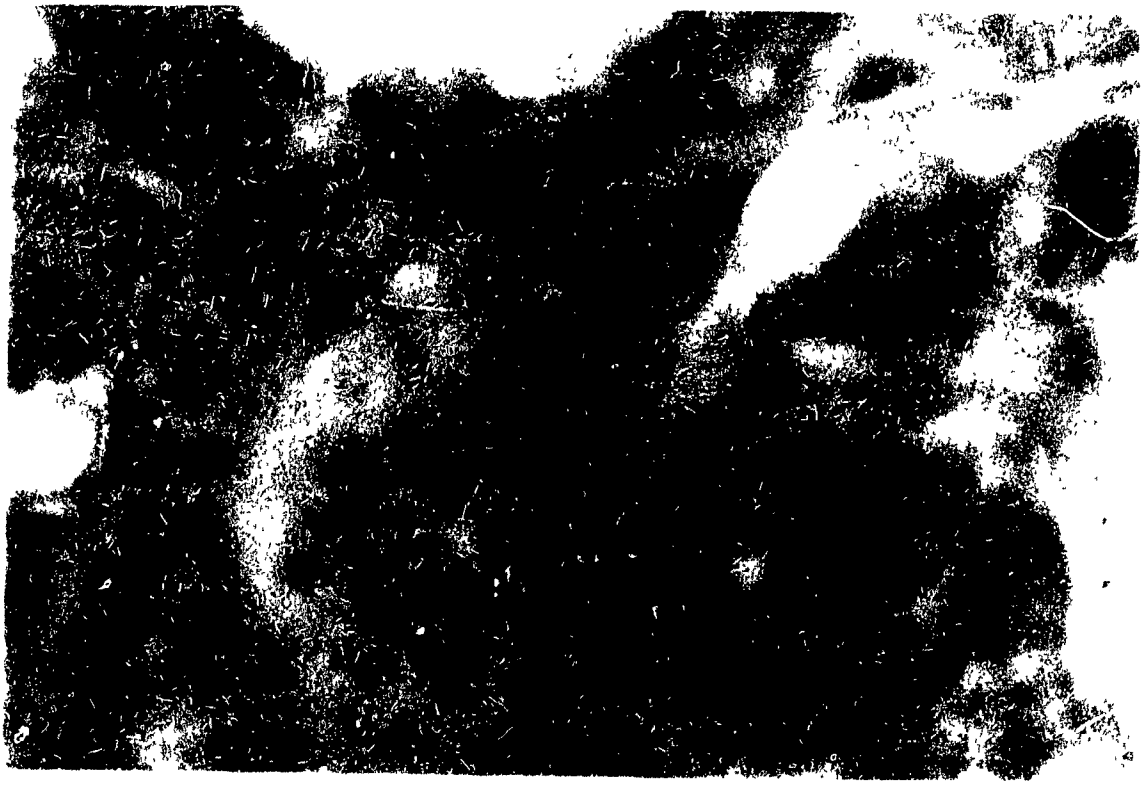

Figure B above gland, higher magn., chromaffin cells of the adrenal medulla, magn. 1340x

resolution. Medullary immunoreactive cells have darkened cytoplasm, indicating the presence of bFGF, while the nucleus of these cells does not stain, indicating the absence of bFGF. This staining is consistent with accounts of bFGF staining in the literature (Grothe and Unsicker, 1989; Grothe and Unsicker, 1990; Westermann, Johannsen, Unsicker and Grothe, 1990). Thus, initial evidence confirmed this protocol for the staining of bFGF in adrenal medullary cells.

Attempts to stain bFGF in endogenous brain tissue proved more difficult. Though the protocol worked well for chromaffin cells of the adrenal medulla, specific staining was evidenced in brain tissue only sporadically over repeated attempts. Despite several manipulations of the protocol, consistent staining of endogenous bFGF in brain tissue could not be obtained. The problem encountered was that of nonspecific staining, that is, staining which does not arise from the antibody/antigen/marker complex. The true immunoreactive signal was hidden in the intense nonspecific background staining in and around the graft. Causes of background staining can arise from many aspects of the methodology. As a result various methodologies were attempted to eliminate the cause of the high background nonspecific staining.

Background staining can be attributed to different sources (see Table C). Endogenous peroxidase activity in the tissue can lead to the inappropriate oxidization of the marker molecule, DAB, in addition to its normal oxidation by the

Table C. Sources of Background Staining			
	Endogenous Peroxidase Activity	Antiserum Concentrations	Fixation of Tissue
Problem	Inappropriate oxidation of the marker molecule	Non-specific interactions between antibody and undesired antigens	Underfixation - decomposition of desired antigen; Overfixation - crosslinking of proteins masks desired antigen
Solution	Preincubate tissue with 0.3% hydrogen peroxide solution	Decrease concentration of antiserum	Alter tissue fixing protocol

visualization complex which incorporates peroxidase. This can be eliminated by preincubating sections with 0.3% hydrogen peroxide solution prior to staining. Using concentrations of antibody thatare too high can also lead to nonspecific interactions between antibody and undesired antigens. This can be easily eliminated by increasing the factor by which an antisera is diluted. In this sense, immunocytochemistry is a challenge of balancing the increase in specific signal with the increase in nondesired nonspecific background staining. Another problem is that poor preparation or over/under fixation of the tissue will lead to problems with background staining indirectly via reducing the qualtiy of the desired signal. Underfixation may lead to denaturing of the desired antigen, while overfixation may lead to the masking of the desired antigen by cross-linked proteins. Changing the protocol of one's tissue preparation procedure is the best remedy for such problems. These examples illustrate the main premise of ICC methodology; a struggle to achieve a balance between increased specific signal and the unwanted but inevitable increased background.

These causes of high background staining being accounted for, led to the conclusion that it was the result of undesired interactions between the tissue antigens and the polyclonal antibody serum. This problem was not encountered when staining the adrenal medullary cells presumably because of the relatively larger amounts of bFGF in the adrenal gland

compared to brain tissue. In an attempt to overcome this problem, immunocytochemistry was again attempted but with a monoclonal antibody to bFGF, instead. Free-floating immunocytochemistry was attempted with the new monoclonal antibody to bFGF, a polyclonal antibody to GFAP, a polyclonal antibody to TOH and a monoclonal antibody to Chromogranin A. This last antibody was used because Chromogranin A is a constituent of endocrine cells and thus may be used as an ideal marker for CC in the graft.

4.3.3 Free Floating Immunocytochemistry

In the present experiments, brains of normal and lesioned (3 months post-lesion), male rats Sprague-Dawley, 400-500g (Charles River Laboratories, Montreal), were sliced on a sliding microtome with a freezing stage to a thickness of 20μm and free floated in 0.1M TBS buffer, pH 7.4. Unless otherwise mentioned this buffer was used throughout this protocol. The primary antibodies used were as follows: Anti-bovine bFGF, Type II (monoclonal $IgG1_k$) antibodies/ Upstate Biotechnology Incorporated, Rabbit immunoglobulins to cow GFAP/Dakopetts, TH antisera against purified bovine adrenal tyrosine hydroxylase/Eugene Tech International, Inc., Anti-chromogranin A (monoclonal IgG1 (mouse))/Cedarlane Laboratories. Sliced sections were placed in primary antisera of the compositions listed in Table D. Slices were incubated in primary antisera for 48 hours at 4°C. After sections were washed for 15 minutes in TBS, sections were incubated for 1 hour, 4°C with

biotinylated secondary antibody (Dakopetts) sera of the compositions listed in Table E.

Following a 15 minute wash sections were incubated with the Avidin-Biotin complex (ABC) Vectastain Elite Standard (Vector Lab) for 2 hours at 4°C. Sections were then washed for 15 minutes in TBS buffer, followed by 10 minutes in 50mM Tris buffer, pH 7.6. Visualization consisted of a 10 minute incubation in solution of 0.05% DAB in 50mM Tris buffer, pH 7.6, followed by 10 minutes in the same solution with 0.3% hydrogen peroxide and 0.032% $NiCl_2$ solution. The $NiCl_2$ solution served to intensify the DAB staining, rendering a blue-black product instead of a brown product. Sections were then washed for 30 minutes in TBS buffer, mounted on slides, dehydrated in graded alcohols, cleaned with xylene and mounted with permount. The same controls used for slide mounted ICC were employed during the free floating ICC. Trials with all antibodies, monoclonal and polyclonal produced excellent results. Initial results with the antibody for bFGF revealed a characterisitic distribution in both the adrenal gland and endogenous brain tissue. As evidenced in Figure C, staining of the adrenal medullary control tissue is in a manner consistent with that produced with the polyclonal antibody. Specific staining was evident in cells of both the adrenal medulla (am) and the glomerulosa zone (gz). Few cells in the intermediary fasciculata zone (fz) and reticularis zone (rz) appear immunoreactive. Under higher magnification in Figure D, these

Table D. Primary Antisera Compositions				
	Primary Antiserum	Normal Serum	Triton X-100	TBS 0.05M pH 7.4
bGFG (Monoclonal)	0.5% (1:200)	1% (horse)	0.05%	98.45%
TOH (Polyclonal)	0.2% (1:500)	1% (goat)	0.05%	98.75%
GFAP (Polyclonal)	0.01% (1:1000)	1% (goat)	0.05%	98.94%
Chromogranin A (Monoclonal)	98.95%	1% (horse)	0.05%	0%

