Transplantation of enteric ganglia and intestinal smooth muscle into the adult rat brain.

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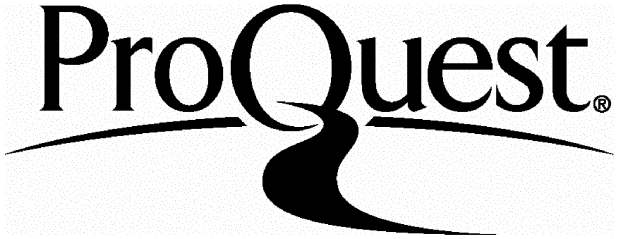

ABSTRACT.

This thesis examines both the survival of grafts of myenteric plexus and intestinal smooth muscle implanted in the adult rat striatum and the interaction between the graft and the surrounding striatum.

Pieces of adult rat ileal muscularis externa (myenteric plexus sandwiched between smooth muscle layers) were implanted into the corpus striatum and examined by electron microscopy. Enteric neurons and glial cells survived for at least 6 weeks and remained morphologically similar to those seen *in situ*. Bundles of small unmyelinated axons, interpreted as CNS axonal sprouts, were seen in the striatum surrounding the grafts. Some passed between the brain and the grafts. Grafts of freeze-killed muscularis externa produced little axonal sprouting in the striatum and were not invaded by axons. Similar live grafts were examined immunohistochemically using an antibody against tyrosine-hydroxylase (TH). Grafts were invaded by TH-containing fibres of CNS origin.

Pieces of freshly dissected myenteric plexus from young donors were implanted in quinolinic acid-lesioned and unlesioned adult rat striatum. Some grafts were histochemically stained for NADPH-diaphorase, three and six weeks after implantation. NADPH-diaphorase-containing enteric neurons were identified within the grafts and extended long processes into the surrounding lesioned or unlesioned striata. The remaining grafts were examined electron microscopically three and six weeks after implantation and were found to contain healthy-looking enteric neurons and glia. Dissected enteric ganglia also appeared to stimulate a sprouting response in both the lesioned and unlesioned striatum adjacent to the grafts. Once again, grafts

were invaded by oligodendrocyte-myelinated and non-myelinated CNS axons.

As part of the investigation of the mechanisms involved in the sprouting response of the striatum, freshly dissected colonic smooth muscle was implanted in the unlesioned striatum and examined electron microscopically, three and six weeks after implantation. A sprouting response was once again observed in the striatum around the grafts. Putative CNS sprouts, associated with CNS glia and Schwann cells, invaded the grafts.

CONTENTS.

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LIST OF ABBREVIATIONS.

AChE - acetlycholinesterase

AD - Alzheimer's disease

BBB- blood-brain barrier

BDNF - brain-derived neurotrophic factor

CCK -cholecystokinin

CGRP - calcitonin gene related peptide

ChAT - choline acetyltransferase

CNS - central nervous system

CNTF - ciliary neurotrophic factor

CSF- cerebrospinal fluid

DA - dopamine

DYN - dynorphin

ECM - extracellular matrix

EM - electron microscopic

ENK- enkephalin

ENS- enteric nervous system

ER - endoplasmic reticulum

bFGF - basic fibroblast growth factor

GABA - gamma-amino butyric acid

GAD - glutamic acid decarboxylase

GAL - galanin

GAP - growth associated protein

GDNF - glial derived neurotrophic factor

GFAP - glial fibrillary acidic protein

GI - gastrointestinal

GRP - gastrin releasing peptide

HD - Huntington's disease

5HT - 5-hydroxytryptamine

IL-1 - interleukin-1

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NADPH - nicotine adenyl dinucleotide phosphate (reduced)

NGF - nerve growth factor

NO - nitric oxide

NOS - nitric oxide synthase

NPY - neuropeptide Y

NT - neurotensin

NT-3 - neurotrophin-3

6-OHDA - 6-hydroxydopamine

PN - peripheral nerve

PNS - peripheral nervous system

PD - Parkinson's disease

QA - quinolinic acid

RT - room temperature

SOM - somatostatin

SP - substance P

TH - tyrosine hydroxylase

VIP - vasoactive intestinal polypeptide

PUBLICATIONS ARISING FROM THE WORK FOR THIS THESIS.

E.M.M. Tew, P.N. Anderson and G. Burnstock. 1991. Preliminary observations of myenteric plexus implants in adult rat striatum. *J. Anat.* **176**: 226-229.

E.M.M. Tew, P.N. Anderson and G. Burnstock. 1992. Implantation of the myenteric plexus into the corpus striatum of adult rat: survival of neurons and glia and interactions with host brain. *Restor. Neurol. Neurol.* **4**: 311-321.

E.M.M. Tew, M.J. Saffrey, P.N. Anderson and G.Burnstock. 1993. An investigation into the fate of NADPH-diaphorase-containing enteric neurons implanted into the corpus striatum of adult rats. *J. Anat.* **183**: 193.

E.M.M. Tew, M.J. Saffrey, P.N. Anderson and G. Burnstock. 1993. Postnatal rat NADPH-diaphorase containing myenteric neurons extend processes when transplanted into adult rat corpus striatum. *Exp. Neurol.* **124**: 265-273.

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CHAPTER ONE.

INTRODUCTION

1A. GENERAL INTRODUCTION.

Over the past 20 years, the intracerebral implantation of neural and non-neural tissue has been investigated as, amongst other things, a strategy for treating neurodegenerative diseases by replacing the lost neurons, or supplying neurotransmitter to specific areas of the CNS. Clinical trials have been almost entirely restricted to Parkinson's disease, utilizing autografts of adrenal medulla (Madrazo *et al.* 1987, Rush Research Group 1990) and allografts of foetal mesencephalon (Freed *et al.* 1992, Spencer *et al.* 1992, Widner *et al.* 1992) with varied results. However there are a number of disadvantages associated with these tissues: grafts of adrenal medullary cells have been demonstrated to produce little dopamine (DA) and appear to act for only a limited period (Lindvall *et al.* 1987), while grafts of foetal mesencephalic cells, although more successful may be subject to problems of immune rejection (see Sloan *et al.* 1991) and to ethical restrictions on their use (see Hoffer and Olson 1991 for review).

These disadvantages have led researchers to search more widely for potential sources of tissue for implantation, and to extensive studies investigating the possibility of utilizing genetically engineered cells, such as fibroblasts (Kawaja *et al.* 1992), astrocytes (Cunningham *et al.* 1991) and skeletal muscle cells (Jiao *et al.* 1993) which are all potentially available in large quantities from the patient, thus avoiding problems of supply and immunological rejection. These cells are then cultured and "injected" with genes for the production of synthetic enzymes (eg: tyrosine hydroxylase used in catecholamine synthesis) or neurotrophic factors (eg: Nerve Growth Factor). So far, it is not clear whether grafts of these cells will continue to produce the injected gene product or whether they

will be able to ameliorate the complex behavioural dysfunctions seen in neurodegenerative diseases.

In this thesis I have examined the possibility of using the enteric nervous system (ENS) as an alternative source of material for intracerebral implantation in models of neurodegenerative disease. The ENS has been suggested by the research group at UCL as explored in this thesis, and to a limited extent by other investigators (Lawrence *et al.* 1991, Jaeger 1993, Jaeger *et al.* 1993), as a fourth category of cells for implantation in models of neurodegeneration for a number of reasons. The ENS has the advantages of containing a wide variety of neurotransmitters and neuromodulators, exhibiting many similarities with the central nervous system (CNS) (see Jessen and Burnstock 1982 and below) and of being available in large amounts, potentially from the patients own gut, thus circumventing ethical dilemmas and potential problems of rejection.

Chapter 1B that follows is a historical background to the subject of intracerebral implantation of neural and non neural material and describes the anatomy, morphology and chemical coding of neurons within the ENS and corpus striatum. Information is also included describing animal models of neurodegenerative diseases associated with the basal ganglia and an outline of what is known about CNS neuronal response to injury and situations where CNS neurons have been observed to form regenerative axonal sprouts.

Chapter 2 comprises information on all the methods used within this thesis and outlines the source of materials

The experimental work is detailed in chapters 3 to 7. Chapter 3 describes the results of an experiment when pieces of muscularis externa (myenteric plexus with two surrounding layers of smooth muscle) was implanted into the corpus striatum and examined electron microscopically after 10 days, three weeks and six weeks. Chapter 4 describes an immunohistochemical investigation of the growth of tyrosine hydroxylase-containing CNS fibres into intrastriatal grafts of muscularis externa. In Chapter 5, intrastriatal grafts of isolated myenteric plexus (without smooth muscle) taken from young rats were examined using reduced nicotine adenyl dinucleotide phosphate (NADPH)-diaphorase histochemistry. This enzyme is found in a subpopulation of enteric neurons and also within a subpopulation of striatal neurons. Since the striatal NADPH-diaphorase-containing neurons are killed off following a quinolinic acid lesion, grafts were examined in the lesioned and unlesioned striatum. Chapter 6 describes an electron microscopic study of grafts of young myenteric ganglia implanted in the lesioned and unlesioned striatum. In Chapter 7, grafts of isolated colonic smooth muscle taken from young rats are implanted into the unlesioned striatum and examined electron microscopically three and six weeks after implantation.

Chapter 8 contains a general discussion of the results contained in this thesis, examining points of a more broad nature, not appropriate for discussion at the end of each experimental chapter.

1B. Historical Introduction.

I have selected the following topics to cover the background literature in the divergent fields covered by the work in this

thesis. The structure of the ENS and corpus striatum and the morphology and neurotransmitter content of enteric and striatal neurons is covered, as is information relating to axonal regeneration within the CNS and much of the work to date within the field of intrastriatal transplantation.

1.1. The Enteric Nervous System.

The ENS is a term used to describe the series of ganglionated nerve plexuses found within the gut wall of most vertebrate species (Campbell and Burnstock 1968, Nilsson 1983). The name was first coined by Langley (1921) who described the ENS as the third division of the autonomic nervous system (along with the sympathetic and parasympathetic). It is composed of 2 ganglionated plexuses; the myenteric or Auerbach's plexus, first described by Auerbach in 1862 (for translation, see Furness and Costa 1987) and situated between the longitudinal and circular smooth muscle layers of the muscularis externa and the submucous or Meissner's plexus, first described by Meissner in 1857 and located within the submucosal layer. Neuronal and glial processes extend from both plexuses throughout the gut wall.

1.1.1. Function of the ENS.

The ENS acts to control and co-ordinate the activity of the gastrointestinal (GI) tract. Functionally, it is independent of the CNS, although the activity of the ENS can be modulated by the sympathetic and parasympathetic nervous systems (Langley 1921). The ENS contains complete reflex pathways which control the propulsion of intestinal contents along the GI tract (by contraction and relaxation of the smooth muscle coats around the intestine), the breakdown of

intestinal contents (by the secretion of acid and GI hormones), and the absorption of fluids and nutrients (by controlling intestinal blood flow and ion transport). The ENS contains sensory neurons (which monitor activity along the GI tract), interneurons (which modulate and relay information) and motor neurons (which innervate targets including smooth muscle and the mucosa). These three types of neurons are found within enteric ganglia. Examination of enteric ganglia has shown that enteric neurons contain a wide variety of neurotransmitters and neuromodulators and so it has proved difficult to match enteric neuronal types with such simple functional categories.

1.1.2. **Structure of the myenteric plexus.**

The myenteric plexus is found lying between the longitudinal and circular smooth muscle layers of the gut wall and is composed of ganglia, small groups of enteric neurons and glia, and nerve strands connecting them (interganglionic strands). There is great variation in the size and shape of myenteric ganglia both along the length of the gut and between the different species described, but in any species, the size and shape of enteric ganglia is fairly constant within each region of the gut (Irwin 1931, Rintoul 1960, Furness and Costa 1987).

The ganglia and interconnecting strands have been called the primary network of the myenteric plexus or the primary plexus (Li 1940, Richardson 1958). Neuronal tracing experiments have demonstrated that many of the nerve fibres in a particular segment of interganglionic strand pass over ganglia to travel in another segment of interganglionic strand. A secondary plexus and a tertiary plexus of finer fibres in the myenteric plexus have also been described (Li 1940, Richardson 1958). The secondary plexus is formed by bundles of

nerve and glial fibres branching off interganglionic strands, running parallel to and into the circular smooth muscle. The tertiary plexus is composed of small bundles of fine diameter fibres arising from the enteric ganglia and interganglionic strands of the primary plexus and the secondary plexus. These fibres are found lying in grooves in the luminal side of the longitudinal muscle and rarely penetrate it (Llewellyn-Smith *et al.* 1993).

1.1.3. Ultrastructure of enteric ganglia.

The enteric ganglia in the myenteric and submucous plexus contain the cell bodies of enteric neurons and glial cells and a neuropil of neuronal and glial processes, all tightly packed within a layer of basal lamina (Richardson 1958, Taxi 1958, 1959, 1965, Hager and Tafuri 1959, Ono 1967, Baumgarten *et al.* 1970, Gabella 1972, Cook and Burnstock 1976, Yamamoto 1977, Wilson *et al.* 1981, Komoru *et al.* 1982). Enteric ganglia are distinct from other types of autonomic ganglia as they contain no blood vessels, collagen fibres or other morphologically identifiable connective tissue elements (Baumgarten *et al.* 1970, Gabella 1972). At the electron microscopic (EM) level, the neuronal cell bodies can be easily recognised by their large, regular shaped electron-lucent nuclei which contain fine grains of chromatin, with condensations of heterochromatin at the nuclear envelope (Baumgarten *et al.* 1970, Gabella 1972, Cook and Burnstock 1976). The perinuclear cytoplasm contains ribosomes, rough and smooth endoplasmic reticulum (ER), Golgi apparatus and mitochondria (Gabella 1972, Cook and Burnstock 1976). Many enteric neurons are completely enclosed by enteric glial cells. However neuronal cell bodies can be observed at the edge of the ganglia, not covered by glial cell processes but in direct contact with the basal lamina (another

characteristic not observed in other autonomic ganglia: Richardson 1960, Taxi 1965, Gabella 1972).

The glial cells of ENS were once described as Schwann cells, identical to the supporting cells of the peripheral nervous system (PNS: Richardson 1958). Like all glial cells, the cytoplasm of these glial cells contains rough and smooth ER, many ribosomes, mitochondria, lysosomes and microtubules (Gabella 1981a). However, EM and immunohistochemical investigations have shown that the enteric glial cells exhibit marked differences to Schwann cells and possess many properties in common with astrocytes (Gabella 1971a, 1981a, 1987, Komoru *et al.* 1982). For example, enteric glia are irregular in shape, with branching processes that adapt to spaces between profiles and enwrap axons in groups, morphological characteristics exhibited by astrocytes, but not Schwann cells (Gabella 1971a). The surface of enteric glia are only covered in basal lamina at the edge of the ganglia (Gabella 1971a, Bannerman *et al.* 1986), unlike Schwann cells which are completely covered in basal lamina (Cornbrooks *et al.* 1983). Enteric glia also contain bundles of 10 nanometer gliofilaments within their cytoplasm (Gabella 1971a, Komoru *et al.* 1982) and a high concentration of immunohistochemically identifiable glial fibrillary acidic protein (GFAP: Jessen and Mirsky 1980, 1983) and S100 (Ferri *et al.* 1982), proteins not observed in myelinating Schwann cells.

Enteric glia act as supporting cells for neuronal cell bodies and neuronal processes (Gabella 1981a). Glial processes surround bundles of nerve profiles in the nerve strands of the plexus, such that a single glial cell may be associated with 600 neurites (Gabella 1981a). The number of nerve processes that are in direct contact with each other, and not shielded by glial processes varies between

species: in small mammals a large number of processes are observed, not separated by glia (Komoru *et al.* 1982), while fewer unshielded processes are observed in larger mammals (Baumgarten *et al.* 1970).

Individual ganglia contain a widely varying number of neuronal and glial cell bodies. A number of studies have attempted to calculate the average number of enteric neurons per ganglion within the guinea pig ileal myenteric plexus: although Wilson and colleagues (1981) estimated a mean of 43 neurons per ganglion, more recently an average of around 100 neurons per ganglion has been reported using two different techniques (Takaki *et al.* 1985, Young *et al.* 1993a). There are no publications of similar investigation in the myenteric plexus of the rat small intestine or colon.

There have been a number of attempts at distinguishing different types of enteric neurons. At the end of the last century, Dogiel described the cellular morphology of enteric neurons in both the myenteric and submucous plexus of a number of different species (Dogiel 1899: described in detail Furness and Costa 1987) and classified the neurons into 3 types.

Type I: flattened with stellate/angular shape with many short dendrites and one long axon that innervated the circular muscle (these are thought to be motor neurons).

Type II: angular, spindle- or star shaped with few long smooth dendrites that rarely branch and one long axon arising from the cell body or a dendrite (thought to be sensory).

Type III: stellate cell body with 2-10 dendrites of intermediate length and a smooth long axon that enters fibre bundles.

Each of these three types of neurons can be found within the same ganglia. However, a number of authors have disagreed with Dogiel's

classification and have attempted to re-classify enteric neurons (see Furness and Costa 1987 for further details).

In the past 15-20 years it has become apparent that the morphology of enteric neurons as described by Dogiel has some correlation with their electrical activity, and to some extent with the neurotransmitters and neuromodulators that they contain. For example, in 1983 Hodgkiss and Lees performed an electrophysiological investigation of myenteric neurons in the guinea pig small intestine and found that 22 of 29 "A.H." cells (neurons that demonstrate an action potential followed by a prolonged after hyperpolarisation on electrical stimulation) had Dogiel type II morphology, while the other 7 had a variety of shapes. 8 of the 14 "S" cells (neurons that give rise to a series of fast excitatory post synaptic potentials on electrical stimulation) had Dogiel type I morphology, while the other 6 had a variety of shapes. This generalization has been confirmed in a number of other investigations (eg: Erde *et al.* 1985, Katayama *et al.* 1986).

Enteric neurons within the myenteric plexus of the guinea pig small intestine have also been categorized, according to their appearance at the EM level (Cook and Burnstock 1976), although it has proven difficult to equate ultrastructurally distinct neurons with different functions.

1.1.4. **Neurochemistry of the ENS.**

Enteric neurons display remarkable neurochemical heterogeneity. There is a wealth of data describing neurotransmitters and peptides within enteric neurons, and it has become clear in the last 2 decades that within the ENS, as with other areas of the nervous system, there is colocalization, corelease and cotransmission of neurotransmitters and neuromodulators. Histochemical and immunohistochemical studies

have demonstrated the presence within enteric neurons of enzymes associated with the synthesis and degradation of transmitters including: acetylcholinesterase (AChE), choline acetyltransferase (ChAT), NADPH-diaphorase and nitric oxide synthase (NOS), and neuroactive molecules including: adenosine triphosphate, calcitonin gene related peptide (CGRP), cholecystokinin (CCK), enkephalin (met- and leu-ENK), dynorphin (DYN), galanin (GAL), gamma-amino butyric acid (GABA), gastrin releasing peptide (GRP), 5-hydroxytryptamine (5-HT), neuropeptide Y (NPY), neurotensin (NT), nitric oxide (NO), somatostatin (SOM), substance P (SP) and vasoactive intestinal polypeptide (VIP) (see Furness and Costa 1987, Belai *et al.* 1992, Llewellyn-Smith *et al.* 1992, Nichols *et al.* 1992, Young *et al.* 1992). In many cases pharmacological and electrophysiological studies have been performed on gastrointestinal tissue, to examine the release and action of these compounds.

1.1.4.1. Neurochemistry and projections of ENS within the guinea pig small intestine.

To date, the only area of the ENS that has been extensively studied by both neurochemical and surgical techniques is the guinea pig small intestine (see Furness and Costa 1987, Furness *et al.* 1992), using the above techniques as well as surgical manipulations, to elucidate the projections of enteric neurons.