Table E. Secondary Antisera Compositions				
	Secondary Antiserum	Normal Serum	Triton X-100	TBS 0.05M pH 7.4
Antimouse IgG (for monoclonal)	3.33% (1:30)	1% (horse)	0.05%	95.62%
Antirabbit IgG (for polyclonal)	0.5% (1:200)	1% (goat)	0.05%	98.45%

Figure C adrenal gland, monoclonal antibody to bFGF, magn. 84x

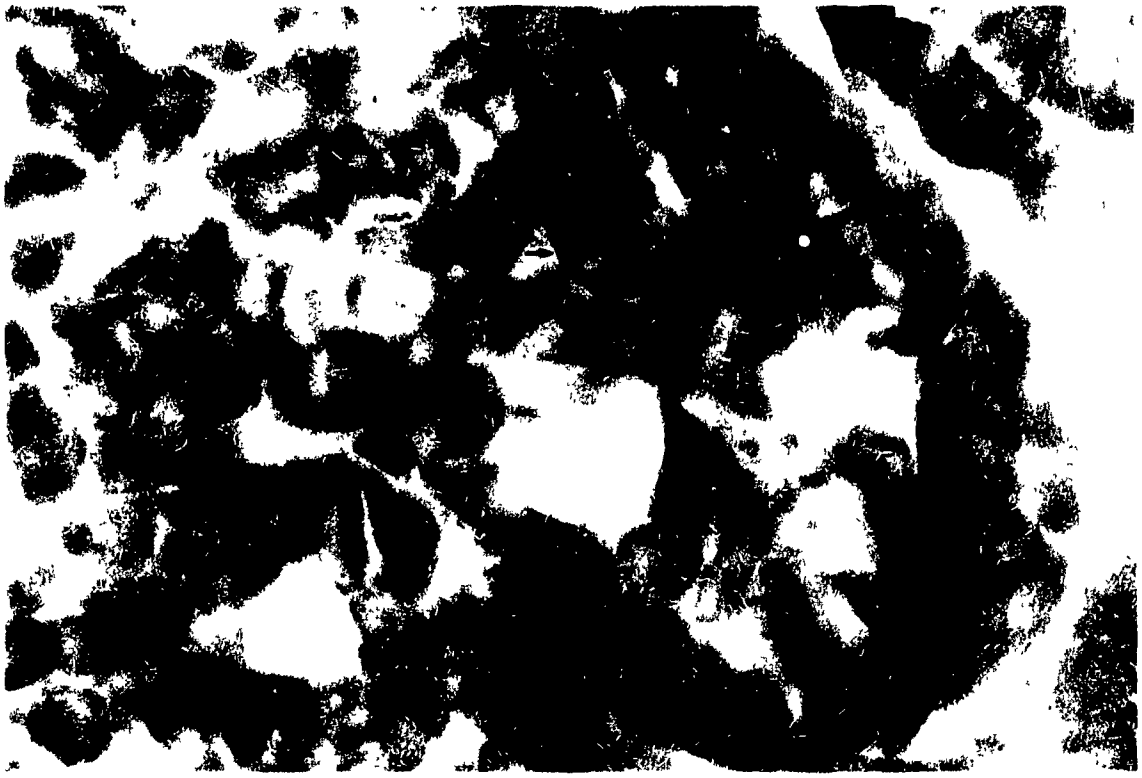

Figure D above gland, higher magn., chromaffin cells of the adrenal medulla, magn. 1340x

immunoreactive cells of the medulla have darkened cytoplasm, while the nucleus of these cells does not stain.

In normal brain tissue, specific staining is evident throughout the brain, occuring almost exclusively in nonneuronal populations of ependymal cells (Figure E) and astroglia, in areas such as the cortex (Figure F), the basal ganglia (Figure G) and the septum (Figure H). Under higher power, Figure I, astroglia are evident, characterized by a dark staining nucleus, and distinctive stellar shaped processes extending away from the nucleus. These extensions are not as well defined as when stained with GFAP, indicating that bFGF predominates in the nuclear and perinuclear space of the astrocyte with lesser amounts being present in the further reaches of the processes. The only neuronal population that appears to constituatively express bFGF is CA2 cells of the hippocampus. Figure J shows large dark region of intensely staining pyramidal cells. Higher magnification of the CA2 region from Figure J, shows that bFGF was concentrated in the nucleus and within proximal dendritic process of pyramidal neurons (Figure K). These findings are consistent with previous findings using this antibody (Gomez-Pinilla et al., 1992; Woodward et al., 1992). In the injured brains, in addition to the normal distribution of bFGF-immunoreactive cells, a marked increase in bFGF staining was evident surrounding the area of injury in the nbm. This increased bFGF immunoreactivity was due to dense accumulations of bFGF-

Figure E ependymal cells of the lateral ventricle, monoclonal antibody to bFGF, magn. 211x