The submucous plexus of the guinea pig ileum has been demonstrated to contain four distinct groups of enteric neuron: one contains ChAT/ CCK/ CGRP/ NPY/ SOM/ DYN/ GAL (Furness *et al.* 1984, 1985, 1987, 1989); a second contains ChAT and SP (Furness *et al.* 1984); a third contains ChAT alone (Furness *et al.* 1984); and a fourth contains DYN/ VIP/ GAL (Costa *et al.* 1980, 1985c, Furness *et*

al. 1987). All four categories of neurons in submucous plexus project to the mucosa (Costa *et al.* 1986) and probably act as secretomotor neurons, the secretomotor action having both a cholinergic and non-cholinergic component (Cooke 1984, Keast *et al.* 1985b). It is likely that not all molecules in any particular neuron will act in the same way, for example, in the ChAT/ CCK/ CGRP/ NPY/ SOM/ DYN/ GAL neurons, ACh provokes water and electrolyte secretion via muscarinic receptors (Keast 1987), while NPY causes a reduction in the amount secreted via a direct action on the epithelium (Keast 1987).

The guinea pig ileal myenteric plexus contains a greater variety of neuronal types. There is evidence of cholinergic and non-cholinergic excitatory motor neurons and of non-adrenergic, non-cholinergic inhibitory motor neurons as well as interneurons and secretomotor neurons (Furness *et al.* 1992). Five types of myenteric neuron have been observed so far based on neurochemical coding and projection studies. One contains CCK/ DYN/ ENK/ GRP/ VIP and projects to the prevertebral ganglia (Dalsgaard *et al.* 1983a,b, Costa and Furness 1983, Costa *et al.* 1984, 1985a, 1986). A second population contains ChAT/ SP/ NPY and projects to the circular smooth muscle where they have an excitatory action (Costa *et al.* 1980, 1985b, Steele *et al.* 1991). A third population contains ChAT/ CCK/ CGRP/ NPY/ SOM and may project to the submucous plexus and mucosa (Furness *et al.* 1983, 1985, Steele *et al.* 1991). A fourth contains DYN/ ENK/ VIP/ NOS and projects into the circular smooth muscle near the ganglion where they have an inhibitory action (Costa *et al.* 1986, 1992a). A fifth population contains DYN/ GRP/ VIP/ NO and projects to the circular smooth muscle further from the enteric ganglia where they also have an inhibitory action (Costa *et al.* 1986, 1992a).

Populations of enteric neurons have been further divided

according to their content of various other proteins, for example the calcium binding proteins calbindin and calretinin, and neurofilament protein triplet (Steele *et al.* 1991). This has led to further subdivisions of cholinergic neurons within the myenteric plexus (see Steele *et al.* 1991).

Organotypic cultures of guinea pig myenteric plexus have been utilized to provide more information on the circuitry of the guinea pig myenteric plexus, especially with regard to motor control (Costa *et al.* 1992b). DiI (a water insoluble fluorescent dye) was used to trace neuronal projections along with immunofluorescent markers for specific subsets of myenteric neurons. Inhibitory motor neurons containing VIP were found to project anally for up to 20mm before innervating the circular muscle, while ChAT/SP-containing excitatory motor neurons were observed to project orally for up to 8mm before innervating circular smooth muscle (Brookes *et al.* 1991). Excitatory motor neurons containing ChAT, SP and calretinin projected for up to 3mm within the tertiary plexus close to but rarely penetrating the longitudinal muscle (Brookes *et al.* 1992, Llewellyn-Smith *et al.* 1993). Orally projecting interneurons contain ChAT, calretinin, SP, ENK, and neurofilament (Brookes *et al.* 1991), while anally projecting interneurons can be divided into four different classes (Costa *et al.* 1992b). Two classes of secretomotor neurons (containing VIP and SOM) have also been described (Furness *et al.* 1985, Song *et al.* 1991). It has been suggested that these organised groups of sensory, inter- and motor neurons within the myenteric plexus are responsible for peristalsis and a computerized model of the enteric reflexes has shown that a simple neuronal circuit deduced from experimental studies can simulate a coordinated behaviour of the entire intestine

(Costa *et al.* 1992b).

1.1.4.2. Neurochemistry and projections of ENS within the rat intestine.

Although there is evidence for the presence in the rat ENS of most of the major neurotransmitters and neuroactive molecules found in the guinea pig intestine (Elde *et al.* 1976, Larsson *et al.* 1976, Schultzberg *et al.* 1980, Buffa *et al.* 1982, Saffrey *et al.* 1983, Sundler *et al.* 1983, Clague *et al.* 1985, Keast *et al.* 1985a, Melander *et al.* 1985, Su *et al.* 1987, Buchan and Baimbridge 1988, Belai *et al.* 1992, Aimi *et al.* 1993), the rat ENS has received far less attention regarding the projections of different sub-types of enteric neurons. Most of the neuroactive molecules have a similar distribution in the rat ENS to that in the guinea pig, although some differences can be found. For example, few GAL-containing neurons are seen in the rat ileal myenteric plexus compared to the numbers seen in the guinea pig small intestine (Melander *et al.* 1985), while the circular muscle in the rat ileum receives more GAL-containing fibres than in the guinea pig (Melander *et al.* 1985). Also there are more SP and ENK-containing fibres innervating rat colonic circular muscle than is seen in the guinea pig small intestine (Schultzberg *et al.* 1980).

Studies of the colocalization of molecules have also been performed on the rat ENS. Buchan and Baimbridge (1988) used the calcium-binding protein calbindin to distinguish two populations of VIP-containing neurons: one, within the myenteric plexus, did not contain calbindin and possibly acted as motor neurons; a second, in the submucous plexus, contained NPY and calbindin, and possibly acted as secretomotor neurons. Later, Su and colleagues (1987) reported that VIP was colocalized with GAL. More recently, NADPH-diaphorase/NOS-containing myenteric neurons within the small

intestine have been shown to contain VIP but not SP or CGRP (Aimi *et al.* 1993), suggesting they are non-adrenergic inhibitory motor neurons. However, without the use of, for example, surgical manipulations and dye tracing of rat enteric neurons as has been performed in the guinea pig small intestine, it is difficult to interpret this data with respect to distinct populations of enteric neurons.

1.1.5. Regenerative capacity of the ENS

The ENS is almost unique in the amount of structural deformation that it undergoes due to the constant movement of the intestinal wall during peristalsis. The shape of enteric neurons and glia within the ganglia varies with the extent of contraction of the muscle layers. Changes in the dimensions of the gut suggest that neurites in the ENS may be constantly remodelled and regenerate (Gabella and Trigg 1984). This notion of remodelling enteric neurites was supported by the observation that enteric neurons contain high levels of the growth associated protein GAP-43 (Sharkey *et al.* 1990, Stewart *et al.* 1992), a protein found at high levels in the developing and regenerating nervous system (Benowitz and Routtenberg 1987). Further evidence of the regenerative capacity of enteric neurons has been produced by Kobayshi and colleagues (1989) who demonstrated that enteric neurons were able to extend neurites across a resection cavity, caused by a laser lesion of the guinea pig small intestine. A separate study demonstrated that 23 days after complete surgical lesion of the guinea pig myenteric plexus, neural fibres could be observed passing over the lesion and the re-connected plexus was able to carry electrical signals (Galligan *et al.* 1989).

It has not been conclusively established whether all types of

enteric neurons are capable of regenerating axons after lesioning, although GAP-43 has been localized in all VIP-containing neurons and in a residual population of non-VIP-containing neurons within the rat ileal myenteric plexus (Sharkey *et al.* 1990). Similarly, immunohistochemical studies have demonstrated that ENK-, SP-, SOM- and GRP-containing enteric neurons are able to regenerate axons after transection (Galligan *et al.* 1989, Kobayashi *et al.* 1989).

1.2. The Corpus Striatum.

The neostriatum (or caudate-putamen, commonly abbreviated to the striatum) forms part of the basal ganglia, a group of forebrain nuclei responsible for the relaying and integration of information from the cortex to the motor (de Long and Georgopoulos 1981) and cognitive system (Iversen and Dunnett 1990). The basal ganglia, and particularly the striatum, are affected in neurodegenerative diseases such as Parkinson's disease and Huntington's disease (Albin *et al.* 1989).

Although it appears to be a homogeneous structure, the corpus striatum has been subdivided into many compartments on morphological, structural and functional grounds. Functionally, it was primarily thought to be a component of the motor system, as suggested by the motor deficits observed in Parkinson's disease (PD) and Huntington's disease (HD), however it now appears that the striatum also deals with cognitive and emotional functions, since dementia and impaired cognitive function are observed as a consequence of dopaminergic degeneration in PD patients (Lange *et al.* 1993, Mindham *et al.* 1993). Shifts in perspective have been needed, regarding the structure of the striatum, as this apparently homogeneous structure can be split into function-related compartments (the dorsolateral striatum has a

mostly sensorimotor function, receiving afferents from the somatosensory and motor cortex, while the ventromedial striatum is mostly involved with motivation, receiving afferents from the allocortex, mesocortex and limbic cortex, amygdala and hippocampus; McGeorge and Faull 1989, Kita and Kitai 1990). Immunohistochemical studies have shown that the striatum contains a wide range of transmitters (see Graybiel and Ragsdale 1983), many of which are grouped into a patch/striosome and matrix compartments which contain different subtypes of neurons, with different projections (Graybiel 1990, Gerfen 1992). Thus, in describing the striatum, it is important to keep all the different levels of compartments in mind in order that a pattern can be observed in a seemly heterogeneous population of neurons.

1.2.1. **Morphology and neurochemistry of striatal neurons**

A large number of studies have been conducted to investigate the different morphological types of striatal neurons in rodents (Mensah and Deadwyler 1974, Dimova *et al.* 1980), and in other species (Kemp and Powell 1971). There is general agreement regarding the different cell types seen, although some variation has been observed, perhaps because of differences in fixation or analytical methods. The most successful studies have combined light and electron microscopy with electrophysiology. In the rat striatum, four basic types of neurons have been described and named for the morphological features displayed in Golgi studies.