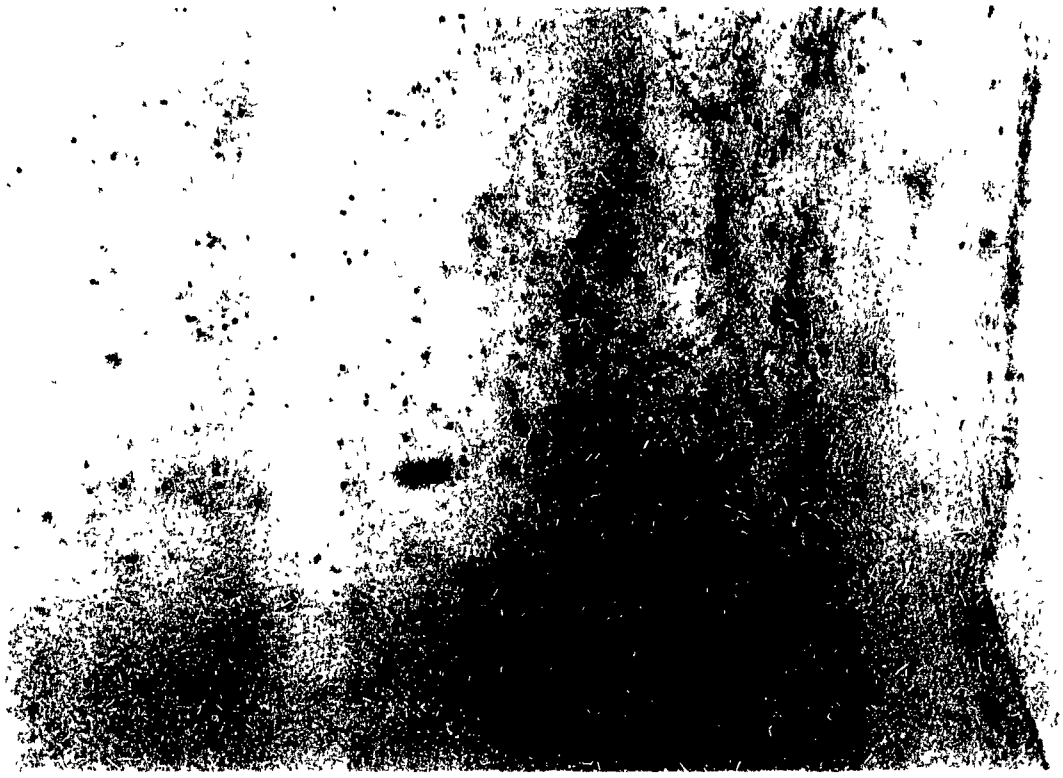

Figure F astrocytes, cortex, monoclonal antibody to bFGF, magn. 134x

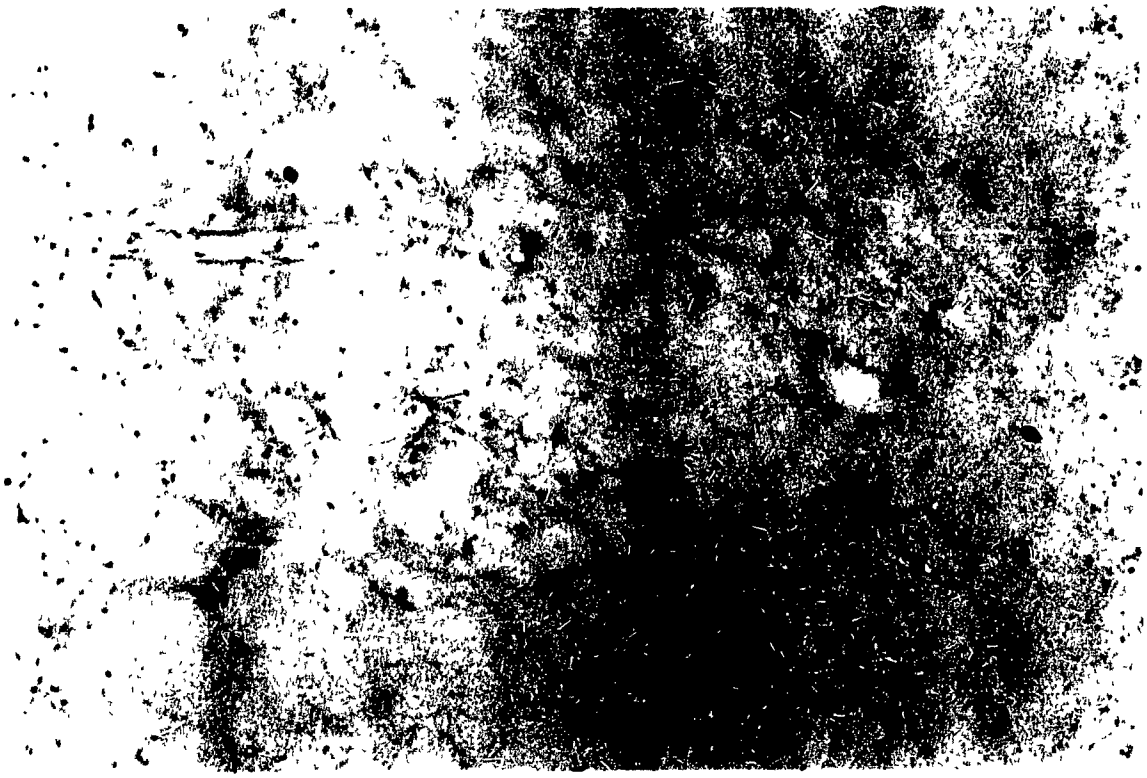

Figure G astrocytes, basal ganglia, monoclonal antibody to bFGF, magn. 134x

Figure H astrocytes, septum, monoclonal antibody to bFGF, magn. 134x

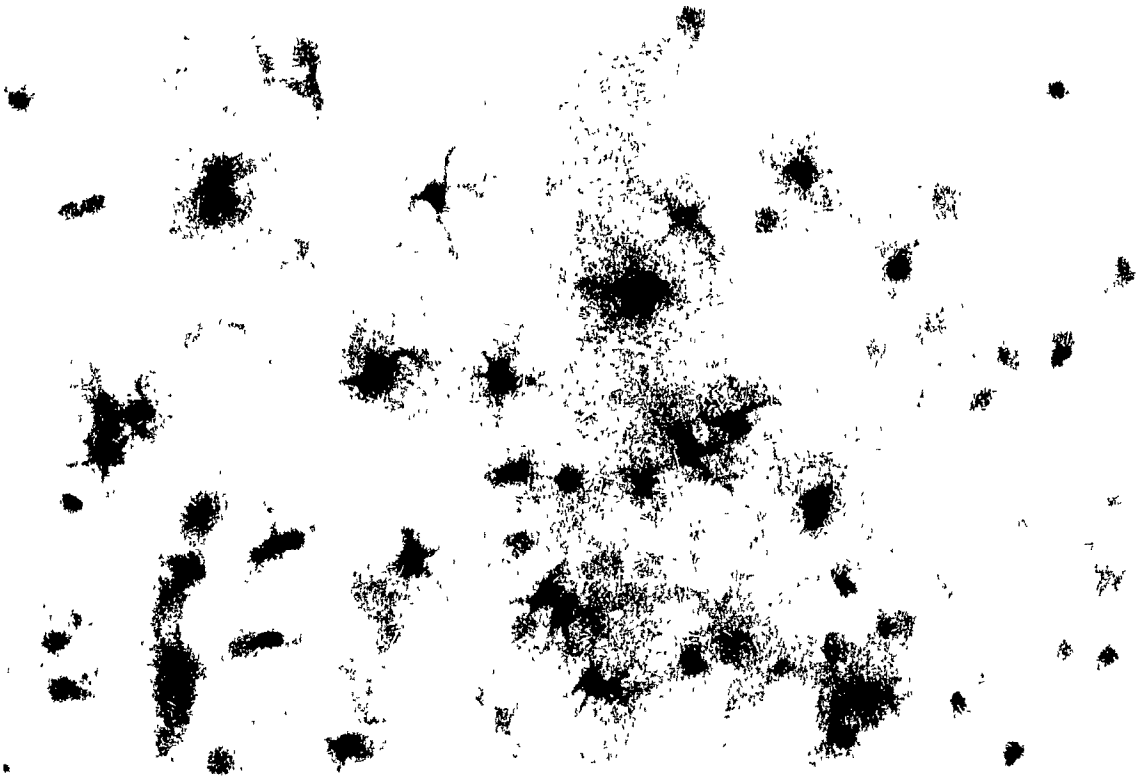

Figure I astrocytes, septum, higher magn., monoclonal antibody to bFGF, magn. 134x

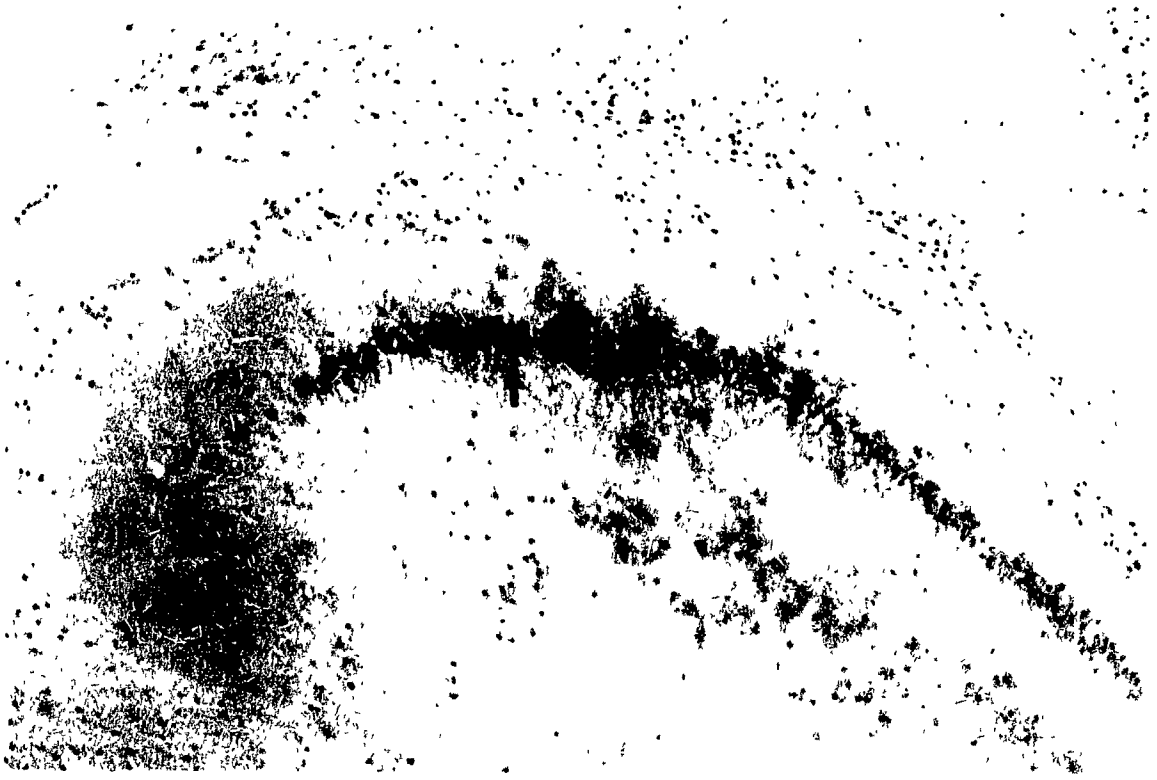

Figure J CA2 neurons, hippocampus, monoclonal antibody to bFGF, magn. 106x

Figure K above neurons, higher magn., monoclonal antibody to bFGF, magn. 675x

positive cells surrounding the injury area, Figure L. As in the undamaged brain, astroglia, characterized by immunoreactive cells with dark staining nucleii and large stellar processes, are evident. Again these findings are born out by previous studies of injury induced expression of bFGF (Finklestein et al., 1988; Gomez-Pinilla et al., 1992; Woodward et al., 1992).

Staining of GFAP confirms the previous findings of bFGF staining. Distribution of GFAP positive astroglia is similar to that found with bFGF, supporting the proposal that these cells stainng for bFGF are indeed astroglia, Figure M. Higher magnification illustrates the extent of arborization of the astroglia, extending well beyond the perinuclear staining seen with bFGF (Figure N). Also as with bFGF, nbm injury resulted in apparent increased concentrations of GFAP (Figure I and J) with positive cells around the lesion site.

The marker for catecholaminergic neurons, TOH, appears to be specifically stained using this protocol. TOH staining is evidenced in areas of catecholaminerginicity in the brain. Specific staining is seen in the arcuate nucleus (AN)(Figure O), the posterior hypothalamus(Figure P) and the efferent projection from the substantia nigra to the caudate that pass through the lateral hypothalamus (LH)(Figure Q).