1.2.1.1. Medium spiny neuron.

The main neuronal type observed in the striatum is the medium spiny neuron, representing 90-95% (Graveland and DiFiglia 1985) of rat striatal neurons. In Golgi preparations, these neurons can be

identified by the characteristic spines on their dendrites, while at the EM level, these cells can be identified by the absence of nuclear indentations, Nissl bodies and nuclear inclusions (eg: Kemp and Powell 1971, Dimova *et al.* 1980). The medium spiny neurons contain GABA (Ribak *et al.* 1979, Oertel and Mugnaini 1984, Kita and Kitai 1988), SP (Bolam *et al.* 1983), met- and leu-ENK (Pickel *et al.* 1980) and DYN (Vincent *et al.* 1982), although the four neuroactive molecules do not coexist in all medium spiny neurons (Penny *et al.* 1986, Izzo *et al.* 1987).

This class of neurons are the major projection neurons of the corpus striatum (Grofová 1975), projecting to the globus pallidus (Niimin *et al.* 1970, Usunoff *et al.* 1974, Cuello and Paxinos 1978, Fonnum *et al.* 1978, Staines *et al.* 1980, Wilson and Groves 1980, Chang *et al.* 1981, Correa *et al.* 1981, del Fiacco *et al.* 1982, Zamir *et al.* 1984), to the substantia nigra pars reticulata (Niimi *et al.* 1970, Usunoff *et al.* 1974, Grofová 1975, Brownstein *et al.* 1977, Gale *et al.* 1977, Hong *et al.* 1977, Kanazawa *et al.* 1977b, Mroz *et al.* 1977, Jessel *et al.* 1978, Staines *et al.* 1980, Vincent *et al.* 1982, Kohono *et al.* 1984, Zamir *et al.* 1984, Fallon *et al.* 1985, Christensson-Nylander *et al.* 1986, Bolam and Izzo 1988), to the substantia nigra pars compacta (Usunoff *et al.* 1974, Bunney and Aghajanian 1976, Brownstein *et al.* 1977, Jessel *et al.* 1978, Somogyi and Smith 1979, Somogyi *et al.* 1979, Kohono *et al.* 1984), to the entopeduncular nucleus (Usunoff *et al.* 1974, Fonnum *et al.* 1978, Staines *et al.* 1980) and to other medium spiny neurons (Wilson and Groves 1980, Bolam and Izzo 1988). Cortical afferent fibres form excitatory asymmetric synapses on dendritic spines (Kitai *et al.* 1976, Hattori *et al.* 1979, Somogyi *et al.* 1981, Dubé *et al.* 1988), while thalamic afferents form asymmetric synapses on dendritic shafts

(Dubé *et al.* 1988). Nigrostriatal afferents form symmetrical synapses on dendritic spines that receive asymmetric synapses (eg: from cortical afferents: Bouyer *et al.* 1984, Freund *et al.* 1984) and also axosomatic synapses (Pasik *et al.* 1976, Somogyi and Smith 1979, Dimova *et al.* 1980, Freund *et al.* 1984). Medium spiny neurons also receive synaptic input from large aspiny cholinergic neurons (Bolam *et al.* 1986, Izzo and Bolam 1988) and medium aspiny interneurons (Smith and Bolam 1990). It has been suggested that medium spiny neurons receive synaptic input in different areas of the dendritic spine, shaft and neuronal cell body, thus allowing interaction and integration of information (see Smith and Bolam 1990 for details).

1.2.1.2. Medium aspiny neuron.

Dimova and colleagues (1980) have identified electron microscopically three types of medium sized aspiny neurons in the rat corpus striatum. Although all had indented nuclei and intranuclear inclusions, it was possible to separate them into groups according to nuclear shape, size of Nissl bodies, the number and shape of mitochondria and the number of microtubules. Immunohistochemical and histochemical studies have demonstrated that medium sized aspiny neurons contain SOM and NPY, and the enzymes NADPH-diaphorase and NOS (DiFiglia and Aronin 1982, Takagi *et al.* 1983, Vincent and Johansson 1983, Vincent *et al.* 1983a, Vuillet *et al.* 1989b, Hope *et al.* 1991). Medium aspiny neurons receive synaptic input from striatal dopaminergic afferents (Kubota *et al.* 1988, Aoki and Pickel 1989, Vuillet *et al.* 1989a, 1989b), corticostriatal afferents (Vuillet *et al.* 1989a) and pallidostriatal afferents (Bennett et aL 1993) and extend axons to, and receive input from, medium spiny GABA-ergic projection neurons (Aoki and Pickel 1989, Vuillet *et al.* 1989a,

1990).

1.2.1.3. Giant aspiny neuron.

EM studies have described one (eg: Dimova *et al.* 1980), two (eg: Mori 1966) or three (eg: Bolam *et al.* 1983, 1984b) types of morphologically distinct giant sized aspiny neurons, however all agree that these cells contain strongly indented eccentric nuclei, large Golgi bodies and intranuclear inclusions (Mori 1966, Dimova *et al.* 1980, Bolam *et al.* 1984a). Immunocytochemical studies have demonstrated that these neurons contain AChE (Bolam *et al.* 1984a) and ChAT (Kimura *et al.* 1980, Bolam *et al.* 1984b, Phelps *et al.* 1985) and make up ~2% of striatal neurons (Phelps *et al.* 1985). Cortical afferents form asymmetric synapses on distal dendrites of giant aspiny neurons (Dimova *et al.* 1993), while nigrostriatal DA-containing afferents circle round these giant cholinergic neurons, sometimes forming symmetric synapses on the cell body or proximal dendrites (Kubota *et al.* 1987, Chang 1988, Dimova *et al.* 1993). Axons from giant aspiny neurons form symmetrical synapses on medium spiny (Bolam *et al.* 1986, Izzo and Bolam 1988) and medium aspiny (Vuillet *et al.* 1992) neurons.

Recent *in situ* hybridization studies locating the mRNA for ChAT, DA D_2 receptors and SP (neurokinin-1) receptors have indicated that large aspiny striatal cholinergic interneurons can be separated into four groups: most (76%) express both receptors, thus receive input from extrinsic nigrostriatal neurons and striatal projection neurons; 16% express receptors for SP alone; 2% express only DA D_2 receptors; 6% express neither receptor (Le Moine *et al.* 1990, Aubry *et al.* 1993). This is one of the many pieces of information that suggest that DA released by nigrostriatal neurons has a regulatory role in the activity of large striatal cholinergic neurons (see Stoof

et al. 1992 for further information).

1.2.1.4. Small aspiny neuron.

Dimova *et al.* (1980) also described small aspiny cells with many processes which they believed to be glial cells, although not astrocytes. However, immunohistochemical studies have demonstrated the presence of a small population of small VIP-containing aspiny neurons within the rat striatum, which appear to be preferentially located close to fibre bundles traversing the striatum (Theriault and Landis 1987). At the EM level, these neurons exhibit a deeply indented, eccentric nucleus, characteristic of aspiny neurons (Theriault and Landis 1987 and see above). Small aspiny VIP-containing neurons receive symmetric axosomatic synapses from VIP-containing and unlabelled fibres and extend to form synaptic contacts with other axons within the striatum (Theriault and Landis 1987). It has been suggested that these neurons are interneurons, possibly projecting to striatal projection neurons, although there is little information regarding their place in striatal circuitry. VIP-containing neurons have also been reported in the feline striatum (Vincent and Reiner 1988), where they constitute a larger population than in the rat striatum (Theriault and Landis 1987).

1.2.2.1.**Subdivisions of the striatum: Patch/matrix**

Although the striatum contains a wide variety of neurotransmitters, they are not uniformly distributed through the striatum, but have been shown to be contained within neurochemically distinct areas (for review see Graybiel 1990, Gerfen 1992). These have been named "striosomes" (or "patches" in the rat) which account for 10-20% of striatal volume (Desben *et al.* 1989) and contain high levels of opioid receptors, but relatively low levels of AChE (Pert

et al. 1976, Graybiel and Ragsdale 1978, Graybiel *et al.* 1981, Herkenham and Pert 1981) and matrices which contain high levels of AChE, calbindin and SOM (Gerfen 1984, 1985, Gerfen *et al.* 1985). ENK and SP appear to be distributed in both compartments (Beckstead and Kersey 1985).

The different structures receive afferents from, and extend axons to, different regions. The matrix receives striatal inputs related to sensorimotor processing (Donoghue and Herkenham 1986), from dopaminergic neurons in the ventral tegemental area, substantia nigra pars compacta and retrorubal area (Gerfen *et al.* 1987, Jimenez-Castellanos and Graybiel 1987, Langer and Graybiel 1989) and from hippocampal afferents from the limbic system and sends projections to the globus pallidus and GABA-ergic neurons in the substantia nigra pars reticulata (Graybiel *et al.* 1979, Gerfen 1984, 1985, Jiménez-Castellanos and Graybiel 1989, Giménez-Amaya and Graybiel 1990, Kawaguchi *et al.* 1990). Neurons within the patches receive input from limbic systems (Donoghue and Herkenham 1986), the amygdala and the substantia nigra pars compacta (Gerfen *et al.* 1987, Jimenez-Castellanos and Graybiel 1987) and send projections to DA-containing neurons in the substantia nigra pars compacta (Gerfen 1984, 1985, Jiménez-Castellanos and Graybiel 1989) and a small projection to the pallidum (Giménez-Amaya and Graybiel 1990). Although it appears that the two compartments are separate, there is some interaction via neurons that extend dendrites between the patch and matrix (Bolam *et al.* 1988, Kawaguchi *et al.* 1989, Smith and Bolam 1990).