The protocol works well with the antiserum for chromogranin A. Figure R reveals staining of the adrenal medullary control tissue is in a manner consistent with their

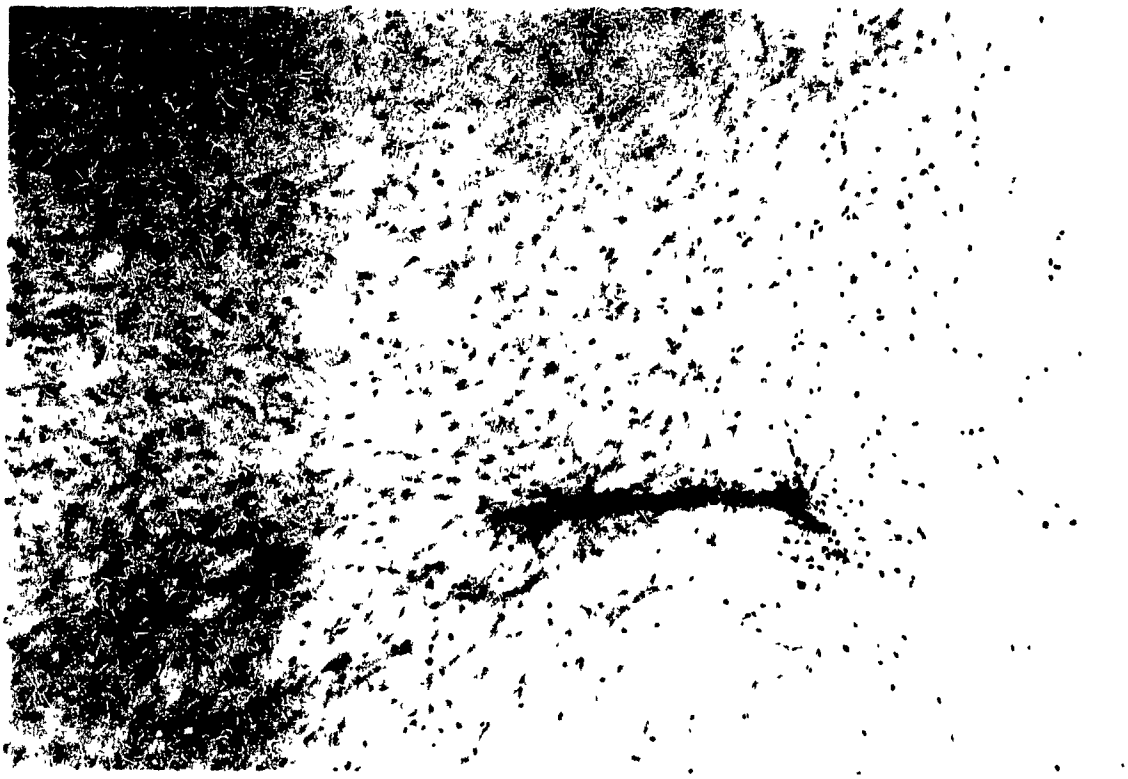

Figure L astrocytic proliferation around lesion site, monoclonal antibody to bFGF, magn. 135x

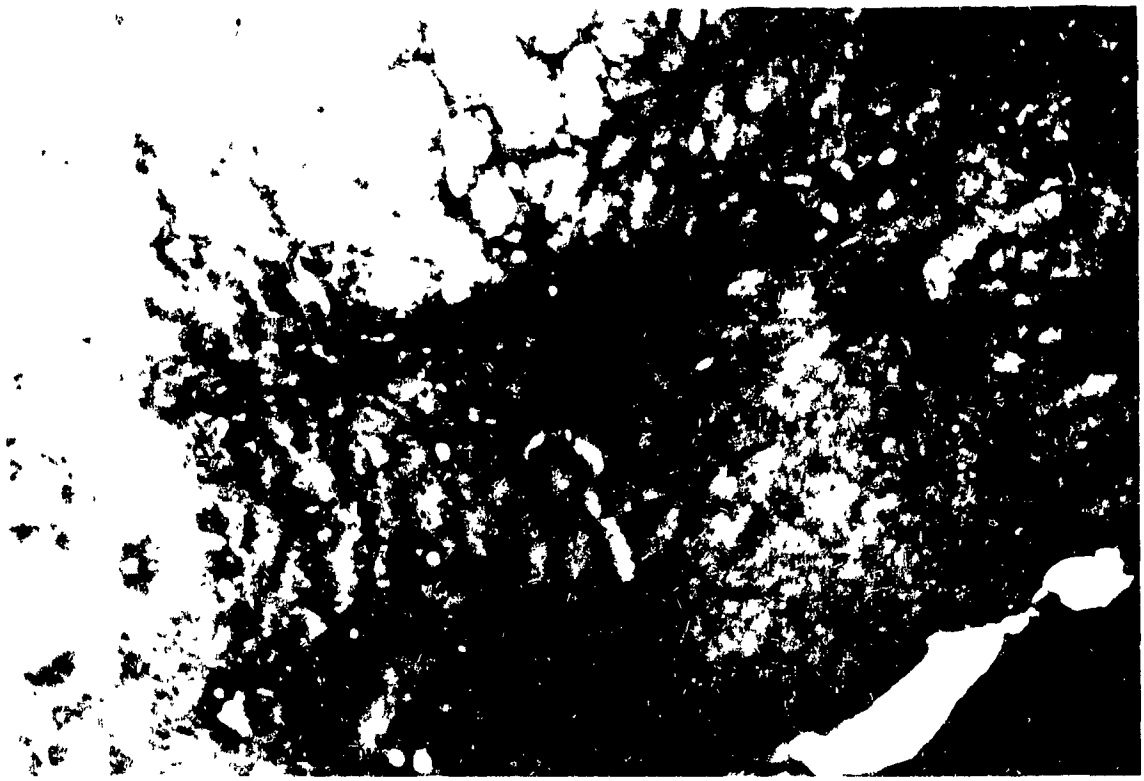

Figure M astrocytic proliferation around lesion site, polyclonal antibody to GFAP, magn. 270x

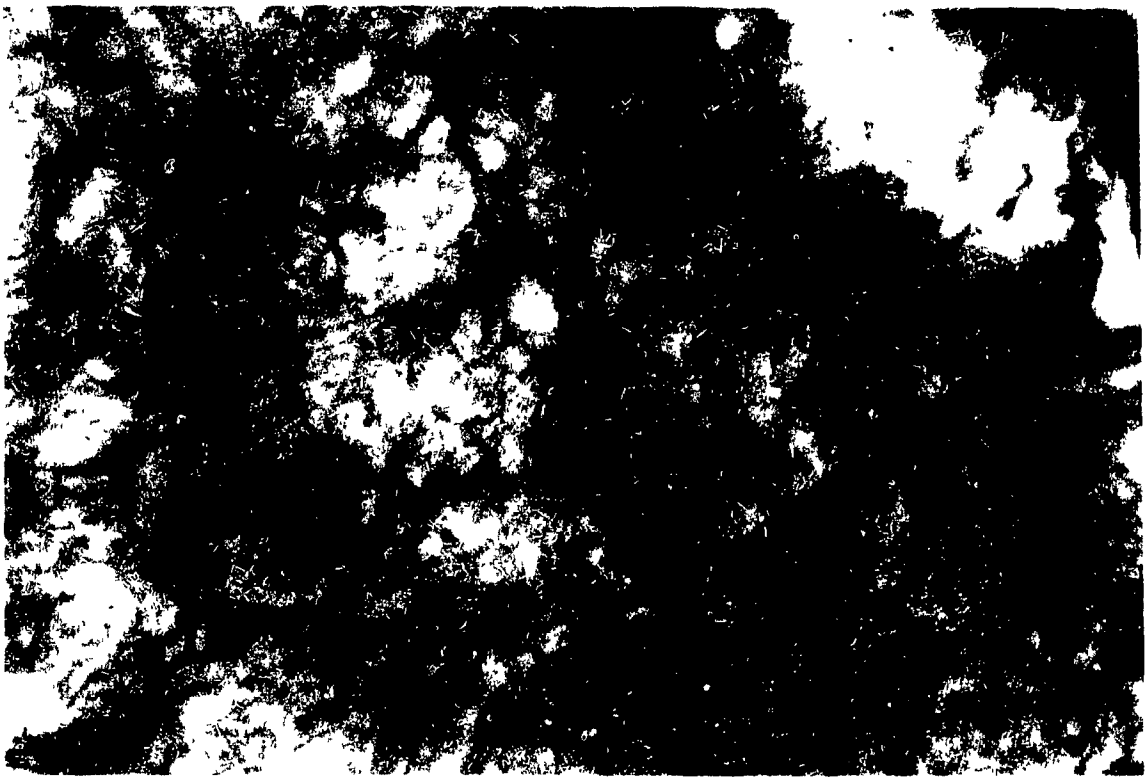

Figure N astrocytes from Figure M, higher magn., polyclonal antibody to GFAP, magn. 1340x

Figure O arcuate nucleus, polyclonal antibody to TOH, magn. 106x

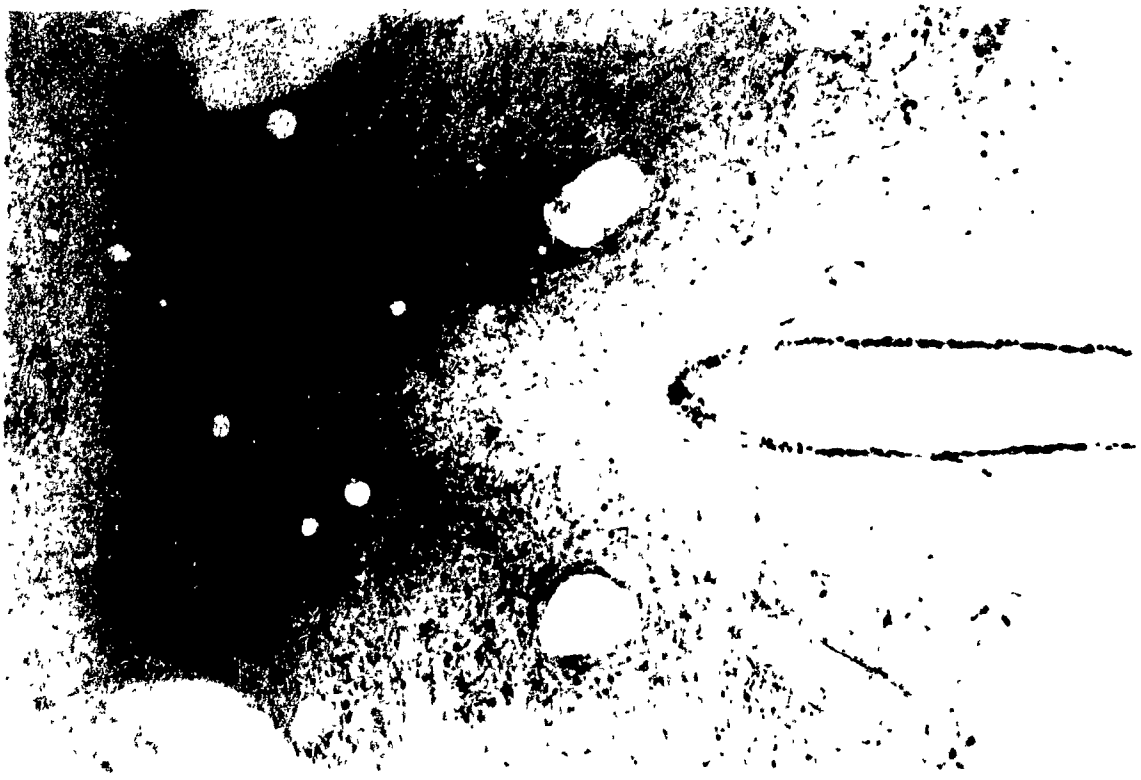

Figure P posterior hypothalamus, polyclonal antibody to TOH, magn. 135x

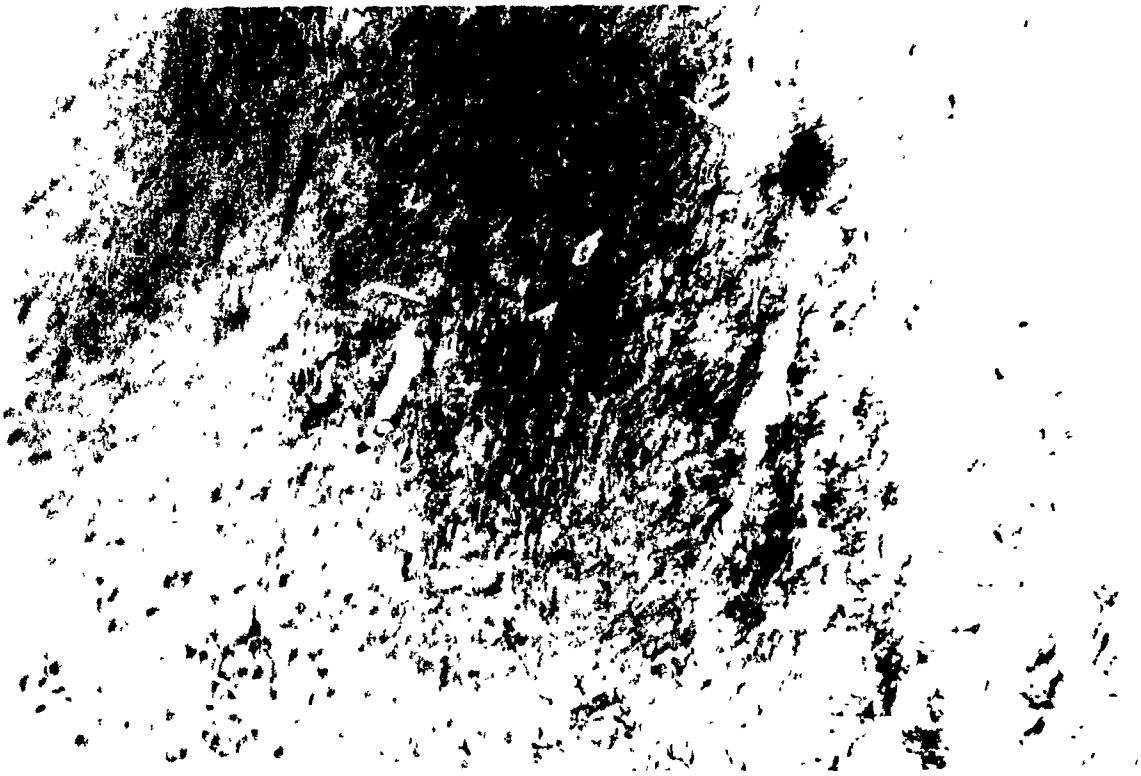

Figure Q efferent axons through the lateral hypothalamus, polyclonal antibody to TOH, magn. 135x

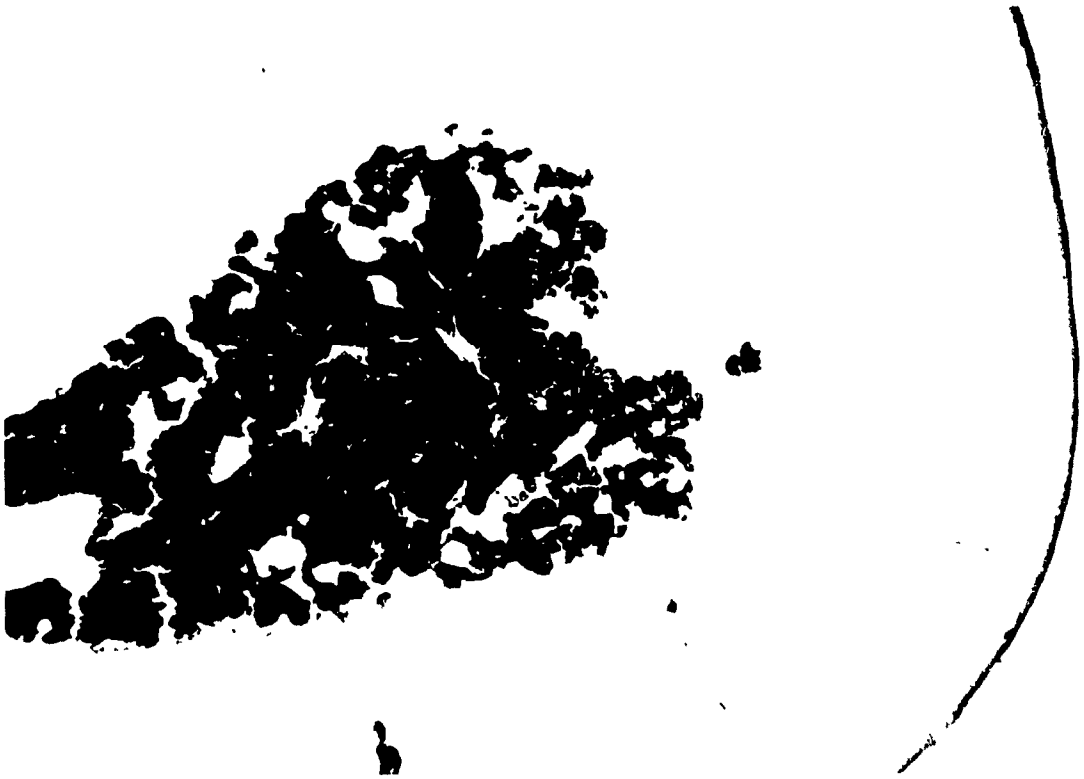

Figure R adrenal gland, monoclonal antibody to chromogranin A, magn. 84x

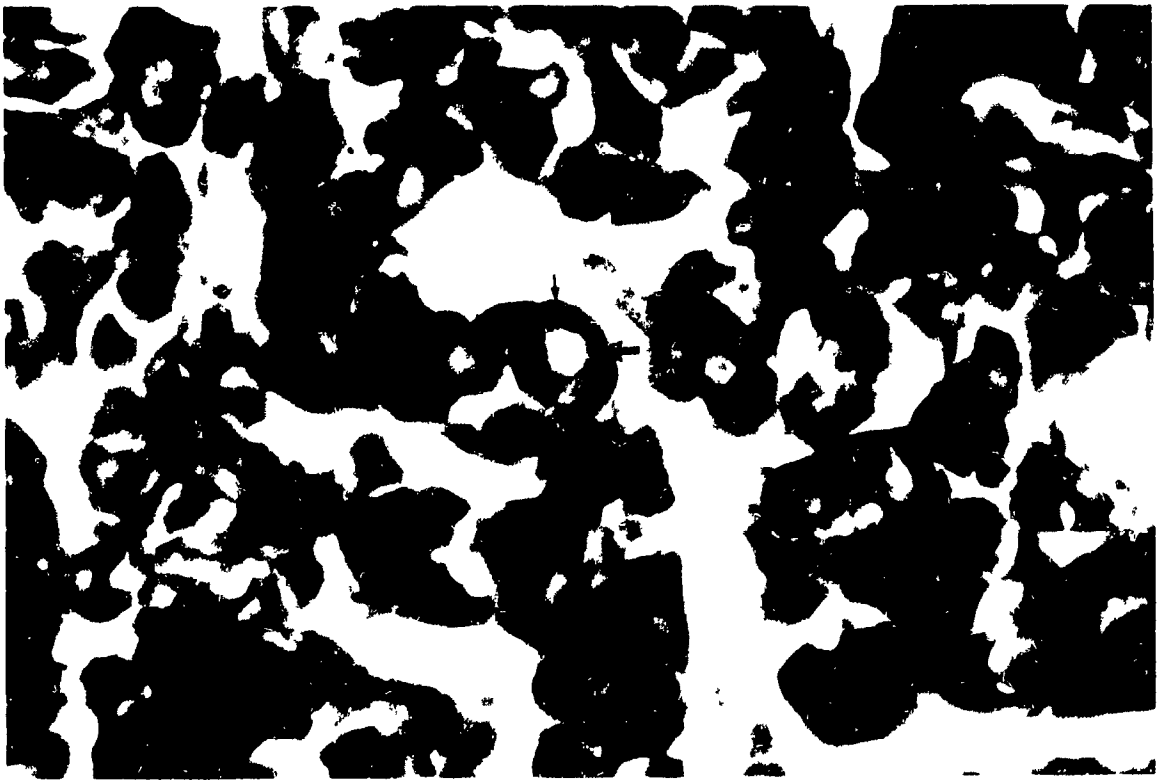

Figure S above gland, higher magn., chromaffin cells of the adrenal medulla, magn. 1340x

endocrine function. Specific staining was evident in cells of the adrenal medulla(am). Under higher magnification (Figure S), these immunoreactive cells of the medulla have darkened cytoplasm, while the nucleus of these cells does not stain.