1.2.2.2. **Subdivisions of the striatum: Neuronal projections**

It has also been suggested that medium spiny striatal projection neurons can be separated according to the structure they innervate; one type extend an axon to the substantia nigra, while

another extends an axon to the globus pallidus that sends collaterals which ramify in the substantia nigra or entopeduncular nucleus (Kawaguchi *et al.* 1990, Gerfen 1992). Immunohistochemical and *in situ* hybridization studies suggest a difference in peptide expression between the two types of projection neuron; those projecting to the globus pallidus contain GABA and ENK and express the D_2 receptor, while those projecting to the nigra contain GABA, SP, DYN and NT, and express the D_1 receptor (Vincent *et al.* 1982, 1983b, Haber and Nauta 1983, Gerfen and Young 1988, Gerfen *et al.* 1990, Le Moine *et al.* 1990, Castel *et al.* 1993, Gerfen 1993). However, this distinction is not absolute, as populations of striatal neurons have been demonstrated to express SP and ENK (Penny *et al.* 1986) and both D_1 and D_2 receptors (Gerfen *et al.* 1990, Pacheno-Cano *et al.* 1993).

These two types of neuron appear to be present in similar amounts in both patch and matrix compartments (Gerfen and Young 1988, Giménez-Amaya and Graybiel 1990), although there is some differential expression in different parts of the striatum (eg: more SP in patch in ventral striatum, more DYN in patch in dorsal striatum: Gerfen and Young 1988). It has been suggested that this comes about because peptide expression is under dopaminergic control (Li *et al.* 1987, 1988, Gerfen *et al.* 1991, Gerfen 1992).

Thus it appears that the corpus striatum is a complex structure with multiple levels of organization, some of which appear to be overlapping.

1.2.3.Degenerative diseases affecting the basal ganglia

There are two main degenerative diseases that affect the basal ganglia: Parkinson's Disease and Huntington's Disease. Parkinson's Disease (PD) is primarily associated with degeneration of DA-

containing neurons in the compacta region of the substantia nigra (see Graybiel *et al.* 1990), although degeneration is also seen in other DA-containing neurons and transmitter and receptor levels are altered in other systems (see Agid *et al.* 1990). Symptoms of PD include a resting tremor in the limbs, postural abnormalities, muscular rigidity and bradykinesia as well as depression, reduced food intake and weight loss.

Huntington's disease (HD) was first described in 1872 and is a genetically linked neurodegenerative disease, the gene responsible having been recently isolated (The Huntington's Disease Collaborative Research Group 1993). Patients suffer chorea and dementia, progressing in severity until they die, 15-30 years after first displaying the symptoms (Martin and Gusella 1986). Post-mortem studies have shown a loss of striatal neurons (Vonsattel *et al.* 1985), especially medium spiny projection neurons (Graveland *et al.* 1985, Reiner *et al.* 1988) which are lost from selective areas. The remaining medium spiny neurons appear to undergo an amount of remodelling, characterized by abnormal dendritic growth (Graveland *et al.* 1985). Medium and large aspiny neurons are somewhat affected in the late stages of the disease (Ferrante *et al.* 1985, 1987, Morton *et al.* 1993). Biochemical studies report similar findings (Perry *et al.* 1973, Bird and Iversen 1974, Kanazawa *et al.* 1977a, Aronin *et al.* 1983, Beal *et al.* 1988, Ellison *et al.* 1987). Although this is a genetically linked disease, investigators have suggested that excitotoxic amino acid (Bruyn and Stoof 1990, DiFiglia 1990, Beal 1992) and alterations in energy metabolism (Albin and Greenamyre 1992, Beal *et al.* 1993, Lees 1993) may be contributing factors in this and other neurodegenerative diseases.

Although L-DOPA has a significant therapeutic effect on PD

patients, the beneficial effects may become outweighed by debilitating side effects after the drug has been taken for many years (Koller and Hubble 1990). Furthermore, there is no effective treatment for HD. Consequently in both PD and HD, there is a possibility that neural transplantation could eventually play a part in the management of the disease.

1.2.3.1. Animal models of Huntington's disease.

Studies into the pathology of HD have shown that the pattern of cell loss is similar to that seen after excitotoxic lesions of the striatum in experimental animals (eg: Coyle and Schwarcz 1976, McGeer and McGeer 1976, Schwarcz *et al.* 1983). Studies using single injections of high levels (see DiFiglia 1990 for review) and chronic infusion of low levels (Forloni *et al.* 1992, Bazzet *et al.* 1993) of quinolinic acid (QA) into the rat striatum have been most successful at replicating the neural deficits seen in HD. Lesions have been shown to kill medium spiny projection neurons while sparing both axons passing through the striatum and a percentage of large aspiny cholinergic neurons (Beal *et al.* 1986, 1991, Norman *et al.* 1991). There has been some debate regarding the extent to which QA spares medium aspiny striatal neurons (Boegman *et al.* 1987, Davies and Roberts 1987, 1988a, 1988b), although a recent report suggests that QA lesions do spare NADPH-diaphorase-containing medium aspiny neurons relative to medium spiny neurons (Roberts *et al.* 1993). Immunohistochemical studies have demonstrated an extensive increase in size and number of GFAP-containing astrocytes in the QA lesioned striatum (Björklund *et al.* 1986, Isacson *et al.* 1987b).

Although the QA-lesioned rat striatum model replicates many of the neurochemical aspects of HD, there are a number of differences

between the model and HD. EM studies have reported aberrant synapses between spared neurons following QA lesions (Roberts and DiFiglia 1990b), and a slow reduction in the number of large cholinergic interneurons (Roberts and DiFiglia 1989). Some of the surviving neurons have also been reported to undergo a regenerative response, which has not been reported in HD (Roberts and DiFiglia 1990b).

Unilateral QA lesions produce specific behavioural deficits in the rodent including locomotor hyperactivity, deficits in T-maze learning and deficits in delayed alternation tasks (Dunnett *et al.* 1988). Bilateral lesions have been shown to produce aphagia and adipsia, as well as locomotor hyperactivity and impairments in spatial memory (Isacson *et al.* 1986, Sanberg *et al.* 1989, Block *et al.* 1993). However, choreic movements, a characteristic of HD are not observed in the rodent model of HD.

Experimental QA lesions in the primate caudate-putamen have been used to produce a model closer to the human disease (Hantraye *et al.* 1991, Ferrante *et al.* 1993), where the behavioural deficits include postural asymmetries, chorea, dystonia, and dyskinesia.

1.2.3.2. Animal models of Parkinson's disease.

The most widely used model of parkinsonism is the 6-hydroxydopamine (6-OHDA) lesioned rat, where the nigrostriatal pathway is lesioned, producing a DA-denervated striatum. This lesion can be performed unilaterally to produce a hemiparkinsonian model, or bilaterally, to produce a lesion more closely resembling PD in man.

Unilateral 6-OHDA lesioned rats exhibit specific behavioural problems: contralateral sensory neglect (neglect of stimuli applied to the side of the body contralateral to the lesion) and spontaneous postural and motor asymmetry (Ungerstedt and Arbuthnott 1970, Ljungberg and Ungerstedt 1976). Although these deficits may be

observed when the animal is quiescent, they are exaggerated when the animal receives apomorphine (DA receptor agonist) or amphetamine (stimulates presynaptic release of DA from DA afferents). Apomorphine produces a contralateral rotation in the affected animal (due to the up-regulation of DA receptors in the DA-denervated striatum) while amphetamine causes ipsilateral rotation (as it stimulates the release of DA from intact nigrostriatal neurons).

Bilateral 6-OHDA lesions of the rodents substantia nigra causes animals to exhibit spontaneous and drug-induced akinesia, as well as sensory neglect, and aphagia and adipsia which result in a severe reduction in body weight (Marshall *et al.* 1971, Ungerstedt 1971, Dunnett *et al.* 1981c).

More recently, 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) has been utilized to produce hemiparkinsonism and parkinsonism in primates and some strains of mice (Heikkila *et al.* 1984, Hallman *et al.* 1985), although rats appear to be resistant to its effects (Boyce *et al.* 1984, Chiueh *et al.* 1984, Sahgal *et al.* 1984). MPTP kills the majority of nigrostriatal DA-containing neurons in primates, although cell bodies may be preserved in mice and the remaining cells exhibit temporary reduction in the enzyme tyrosine hydroxylase (TH: Hallman *et al.* 1985, Ricaurte *et al.* 1986). Animals with MPTP-lesioned striata exhibit behavioural deficits similar to those seen in PD patients, including akinesia, flexed posture, muscular rigidity, postural tremor, cognitive deficits and bradykinesia (Burns *et al.* 1983, Langston *et al.* 1984, Bankiewicz *et al.* 1986, deLong 1990, Sun *et al.* 1993).

1.3. Neural Transplantation.

The technique of intracerebral implantation has been widely utilized in the past 15-20 years, both to answer basic neurobiological questions and to examine the suitability of different types of neural and non-neural cells and structures as sources of tissue for use in treating neurodegenerative diseases. This has led to important clinical work, though still in an experimental stage, using intrastriatal grafts of foetal mesencephalon or adult adrenal medulla in patients suffering from PD and MPTP-induced nigrostriatal lesions (Rush Report Group 1990, Freed *et al.* 1992, Spencer *et al.* 1992, Widner *et al.* 1992).

1.3.1 History of neural transplantation.

The first recorded attempt of neural implantation was performed by Thompson in 1890, when he transplanted adult feline neocortex into the adult canine brain. Although he reported the event as being successful, it is likely that the grafts examined seven weeks after implantation contained no neurons. However, it was not until early this century that the first successful intracerebral grafts of spinal ganglia (Ranson 1909), peripheral nerve (PN) (Tello 1911) and neonatal CNS (Dunn 1917) were demonstrated. Ranson (1909) demonstrated that neonatal sensory neurons from spinal ganglia were capable of surviving in young adult cerebral cortex, while Tello (1911) demonstrated that pieces of predegenerated PN implanted in the adult rabbit cerebral cortex were invaded by fibres, possibly of CNS origin (although it was not possible to elucidate the origin of such fibres until the advent of reliable tracing techniques). Dunn (1917) implanted the neocortex from neonatal rats into the neocortex of littermates and observed a 10% survival rate; the surviving grafts retained their cortical features and became laminated.