4.3.4 Double Labelling Immunocytochemistry (Free-Floating)

In an attempt to see if the cells staining for bFGF were indeed astroglia, following staining for bFGF, ICC was then performed on the same tissue but using GFAP as the primary antiserum instead. The bFGF staining was carried out as above up until the final washes with buffer for 30 minutes prior to mounting. Instead, slices were washed for 60 minutes in TBS and stored overnight at 4°C. Sections were then incubated with 0.03% hydrogen peroxide for 10 minutes to react any unreacted peroxidase left over from the previous staining procedure. Then staining was carried out as above but with GFAP as a primary antibody serum. No $NiCl_2$ was added to the DAB/hydrogen peroxide solution for visualization which then yielded the brown reaction product characteristic of DAB staining.

The double labelling experiment indeed confirmed that many of the bFGF immunoreactive cells in brain tissue were astroglia. Double-labelled cells were evident in and around the lesion as well as areas of the cortex, septum and hippocampus, figure T. Under higher power examination all astroglia are evident from their dark brown stained astrocytic process, Figure U. However, of these only a portion appear to

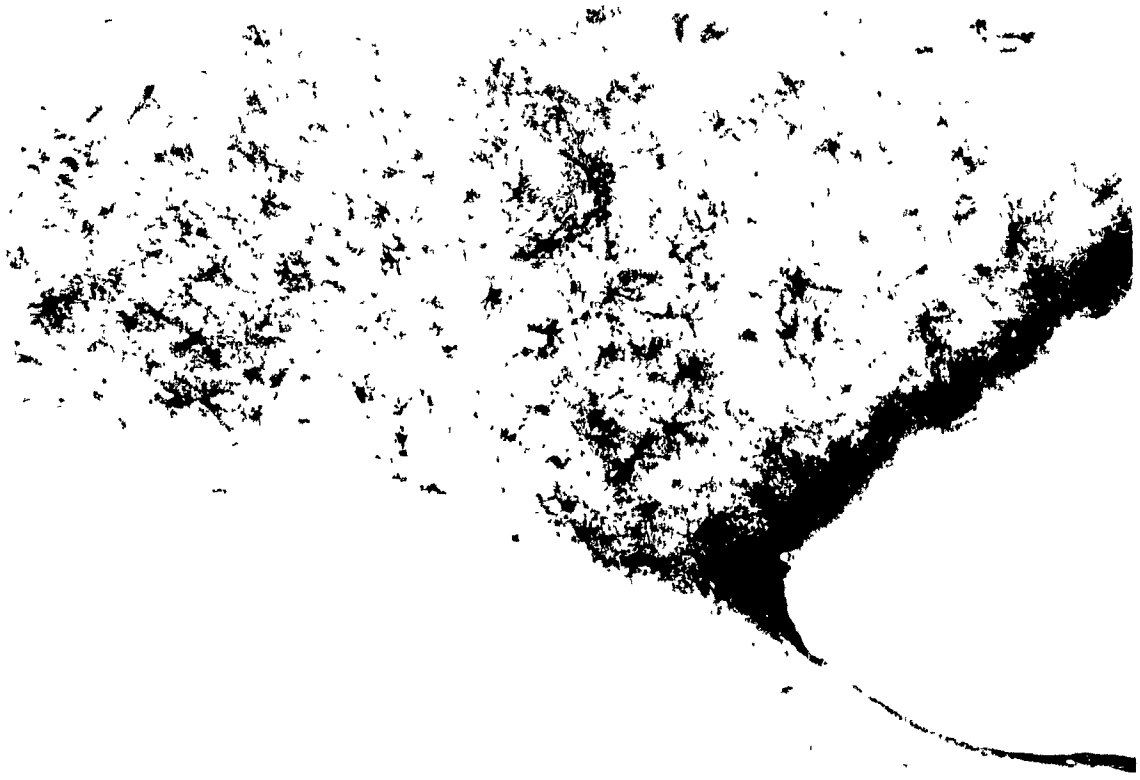

Figure T astrocytes, septum, double staining of polyclonal antibody to GFAP and monoclonal antibody to bFGF, magn. 268x

Figure U above astrocytes, higher magn., note differential expression of bFGF as indicated by the darkened soma, magn. 1340x

have the blue-black nuclear and perinuclear area representative of bFGF staining. Thus, astroglia do not appear to express bFGF constituitively, but may be instead getting turned on or off temporarily.

4.4 Immunocytochemistry Discussion

Establishment of the ICC protocols for the use of these antibodies is complete. As evidenced in the staining of all antigens in their respective positive control tissues. bFGF was stained succesfully in both the adrenal medullary cells and in brain tissue. GFAP was evident in the stellar shaped support cells of the brain, the astrocytes; its distribution is coincidental with lesion sites as well as being found in areas such as the septum, the cortex and the basal ganglia. Finally, chromogranin A, definitely, and TOH, possibly, were found to be useful markers for adrenal medullary cells. Anti-chromogranin A marked the adrenal medulllary cells because of their endocrine function. TOH staining, as indicated by its staining in positive control tissue, may label the adrenal medullary cells because of their catecholaminergincity. The protocol was also found to successfully stain TOH in endogenous brain tissue.

The observation that bFGF was localized to astrocytes is significant with respect to the influence of grafts. As mentioned, though grafts may act directly on the surrounding neuronal populations, they might also exert their effects indirectly via an intermediary cell. Support for this is

evident in that bFGF seems to be localized to a great extent in support cells following neuronal injury. BFGF has been localized in microglia (Gomez-Pinilla et al., 1992), macrophages(Frautschy et al., 1991) and astroglia(Yoshida and Gage, 1991). Further evidence of bFGF's intricate link with astroglia coems from cell culture work. Utilizing cultures of astyrocytes, bFGF significantly increased both the expression of GFAP(Morrison et al., 1985) and the proliferation of astrocytes (Pettman et al., 1985). The presence of bFGF has even been seen to increase the expression of nerve growth factor in cultured astrocytes (Yoshida and Gage, 1991). The link between NGF and the basal forebrain cholinergic nucleii is well documented. Levels of NGF and its mRNA in the central nervous system of the rat correlate with cholinergic innervation (Korsching et al., 1985) and NGF receptor is colocalized with choline acetyltransferase in neurons in the rat forebrain (Batchelor et al., 1989). Human studies confirm the link; receptors for NGF are expressed in high levels in neurons of the cholinergic magnocellular complex (Hefti et al., 1986; Hefti and Mash, 1989).

These studies are highly suggestive of a sequence of events by which the graft may exert its effects on the cholinergic system. Localized release of bFGF may increase astrocyte proliferation or growth. This may in turn initiate or increase the release of NGF and other supportive factors from these cells which in turn supports the cholinergic

recovery in the cortex.

That bFGF is present in astrocytes suggests two other possibilties. BFGF release by astrocytes may serve as an autotrophic factor. It may be released by astrocytes to stimulate their own growth in areas of tissue distress, to facilitate the many roles atrocytes play in wound healing. Alternatively, bFGF itself, in addition to or exclusively, may be the factor released by the astrocytes which may support the evident neuronal recovery. Evidence that bFGF is supportive of cholinergic neurons form basal forebrain cholinergic nucleii is found in cell culture work (Matsuda et al., 1986; Morrison and De Vellis, 1986; Kornblum et al., 1990; Walicke, 1988). Also, both of these prospects may act in concert with bFGF playing a primary role in wound healing by coordinating the response of supoort cells, primarily astrocytes but also miacrphages and micrglia, while supporting neuronal recovery directly, as well.

This work opens the door for a great deal of further studies. Firstly, ICC detection of chromogranin A in adrenal medullary CC enables our lab to use this as a marker of CC for the early, 5 day, time points, and possibly longer time points, in graft studies. As there are no endocrine cells in cortical tissue, chromogranin A is well suited as a marker, staining endocrine CC quite strongly. While it is possible that these cells may change their phenotype when placed in the graft site, preliminary evidence from our lab of bFGF staining

CC at the 1.5 month time point makes this prospect seem unlikely. Therefore use of this antibody would serve to characterize graft survival over time as well as allowing quantification of initial CC graft concentrations.

TOH staining also provides another marker of CC at least for the 5 day time point. More importantly, it provides the valuable feature of allowing one to observe the phenotype of the graft. Though earlier studies in our lab have suggested that the possibilty of the graft changing its phenotype to cholinergic is unlikely (Welner et al., 1990), it would prove useful to evaluate whether the CC lose their catecholaminerginicity.