The first successful attempt at grafting foetal tissue into the neonatal brain was reported by Le Gros Clark (1940), who demonstrated that neocortical tissue transplanted into the neonatal cortex was capable of surviving, differentiating and developing its characteristic laminar organization. The survival rates of Le Gros Clark's neocortical grafts were better than those reported by Dunn, the first indication that foetal CNS tissue survives implantation better than neonatal CNS tissue. These early studies also shed light on other aspects affecting graft survival rates, such as the need for an adequate vascular supply for the graft (Dunn 1917).

Even though a substantial number of papers had been published on the subject of grafting neuronal tissue into the CNS, it was not until the early 1970's that the subject took-off, when a number of new techniques were used (such as implantation of material in the anterior eye chamber, thymidine incorporation into cells prior to grafting and histochemical fluorescence techniques to identify CNS axons invading grafts) that enabled grafts to be examined more clearly (eg: Björklund and Stenevi 1971, Das and Altman 1971, Olson and Seiger 1972), and thus enabled investigators to determine the conditions necessary for optimum graft survival (Stenevi *et al.* 1976, Björklund *et al.* 1983a). The first evidence that foetal CNS tissue was capable of extending processes into, and receiving processes from, the host brain was reported by Lund and Hauschka in 1976. Later, Perlow and colleagues (1979) demonstrated, that these connections were behaviourally functional.

1.3.2. Types of intrastriatal grafts.

The technique of intracerebral implantation has been utilized for a number of different reasons: to replace afferents to a

structure (eg: intrastriatal grafts of foetal mesencephalon); to replace neurons in specific CNS areas (eg: intrastriatal implantation of foetal striatal tissue); to provide a "global" release of neurotransmitter or trophic factor (eg: adult adrenal medullary cells or genetically engineered cells implanted in the striatum or ventricle); to act as a conduit for regenerating axons (eg: peripheral nerve grafts on severed optic nerve). Thus in order to examine the efficacy of intrastriatal grafts, it is necessary to separate grafted material into different categories.

1.3.2.1 Grafts containing nigral neurons.

1.3.2.1.1. Rodent model.

Intrastriatal grafts of foetal substantia nigra neurons have been extensively investigated in a rat model of Parkinson's disease, where the animal receives a 6-OHDA lesion of the DA-containing nigrostriatal pathway, thus destroying DA-containing afferents to the striatum (see section 1.2.3.2.). The host animal is left with a series of behavioural abnormalities. Using such a model, Björklund and Stenevi (1979) were able to demonstrate that the neurons within a solid pieces of mesencephalic tissue taken from embryonic rats and implanted in the adult rat cortex, survived for up to 7 months and extended fibres into the DA-denervated striatum. These grafts also had a beneficial effect on the lesion-induced turning behaviour observed after systemic application of amphetamine (Björklund and Stenevi 1979) and apomorphine (Perlow *et al.* 1979).

1.3.2.1.2. Properties of grafted neurons.

Histofluorescence and immunohistochemical studies have demonstrated the presence of DA-containing (eg: Björklund and Stenevi 1979) and non-DA-containing (Bolam *et al.* 1987, Doucet *et al.* 1989, Mahalik and Clayton 1991) neurons within grafts of foetal mesencephalon in the

DA-denervated adult rat striatum. Grafted DA-containing neurons develop in a similar manner to normal DA-containing neurons (Jaeger 1985) and display the electrophysiological characteristics of typical nigral dopaminergic neurons (Wuerthele *et al.* 1981, Arbuthnott *et al.* 1985). Biochemical studies demonstrate that grafted dopaminergic neurons can produce DA and can partially restore levels of DA synthesis and release in the DA-denervated striatum (Schmidt *et al.* 1982, 1983, Zetterström *et al.* 1986).

1.3.2.1.3. Afferent innervation.

Anterograde labelling of neurons in the host frontal and cingulate cortex and immunohistochemical studies with antibodies raised against 5-HT have demonstrated that grafts of foetal nigral cells are innervated by fibres from both the cortex and raphe nuclei and that the ingrowing fibres form synaptic contacts with grafted DA-containing neurons (Doucet *et al.* 1989). Electrophysiological studies have demonstrated that grafted nigral neurons respond following stimulation of the locus coeruleus and cerebral cortex (Arbuthnott *et al.* 1985, Fisher *et al.* 1988).

1.3.2.1.4. Outgrowth of grafted neurons.

Grafted neurons extend axons when implanted into normal target areas such as the striatum or nucleus accumbens, but axons do not extend outside grafts implanted in non-target areas such as the hypothalamus (Björklund *et al.* 1983b). The extent of outgrowth is also affected by whether the target site has received a prior lesion; the density of nigral graft-derived innervation of the DA-denervated striatum is reported to be twice that seen around similar grafts in the unlesioned striatum (Doucet *et al.* 1990). When examined using EM-immunohistochemistry, grafted DA neurons were found to make

appropriate synaptic contacts on the dendritic shafts and spines of medium spiny striatal projection neurons (Freund *et al.* 1985, Mahalik *et al.* 1985, Mendez *et al.* 1991), but also inappropriate synapses on the cell bodies of giant aspiny neurons (Freund *et al.* 1985).

Electrophysiological studies on nigral neurons grafted into the DA-denervated striatum have demonstrated that the DA-containing grafted neurons innervate the striatum (Arbuthnott *et al.* 1985), and have a modulatory effect on host striatal neurons, reducing the rate of spontaneous firing to that recorded in the normal striatum (Strömberg *et al.* 1985b, van Horne *et al.* 1990).

Grafted neurons have other effects on target neurons in 6-OHDA lesioned rats, reducing rates of glucose uptake (Schmidt *et al.* 1982) and normalizing the post-lesion DA-containing receptor super-sensitivity in D_1 and D_2 receptors (Sirinathsinghji *et al.* 1990b, Dawson *et al.* 1991b, Rioux *et al.* 1991, Chritin *et al.* 1992, Savasta *et al.* 1992). Levels of transmitter and synthetic enzyme within GABA-containing and ENK-containing striatopallidal projections, which are up-regulated after the 6-OHDA lesion, are down-regulated after implantation of DA-containing nigral neurons (Segovia *et al.* 1989, 1990, 1991, Sirinathsinghji and Dunnett 1991, Cadet *et al.* 1991, Manier *et al.* 1991, Cenci *et al.* 1993, Mendez *et al.* 1993). Grafts also act to reverse changes in DA receptor mediated production of intracellular proteins (such as Fos) following DA-denervating lesions (Cenci *et al.* 1992).

1.3.2.1.5. Functional/behavioural effects.

As stated earlier, the primary concern of many investigators has been to examine the functional actions of grafts in the DA denervated striatum (for review see Iversen and Dunnett 1990). To date, the tests where grafts have shown to be beneficial in

ameliorating the lesion-induced behavioural deficits include:-

spontaneous-/tail pinch-/drug-induced rotation (Dunnett *et al.* 1981a)

side bias in maze exploration (Dunnett *et al.* 1981a)

contralateral sensory neglect (Dunnett *et al.* 1981b)

akinesia (bilateral) (Dunnett *et al.* 1981b)

conditioned rotation (Dunnett *et al.* 1986)

locomotor activity (Hattori *et al.* 1993)

However, grafts have been demonstrated to have no beneficial effect on a number of behavioural problems associated with 6-OHDA lesions including aphagia, adipsia (Dunnett *et al.* 1983), and skilled paw reaching (Dunnett *et al.* 1988).

It would appear that the placement of the graft is vital for the observed functional effect: animals with grafts in dorsal striatum show improvements in rotational and motor asymmetry, while animals with grafts in the lateral striatum show improvements in recovery from akinesia (Dunnett *et al.* 1981b, 1983, Mandel *et al.* 1990). The complexity of the task is also an important determinant of whether a graft will be able to ameliorate the deficit; the ability to undertake more simple tasks probably requires less complex input, and is generally easier to restore (Mandel *et al.* 1990).

Thus it would appear that experimental studies of grafts of foetal mesencephalon using the rodent model of PD (6-OHDA lesion of the nigrostriatal pathway) demonstrate that the grafts restore some of the missing innervation of the striatum and are able to ameliorate many (although not all) of the behavioural deficits associated with the DA-denervated striatum.

1.3.2.1.6. Primate model.

Experiments in non-human primates have also demonstrated that foetal nigral tissue can survive in the striatum of animals with 6-OHDA and MPTP model of PD (Annett *et al.* 1990, Taylor *et al.* 1990, Ridley and Baker 1991, Annett *et al.* 1994). The grafted tissue ameliorates a number of the behavioural deficits associated with the disease model (Redmond *et al.* 1986, Sladek *et al.* 1988, Annett *et al.* 1990, Taylor *et al.* 1990, Annett *et al.* 1994) and can survive for up to 20 months (Taylor *et al.* 1993). Subjects with the least severe lesions have been observed to exhibit the greatest improvements (Taylor *et al.* 1993).

1.3.2.1.7. Clinical trials.

In the past 5 years, a number of groups have undertaken clinical trials to determine whether grafts of foetal mesencephalon placed in the caudate and/or putamen are capable of having a beneficial effect in patients suffering from PD (eg: Freed *et al.* 1992) and MPTP-poisoning (Widner *et al.* 1992). In this time a number of different methods have been utilized, including the implantation unilaterally (eg: Lindvall *et al.* 1992) or bilaterally (eg: Freed *et al.* 1992), of single (eg: Henderson *et al.* 1991) or multiple grafts (eg: C.Freed *et al.* 1990), taken from a single foetus (eg: Spencer *et al.* 1992) or using pooled tissue from up to four foetuses (eg: Lindvall *et al.* 1992). There is also debate over whether patients require immunosuppression (see Freed *et al.* 1992). Likewise, there have been a number of different ways of assessing the clinical effects of grafts. Consequently, it has proven difficult to compare results in the different trials and to come to firm conclusions about the efficacy of particular techniques. However, many groups have reported that grafts have a beneficial effect on patients behaviour (eg: C.Freed *et al.* 1990, Madrazo *et al.* 1990, Henderson *et al.* 1991,

Freed *et al.* 1992, Lindvall *et al.* 1992, Spencer *et al.* 1992), although in some cases the effect has been reduced with time (Hoffer *et al.* 1992). Post mortem studies have reported grafted neurons are of normal appearance and form appropriate synapses (Redmond *et al.* 1990), although not all grafts examined contained the TH-positive cells (Redmond *et al.* 1990, Bankiewicz *et al.* 1993). Some grafts have been reported to stimulate the sprouting of TH-immunoreactive fibres (Bankiewicz *et al.* 1993) and rectify disease-induced changes in the levels of peptide precursors (Foster *et al.* 1993). Positron-emission tomography scans have shown an increase in DA synthesis in the area around the graft (Lindvall *et al.* 1989, Sawle *et al.* 1992, Spencer *et al.* 1992, Widner *et al.* 1992), although there are conflicting reports regarding whether grafts reinnervate the caudate or putamen (Lindvall *et al.* 1989, Sawle *et al.* 1992, Samson *et al.* 1993).