Perhaps the most valuable accomplishment of the ICC section of this study was the development of the protocol for staining bFGF. In pursuing the characterization of graft expression, elucidation of any of the appropriate protocols required a great deal of effort and time. This attests to the difficulty in establishing protocols for immunocytochemistry even when published techniques exist and expert advice is avilable. With the theory heavily implicating the CC graft's high concentrations of bFGF being involved in its ameliorative properties, bFGF's detection was especially critical.

The most difficulties that were encountered centered around the initial trials with polyclonal antibody to bFGF. The antibody was not commercially available but rather as mentioned was developed by Dr. Andrew Baird of The Whittier

Institute for Diabetes and Endocrinology, La Jolla, California. As such the characterization of this antibody was not subject to the same rigours as that encountered with a commercially available antibody. Therefore, despite my efforts to follow Dr, Baird's published protocols using this antibody, continual pitfalls were encountered. In my personal correspondence with Dr. Baird, it was suggested by him that the antibody was indeed difficult to work with and that its use in his lab still often met with unsatisfactory results. He stated that the protocols for obtaining immunoreactivity varied from tissue to tissue and as such consistency was very difficult to achieve. Therefore, procedural differences were most likely the cause of the difficulties encountered using this antibody. These problems coupled with the aforementioned technical problems encountered when utilizing any polyclonal antibody, contributed to much of the lack of progress with this protocol. Barring the prohibitively expensive option of learning the technique in Dr. Baird's lab, the trial of a different antibody was decided upon, with successful staining achieved as reported above.

5 GENERAL DISCUSSION

As recently as 10 years ago, the traditional view regarding cortical plasticity in the adult central nervous system(CNS) was that it was minimal at best. The adult cerebral cortex was "hardwired" with stable organizations being established by developmental processes that were completed by adulthood. Recently, the adult cerebral cortex has been found to have a great potential for remodelling or reorganizing. At the forefront of neuroplasticity research has been the technique of neuronal grafting, the process of implanting a piece or suspension of neuroactive tissue into the CNS. This technique has been essential in studying neural development, regeneration and degeneration, basic neural mechanisms, understood in light of experimental transplant manipulations that enhanced or inhibited development. The great potential for plasticity in the CNS has become apparent from this research. With the newfound awareness of brain plasticity came proposals that treatments for neurodegenerative disorders like AD could be found in grafting techniques.

The principles upon which this work is based are not without conflict. One of the principal concerns is the applicability of nbm-lesioning as a model of AD. Despite evidence which supports the role at this model plays in mimicking at least some behavioral deficits of AD, there are a variety of criticisms which must be considered when

interpreting results using this model.

AD is characterized by an incredibly complex array of neurophysiological and behavioral pathology. The nbm-lesion model, however, mimics only one aspect of the disease, the neuronal loss of the nbm and the deficiencies of cortical cholinergic activity. Furthermore, the disease is a progressive degenerative disorder and, as such, the pathology is ever-changing. To mimic the cholinergic deficiencies in AD, manipulations are performed that destroy the specific brain structure. This abrupt insult occurs over a period of days, reflecting a very acute cell loss, when compared to the chronic gradual neurodegeneration seen in the human condition. Thus, the model may mimic only one stage of the progression of AD. Furthermore, extensive neuronal loss in the nbm may reflect only the very end stages of the disorder, a time when there are also a great many other pathological phenomena occurring. It is this end stage that is most likely resistant to treatment.

Evidence has indicated that such abrupt changes are likely to initiate a series of compensatory changes within the remaining intact CNS structures that may not occur during the long term neurodegenerative process in AD. This may account for some nbm-lesion studies which have evidenced spontaneous behavioral (Bartus et al., 1985; Saghal et al., 1990) or cholinergic (Wenk and Olton, 1984; Casamenti et al., 1988; Hohmann and Coyle, 1988; Stephens et al., 1988) recoveries.

This is especially true of younger rats (Stephens et al., 1988).

Perhaps one of the strongest criticisms of the nbm lesion as an animal model of AD is evident from studies of pharmacological manipulation of the cholinergic system. Studies of systemic injections of scopolamine show similar behavioral hypersensitivity in both nbm-lesioned animals (Hughey et al., 1986) and AD patients (Smith, 1987). Despite this evidence, when nbm-lesioned animals are used to investigate the potential effectiveness of various pharmacotherapeutics designed to alleviate the behavioral symptoms associated with AD, the similarity between the two ends. AChE inhibitors and ACh agonists, when administered to the nbm-lesioned animal are seen to alleviate memory deficits (Wenk and Olton, 1984; Murray and Fibiger, 1985). However, these cholinergic enhancement therapies do not produce consistent or significant improvements in the behavior of AD patients (Bartus et al., 1982).

Reasons for these pharmacological descrepancies between the nbm-lesion model and AD are unclear. More detailed knowledge of receptor changes in the cortex would help to clarify these discrepancies. One possibility is that there are differences in the cholinergic component of the rat nbm and the human nbM. In other words, the primary behavioral deficit that has been attributed to the decrease in ChAT levels in the nbm-lesioned animal, and the human pathology it supposedly

represents, may in fact, be due to other neurotransmitter systems in the nbm or other neuroanatomical structures that lie close to the nbm. Evidence for this has been shown by Reiner et al. (1987) who noted a subpopulation of noncholinergic nbm neurons constituting 13% of nbm cells. One possible candidate as an alternative nbm neurotransmitter that may play a role in the behavioral deficits in humans is neurotensin (Wenk et al., 1989). Support for the proposal that non-cholinergic nbm projections may play a role in the deficits lesioning produces is evident in studies which have found that quisqualic acid and AMPA lesions result in greater destruction of cortically projecting cholinergic neurons of the nbm than that seen with ibotenate or kainate lesions, but fail to induce the majority of profound learning and memory deficits produced by the latter (Dunnett et al., 1987).

A corollary to this criticism is evident in that preferential loss of nbm neurons is not an exclusive neuroantomical feature of AD. Degeneration of nbm neurons occurs in other diseases such as the neurodegenerative disease, PD, and the transmissable dementia, Creutzfeldt-Jakob Disease (Rogers et al., 1985). This is supported by a recent finding in which deficits of ChAT activity were found to be no different between AD, PD or AD/PD brains (Aubert et al., 1992). The fact that loss of nbm cells and concurrent loss of cortical cholinerginicity occurs in disorders in which there is no dementia suggests that current assumptions of the role

of this cholinergic system and memory disorders may need revision.

Recent studies have compromised the traditional association between the cholinergic system and the assumed mnemonic nature of the behavioral deficits. Lesioning of the nbm in rats produces behavioral deficits on a variety of cognitive and sensory tasks which, in turn, cause deficits on a variety of behavioral tasks. Orthodox thought assumes that these deficits are cognitive in nature and that they reflect the deficits in cholinergicity of the nbm and its corresponding projections to the cortex. As such it was believed that this technique could provide a simple animal model of the behavioral and neurological pathology evidenced in AD in which the nbm degeneration is concurrent with both a decrease in cholinerginicity in the cortex and behavioral deficit. Despite observations that behavioral deficits are induced in the performance of tasks involving discrimination learning and memory, it has recently been proposed that this may reflect damage to attentional or sensorimotor systems. This is based on evidence that in addition to the deficits on mnemonic behavioral measures, deficits are also witnessed on tests of sensory attention and sensorimotor abilities (Dunnett et al., 1985; Whishaw et al., 1985; Welner and Koty, 1993).

While the resolution of the nbm/cholinergic functionality debate is extremely important with respect to both basic science and potential pharmacotherapeutics of AD, its

resolution for the purposes of this research is not as critical. As AD has a significant behavioral component which involves deficits in attentional abilities (Nebes and Brady, 1989; Sahakian et al., 1990), the relevance of the nbm-lesion model functional deficits may indeed be appropriate. When considered in light of recent findings from our lab evidencing the therapeutic value of CC grafts in alleviating attentional and sensorimotor deficits (Welner and Koty, 1993), the exact nature of the functional deficits is less important. More important is that the CC grafts can induce recovery as measured by a diverse array of behavioral measures.

6 CONCLUSION

These experiments in brain plasticity suggest the potential for treatment of CNS injury, slowing or even halting the progress of the injury induced degeneration and inducing or directing neuronal regeneration. Non-cholinergic, non-embryonic CC grafts were evidenced to induce a behavioral recovery in a mnemonic measure, coincidental to inducing a cholinergic amelioration in the cortical tissue in which the graft was placed. Thus CNS transplantation, while originally perceived as a tool for studying plasticity, may be developed into a very promising approach as a therapeutic method to treat brain injury and degeneration. With further development and technical refinements, intracerebral grafting is likely to provide new possibilities for intervention in neurodegenerative processes, and for the stimulation of regeneration and functional recovery in human beings.

7 FUTURE DIRECTIONS

One of the primary directions in which this research should expand is further characterizing the proliferation of astrocytes in and around the graft. As mentioned astrocytes may play a key role in the graft effect. As any insult to CNS tissue results in changes in GFAP expression, it is essential to separate any difference between astrocytic proliferation or growth resulting from the grafting procedure, from that arising from the CC grafts themselves. As a result, characterizing GFAP expression in CC grafts as well as sham tissue graft described in the functional section of this report would be critical. This would allow one to discern if the concentrations of bFGF within the CC graft was enough to influence support cell proliferation above and beyond that arising from the tissue insult of grafting. Only if differences in GFAP expression exist between the two graft groups can one further develop the hypothesis that astrocytes are an essential player in the graft effects.