A number of clinical groups have now performed a series of clinical trials, attempting to clarify methodological problems (eg: Lindvall *et al.* 1989, 1992). However, it does appear that although grafts of foetal nigral neurons placed in the caudate and putamen of patients with Parkinson's disease produce some beneficial effects within these patients, such effects are not as dramatic as was first hoped, and in some cases may be short-lived (Hoffer *et al.* 1992). It remains to be seen whether the way forward is to carry out further, carefully planned, clinical trials attempting to optimize graft effects, or whether it would be more ethical to return to non-human primate studies, in order that the actions of grafted neurons could be more carefully assessed.

1.3.2.2. Adrenal medulla grafts in Parkinson's disease.

1.3.2.2.1. Experimental models.

The practice of implanting adrenal medullary chromaffin cells was first reported by Freed and colleagues (1981), who demonstrated that adrenal medulla grafts placed in the lateral ventricle were capable of significantly reducing the apomorphine-induced rotation associated with the rodent 6-OHDA model of PD. Adrenal medullary cells were utilized as they produce and secrete catecholamines (Coupland 1989), and have been shown to alter their morphology in culture and after implantation in the anterior eye chamber, growing new processes and extending them into the surrounding culture medium, CNS or into co-transplanted CNS material (Olson 1970, Unsicker *et al.* 1978, 1982, Olson *et al.* 1980, Strömberg *et al.* 1985a). This formation of processes by adrenal medulla chromaffin cells has been shown to be stimulated by nerve growth factor (NGF) and inhibited by glucocorticoids (Unsicker *et al.* 1978, Tischler *et al.* 1980, Strömberg *et al.* 1985a).

Preclinical and clinical investigations have examined adrenal medullary grafts in PD models at 2 sites, intraventricular and intrastriatal. Of these it has been the intraventricular grafts that have proven more successful. Adrenal medullary chromaffin cells have been demonstrated to survive within the ventricle (Freed *et al.* 1981, Freed 1983, Simonds *et al.* 1990), and display a variety of morphologies, including cells with processes, although processes derived from adrenal medullary cells do not enter the brain (Freed *et al.* 1981). Histofluoresence studies of the grafts reveal mainly adrenaline and noradrenaline-containing cells (Freed *et al.* 1981), while biochemical investigations have shown that intraventricular grafts of adrenal medullary chromaffin cells contain variable amounts

of DA, adrenaline and noradrenaline (Freed *et al.* 1983). It would appear that the catecholamine levels were affected by the surrounding brain, such that grafts near DA-denervated striatum contained a greater concentration of adrenaline and less DA (Freed *et al.* 1983).

Functionally, intraventricular grafts of adrenal medullary chromaffin cells have been demonstrated to decrease amphetamine- and apomorphine-induced rotational behaviour (Freed *et al.* 1981, Strömberg *et al.* 1985b, Becker and Freed 1988a, Bing *et al.* 1988, Pezzoli *et al.* 1988, Fitzgerald *et al.* 1989, Becker *et al.* 1990, Curren and Becker 1991, Takashima *et al.* 1992). Additionally, bilateral grafts in neonatal rats have been shown to have a partial protective effect on the aphagia and adipsia observed after bilateral 6-OHDA lesions (Simonds *et al.* 1990), although the protective effect observed after implantation of adrenal medullary chromaffin cells appears to be less than that observed with intrastriatal grafts of foetal nigral tissue (Schwarz and Freed 1987).

Following the success of intraventricular grafts, investigations have been carried out to examine intrastriatal grafts of adrenal medullary cells. Most of the intrastriatal grafts examined were found to contain few, if any, grafted adrenal medullary cells (Freed *et al.* 1986a, Strömberg *et al.* 1988, Decombe *et al.* 1990, Jedrzejewska and Dymecki 1990) and detailed examination showed the majority of cells died within the first few hours after transplantation (Strömberg *et al.* 1984). Grafts had little functional effect on the behavioural impairments caused by 6-OHDA lesions (Brown and Dunnett 1989, Decombe *et al.* 1990). On the whole, intrastriatal grafts of dissociated chromaffin cells also showed poor survival (Patel-Vaidya *et al.* 1985, Bing *et al.* 1988, Brown and Dunnett 1989)

although Ortega and colleagues (1992) have reported that dissociated and isolated (Schueler *et al.* 1993) bovine chromaffin cells survived well in the immunosuppressed rat.

Functionally, intrastriatal grafts of dissociated chromaffin cells have been reported to have a positive effect on the amphetamine-(Bing *et al.* 1988) and apomorphine-(Brown and Dunnett 1989) induced rotation, although it has been reported that purified chromaffin cells are unable to reduce amphetamine-induced rotation in hemiparkinsonian rats, even though grafted cells were observed (Bresjanac *et al.* 1993). While no extensive investigations have been undertaken, the poor survival of intrastriatal grafts of adrenal medullary cells has been blamed on too low a concentration of NGF in the striatum (Strömberg *et al.* 1988), poor nutritional conditions, accumulations of blood products, and compression of grafted cells (see W.Freed *et al.* 1990 for details), however the recent reports of extensive survival of isolated adrenal chromaffin cells (Schueler *et al.* 1993) suggests that the presence of non-chromaffin cells has a deleterious effect on graft survival.

Few investigations have been performed to determine the survival of intrastriatal grafts of adrenal medullary cells in primate model of PD. Bankiewicz and colleagues (1988) reported that grafted cells produced an ameliorating effect on both apomorphine-induced turning behaviour and skilled paw movement. However, this positive effect was short-lived (3 months) and morphological examination found no evidence of grafted cells. While there are other reports of the limited survival of adrenal medullary cells after implantation into the primate caudate (eg: Morisha *et al.* 1984, Hansen *et al.* 1988), more extensive survival of grafted cells has been reported when grafts were implanted as ribbons of tissue (Dubach

and German 1990). Such grafts are reported to produce behavioural amelioration in proportion to the amount of surviving grafted tissue (Dubach 1992).

It is interesting to note that a number of studies in different animals have demonstrated an improved rate of survival of adrenal medullary cells when co-grafted with PN (Date *et al.* 1990, Kordower *et al.* 1990, Doering 1992). This increased survival rate has been put down to the trophic support provided by the PN. While no data is available regarding any beneficial behavioural effects of such grafts, biochemical studies of the DA-denervated striatum have shown that animals with PN and adrenal medulla grafts utilize DA more efficiently (Junn *et al.* 1993).

A number of mechanisms have been suggested, to explain the action of grafted adrenal medullary cells. Since no processes extend into the DA-denervated striatum, it has been suggested that intraventricular grafts of chromaffin cells release DA into the cerebrospinal fluid (CSF), however examination of grafts found no evidence of DA within the CSF (Becker and Freed 1988a, 1988b) and only a small number of DA metabolites were detected.

A second possible mechanism of graft action is via DA release into the blood (Becker and Freed 1988b). The blood-brain-barrier (BBB) is impaired around adrenal medullary grafts (Rosenstein 1987, Curran and Becker 1991) and is permeable to pharmacological agents (Sagen and Pappas 1988), thus allowing molecules to leak out at the interface between graft and surrounding brain. Experiments have shown a positive correlation between the DA concentration in peripheral blood and the behavioural efficacy of grafts (Becker and Freed 1988a, Becker *et al.* 1990). Recently, investigators have reported that the

behavioural amelioration associated with intraventricular grafts of adrenal medullary chromaffin cells is greater in animals with intact adrenal glands than in those without, suggesting that catecholamines produced by the adrenal gland may be able to enter the CNS at the graft site (where the BBB is disrupted) and have an effect on behaviour (Takashima *et al.* 1992).

A third possible mechanism of action of adrenal medullary grafts is via the release of trophic molecules, that may cause sprouting of any remaining axons. Candidate molecules include basic fibroblast growth factor (bFGF), which is produced by adrenal medullary chromaffin cells (Blottner *et al.* 1989) and has a supportive effect on cultured embryonic dopaminergic neurons (Otto and Unsicker 1993), transforming growth factor β, interleukins, neurotrophins and ciliary neurotrophic factor (CNTF) (see Unsicker 1993 for details).

Recently it has been suggested that the amelioration of amphetamine- and apomorphine- induced rotational behaviour occurs by different mechanisms, as recipient animals exhibited amelioration in one test but not the other. Curran and colleagues (1993) report that amelioration of amphetamine-induced rotation is associated with the restoration of striatal DA-containing function, as indicated by the normalization of striatal DA concentrations and DA D_2 receptor sensitivity, while amelioration of apomorphine-induced rotation is associated with increased levels of DA, but not normalization of striatal function.

It is interesting to note that in the more recent model of PD, utilizing the toxin MPTP, the mechanism of action is such that dopaminergic nigral neurons are not killed but causes a temporary reduction in the TH enzyme (Hallman *et al.* 1985). Adrenal medullary

chromaffin cells implanted into the MPTP-lesioned mouse brain produce an up-regulation of the TH enzyme in the MPTP-sensitive nigral neurons (Bohn *et al.* 1987).