Positive results with bFGF ICC in both adrenal tissue and endogenous brain tissue, will now lead to this protocol being used to determine graft and cortical expression of bFGF. Of primary use would be the elucidation of bFGF expression by the graft at different time points. This could be used as a measure of graft viability and trophic potential on surrounding tissue a time goes by. Assuming that this is indeed key to graft effects it could be used as a marker of

therapeutic index to discover whether graft effects are acute or are more chronic, occurring over months. It would also be of interest to discern differences in bFGF expression between graft types on the surrounding astrocytic population. Much as with the measurement of GFAP surrounding the CC grafts and sham grafts, this is a necessary step in sparating procedural effects of grafting on surrounding tissue and a tissue specific effect of the CC graft itself. Furthermore, pending the development of an adequate marker of NGF, bFGF staining could be examined in relation to NGF levels by astrocytes to primary effects of one growth factor over the other.

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Appendix A - Overview of the Immunogenic Response

Lymphocytes belonging to a group of cells, called mononuclear cells are fundamental in recognizing immunogenic stimuli, stimuli foreign to the host (antigens), produce substances that participate in immunological reactions (antibodies) and coordiante cellular interaction during antibody formation. The most prominent product of a specific type of lymphocytes, B cells, are immunoglobulins. Immunoglobulins can undergo immunological reaction, binding to a specific antigen independent of the lymphocyte in which it was made. Immunoglobulins are produced and released when a foreign body or antigen is recognized by its specific antigenic determinants or epitopes. Depending on its size, any given antigen will have several epitopes each of which can trigger the production and release of a particular antibody which recognizes and binds to that epitope. This induces an immune response in which the host animal produces immunoglobulins to the antibody. Different epitopes on the antibody will trigger several specific lymphocytes to each produce a single specific antibodies to one particular epitope on the antigenic molecule. Thus an antigen will induce the production of many different antibodies from different immunoactive lymphocytes to its different epitopes. It is the <u>in vitro</u> immunological reaction between these antibodies, the molecule to be visualized and various marker compounds that constitutes the basis of ICC.

Appendix B - Peroxidase-Antiperoxidase Immunocytochemistry

This method involves the application of a peroxidase-antiperoxidase (PAP) complex following the application of the secondary antibody. This PAP complex consists of three peroxidase molecules bound by two antibodies to the peroxidase. These antiperoxidase antibodies are raised in the same species in which the primary antigen was raised. Therefore, the bound secondary antibody will recognize the PAP complex and immunologically bind to it as well. The peroxidiase is essential to the visualization procedure as, when reacted with its substrate, hydrogen peroxide, in the presence of an electron capturing agent, 3',3'-diaminobenzidine(DAB), a brown precipitate is formed. This precipitate is the marker or label, indicating immunoreactivity.

Appendix C - Avidin-Biotin Complex Immunocytochemistry

The second technique utilizes the high affinity of two molecules, avidin and biotin, have for one another. In this process, the secondary antibody is conjugated with biotin. Instead of a PAP complex being added, a complex of avidin and biotin-conjugated peroxidase is used. This avidin-biotin complex (ABC) then becomes bound to the biotinylated secondary antibody. The ABC method is advantageous to the PAP as the affinity between the avidin and the biotin is greater than the affinity between the secondary antibody and the antiperoxidase. This leads to more specific binding of the marker to the antigen complex. Furthermore, the ABC method leads to a greater number of peroxidase molecules being bound to the complex, increasing the intensity of the labelling by the DAB.

Appendix D - Polyclonal vs. Monoclonal Antisera

Primary antisera consist of two types, polyclonal and monoclonal. The differences lie in their production and determine their application. Upon introduction of an antigen to a host animal, serum antibodies to the antigen are produced. The antigen has several epitopes or recognition sites and as such antibodies to the antigen are of heterogenous idiotypes. In other words, different antibodies are produced by several clones derived from several antigen stimulated cells.

Extraction of these antibodies for use in immunocytochemistry is accomplished by affinity purifying the animal's blood on a column to which the antigen has been bound. The result is a ploYclonal primary antisera consisting of a heterogenous mix of different antibodies to a single antigen, the separation of which is exceedingly difficult.

One drawback of the use of polyclonal antisera is the difficulty controlling for the inevitable nonspecific binding which always occurs to some extent in any binding procedure. The more heterogenous the mix of antibodies to an antigen, the greater chances of nonspecific binding to molecules resembling that antigen and the greater the nonspecifc or background signal. Thus, when using polyclonal antisera, some may react specifically, and others may crossreact with unknown tissue constituents.

Most of this type of background staining can be

eliminated by using normal sera from the animal in which the secondary antibody was made. By pretreating the tissue with this serum and then adding it to the antibody sera themselves, one will eliminate much nonspecific binding. This is because the antibodies taken from a particular animal will not bind to antigens of the same animal type. Thus, many nonspecific interactions are prevented as the tissue antigens are 'protected' by the antigens in the normal serum. Because of the higher chances of nonspecific staining inherent in their use, polyclonal antisera are preferred when antigen concentrations are high and, consequently, the labelling of the antigen, or signal, will be clear.

Monoclonal antibody sera possess a higher degree of specificity then polyclonal antibodies. The reason for this is, as with polyclonal antisera, inherent in their production. Essential to the production of monoclonal antibodies is the preparation of clones derived from single antibody producing cells. The principal behind the preparation of such cells involves the fusion of immune spleen cells (lymphocytes) with myeloma cells from mouse lines, so as to confer the spleen cells the replicative ability of the myeloma cells while retaining their antibody producing capabilities.

Its preparation is as follows. As with primary antisera production the desired antigen is injected into the host animal, in this case usually a mouse but may also be a rat. The spleen cells are then attempted to be fused with myeloma

cells. Fusion efficiency is low and of 2 x 10^8 cells of spleen and an equal number of myeloma cells, only 400 viable fused cells will be produced, out of which only 80 will be producing antibodies to the antigen. As the remaining cells that survivie in culture will be myeloma cells, fused or not, they must be selected out for. For this reason mutant cell lines have been utilized for monoclonal antibody produciton. Cells possess two pathways for deoxynucleotide synthesis, a de novo pathway, in which nucleotides are synthesized from the single building blocks, and a recovery pathway, in which metabolites of spent DNA are reincorporated into new deoxynucleotides. Myeloma cells have a mutation in the recovery pathway and as such can be poisoned by growth on selective media which inhibits the de novo pathway. As spleen cells still possess this pathway only myeloma cells succesfully fused with spleen cells will suvive thanks to the addtion of the spleenic genome. Therefore after growth on selsctive media, only the hybridomas cells will survive.

The hybridomas are a collection of different immunocompetent cells stimulated by the same antigen but producing different antibodies to the differnt epitopes on the antigen. Selective plating of these cells on media, separates out the surviving hybridomas and ensures that observed colonies will all have arisin from a single fused spleen/myeloma cell with the same idiotype. Selection of the desired antibody producing cell line may then be accomplished

via different techniques such as radioummunoassay, immunofluoresence, enzyme-linked immunosorbent assay or immunocytochemistry.

Thus monoclonal antibodies, are considered so as they are all produced by clones deriving from a single, antigen stimulated cell, recognize only one epitope on the antigen molecule. While this increases the clarity of the specific staining signal, due to the reduced background staining, a new problem is faced which may counteract any benefit in staining incurred from the primary antibodies specificity. As mentioned, a primary antibody serum is comprised of several different antibodies each of which recognizes different parts of the epitopes of the antigen. This increases the odds that any antibody in the serum will recognize one of the epitopes on the desired antigen and bind to it. However, the odds that one of these antibodies will incorrectly recognize a similar epitope on a nondesired antigen also increases, thus increasing the nonspecific binding or background signal. As a serum of monoclonal antibodies recognizes only one epitope on the desired antigen, one decreases the odds of nonspecific binding to 'similar' appearing epitopes on undesired antigens, while at the same time decreases the odds that the antibodies will locate and recognize the one epitope on the desired antibody and bind specifically. Therefore, in increasing the specificity of the signal, the confidence a particular signal is specific, one may compromise the strength of that signal.

Appendix E - Slide-Mounted vs. Free-Floating Tissue

Immunocytochemical procedures can be performed on slide mounted tissue or on slices free-floating in buffer. Slide-mounted slices as the name implies are mounted directly on to the slide once cut. During the free-floating technique, the brain slice is subjected to the the particular antibody serums and buffers of the staining protocol by free-floating in them.

Slide-mounting offers the advantage of maintaining tissue integrity, as slices are not manipulated once fixed to the slide. The antibody solutions are poured directly on to and washed off the slide during the procedure. This is particularly important when working with unfixed tissue. Free-floating immunocytochemistry allows both sides of the tissue exposure to the solutions. As such, it allows much greater accessibility of the antibodies to the antigens. This increases the odds of the antibodies locating and binding to the particular epitope on the desired antigen and specifically labelling it. Therefore, while this technique can only be performed on fixed tissue, due to the mechanical manipulation of the slices, greater antibody binding leads to increase in the intensity of the antigen labelling. This makes it particularly well-suited for use with monoclonal antibodies in which greater specificity of binding is comprimised with a lessened signal strength.