1.3.2.2.2. Clinical trials.

To date, there have been a number of clinical trials utilizing grafts of adrenal medullary cells in patients with Parkinson's disease. Although some investigators have reported that grafts produce remarkable effects in patients (eg: Madrazo *et al.* 1987, Jiao *et al.* 1988), the duration and extent of such improvements is as varied, as are the clinical tests used to determine functional improvements. Standardization of behavioural testing has demonstrated that adrenal medulla grafts do have a moderate beneficial effect in patients suffering from PD, although it has become clear that such patients are also prone to a range of clinical complications (Bakay *et al.* 1990, Goetz *et al.* 1990). Post-mortem studies have demonstrated a low incidence of surviving chromaffin cells (Dohan *et al.* 1988, Forno and Langston 1989, Hurtig *et al.* 1989, Jankovic 1989, Peterson *et al.* 1989, Hirsch *et al.* 1990) and biochemical studies indicate that grafts do not increase CSF levels of DA (Rush Research Group 1990). The mechanism of action of such grafts is unknown, although their long-term efficacy would appear to rule out a placebo effect (Goetz *et al.* 1990). Other suggested mechanisms include lesions of the caudate nucleus producing behavioural effects (Rush Research Group 1990); a local inflammatory response causing production of trophic factors (Riopelle 1988); grafts producing a factor that inhibits an autoimmune response (Carvey *et al.* 1990); tissue implantation causing striatal neurons to re-regulate DA receptor density (Joyce and Hurtig 1990). However, further

information is required to determine how the implantation of adrenal medulla cells produces the behavioural effects seen in patients suffering from PD. Furthermore, it remains to be seen whether the clinical effects produced by grafts of adrenal medullary chromaffin cells may not be better be produced by the implantation of different type of material.

1.3.2.3. Implantation of cell lines and genetically engineered cells.

Cell lines, such as PC12 pheochromocytoma cells (Greene and Tischler 1976), have received some attention with regard to their possible utilization in neurodegenerative diseases, as they produce catecholamines and are available in large quantities. PC12 cells survive for long periods within the rat striatum (Freed *et al.* 1986b) and reduce apomorphine-induced rotation in rats with unilateral 6-OHDA lesions (Emerich *et al.* 1993), but do not affect the aphagia, adipsia or alterations in locomotor behaviour observed in bilaterally 6-OHDA lesioned rats (Emerich *et al.* 1993).

The prospect of utilizing genetically engineered cells as a source of material to combat neurodegenerative diseases was first proposed in 1987 (Gage *et al.* 1987b). By implanting non-neural cells, taken from the patient, that have been genetically engineered to produce a certain neurotransmitter or trophic factor, it is theoretically possible to provide an affected area with the desired chemical, without any problems regarding the ethics of tissue procurement or possible immunological rejection (for review of strategy see Gage *et al.* 1990). To this end, trials utilizing immortalized fibroblast (eg: Wolff *et al.* 1989), neuroblasts and neuroendocrine cells (Horellou *et al.* 1990a, 1990b) have been reported. More recently, investigations have begun, utilizing primary

fibroblasts (Kawaja *et al.* 1992), astrocytes (Cunningham *et al.* 1991) and skeletal myocytes (Jiao *et al.* 1993).

Although it was first believed that all cell types would be equally effective as carriers for introduced genes (Gage *et al.* 1987b, Horellou *et al.* 1990b), grafts of immortal cell lines have been found to produce tumours two weeks after implantation into the rat brain (Horellou *et al.* 1990a, 1990b). Experiments utilizing primary rat fibroblasts engineered to make the TH enzyme have demonstrated that grafted cells reduce apomorphine-induced turning in the unilaterally DA-denervated striatum and do not form tumours (Chen *et al.* 1991, Fisher *et al.* 1991, Gage and Fisher 1991). However, this behavioural effect is short-lived (Fisher *et al.* 1991, Gage and Fisher 1991).

More recent experiments involving the implantation of primary skeletal muscle cells transfected with the TH gene into the striatum of a 6-OHDA lesioned rat have been more promising (Jiao *et al.* 1993). The grafted cells partially restored DA levels in the DA-denervated striatum, and reduced apomorphine-induced rotation up to 6 months after implantation (Jiao *et al.* 1993). This is the most successful report of the use of genetically engineered cells in an animal model of neurodegenerative disease to date, but the grafts were unable to completely eliminate the apomorphine-induced turning behaviour. While it is encouraging that these cells can have such a profound effect, it remains to be seen if technical advances will enable grafts to totally eliminate apomorphine-induced turning, or any of the other more subtle behavioural deficits associated with PD and its animal model.

1.3.2.4. Striatal grafts.

1.3.2.4.1. Rodent model.

As has been described earlier, excitotoxic amino acid lesions of the rat corpus striatum have been put forward as a rodent model of the neurological disorder, Huntington's Disease (HD: see DiFiglia 1990 for review and section **1.2.3.1.**). This lesion model leaves the animal with a specific set of behavioural abnormalities and defined neuronal loss within the striatum (see section **1.2.3.1.**). Schmidt and colleagues (1981) were the first to describe the survival of grafts of embryonic rat striatal tissue implanted in the lesioned striatum and the subsequent restoration of glutamic acid decarboxylase (GAD) and ChAT levels in the striatum. This was then followed by two reports of intrastriatal grafts of embryonic striatal tissue ameliorating a behavioural deficit produced by the excitotoxic lesion (Deckel *et al.* 1983, Isacson *et al.* 1984).

1.3.2.4.2. Morphology of grafted neurons.

To date immunohistochemical and *in situ* hybridisation studies have demonstrated that grafts of foetal striatal tissue develop within the lesioned and unlesioned striatum and express the enzymes AChE, ChAT, GAD and NADPH-diaphorase, and the transmitters GABA, met-ENK, NPY, SOM and SP (reviewed by Wictorin 1992, and see Isacson *et al.* 1985, 1987a, Walker *et al.* 1987, Roberts and DiFiglia 1988, 1990a, Graybiel *et al.* 1989, Wictorin *et al.* 1989c, Zhou and Buchwald 1989, Zhou *et al.* 1989, Clarke and Dunnett 1990, Giordano *et al.* 1990, Liu *et al.* 1990, Sirinathsinghji *et al.* 1990a, K.Campbell *et al.* 1992, Helm *et al.* 1992). The grafts also contain high levels of muscarinic and D_1 and D_2 receptors (Deckel *et al.* 1986b, Isacson *et al.* 1987a, Liu *et al.* 1990, Mayer *et al.* 1990, Helm *et al.* 1991). Detailed morphological studies of the grafts have also demonstrated

the presence of morphologically typical striatal cells (McAllister *et al.* 1985, Clarke *et al.* 1988, DiFiglia *et al.* 1988, Helm *et al.* 1990, Xu *et al.* 1992). Electrophysiological studies have demonstrated that grafted striatal neurons express patterns of ionic currents identical to normal striatal neurons (Surmeier *et al.* 1992) and respond in a similar manner to adult striatal neurons (Rutherford *et al.* 1987, Xu *et al.* 1991b). However, the distribution of such striatal markers is patchy, and the non-striatal regions have been shown to contain some cells typical of the globus pallidus, cortex and amygdala (Walker *et al.* 1987, Graybiel *et al.* 1989, Wictorin *et al.* 1989c, Sirinathsinghji *et al.* 1993, Usui *et al.* 1993), which may be included during the dissection process.

1.3.2.4.3. Innervation from afferents to the striatum.

There is now a wealth of evidence that foetal striatal tissue implanted into excitotoxin-lesioned striatum is able to receive "normal" afferents and extend fibres to many of the striatal target areas (for review see Wictorin 1992).

1.3.2.4.3.1. Nigrostriatal afferents

Histofluorescently labelled DA-containing fibres have been demonstrated to form a patchy distribution within intrastriatal grafts of foetal striatal tissue (Pritzel *et al.* 1986), the density of DA-containing fibres within the patch being very similar to that seen in the striatum. When examined using EM-immunohistochemistry, TH-containing fibres were demonstrated to form "normal looking" symmetric synapses on the dendritic shafts and spines of grafted medium spiny neurons (Clarke *et al.* 1988). Studies using retrograde axonal tracers have demonstrated that foetal striatal grafts receive afferents from the substantia nigra pars compacta (Pritzel *et al.*

1986, Wictorin *et al.* 1988, Wictorin and Björklund 1989) and these represent 50-75% of cells labelled by similar injections in the normal striatum (Wictorin and Björklund 1989), although one group of workers have been unable to identify nigral fibres innervating the grafts (Walker and McAllister 1987, McAllister *et al.* 1989).

A number of studies have attempted to demonstrate the connectivity between the host substantia nigra and grafted striatal neurons. Using a push-pull perfusion technique to constantly perfuse and collect from the globus pallidus and substantia nigra, Sirinathsinghji and colleagues (1988) demonstrated that stimulation of DA systems (via systemic application of amphetamine) led to an increase in GABA release in the striatal projection areas. More specific experiments have utilized the fact that the proto-oncogene *c-fos* is turned on to express Fos protein following the activation of D_1 DA receptors (Robertson *et al.* 1989) and grafts have been examined immunohistochemically for Fos, following activation of DA-containing systems. Grafted striatal neurons show increased Fos immunoreactivity following injections of haloperidol (Dragunow *et al.* 1990), cocaine (Liu *et al.* 1991), amphetamine (Mandel *et al.* 1992) and areas of increased Fos immunoreactivity correlated with localization of striatal markers (Liu *et al.* 1991, Mandel *et al.* 1992), thus demonstrating that DA-containing inputs into grafts are functional and that striatal-like regions of the graft respond in the same manner as the surrounding striatum.

1.3.2.4.3.2. Corticostriatal afferents

Retrograde labelling of intrastriatal grafts of foetal striatum has labelled cortical cells, although only approximately a third of the number seen after similar injections in the normal striatum (Wictorin and Björklund 1989), while anterograde labelling from the

frontal cortex has shown that cortical afferents densely innervate the periphery of grafts (Wictorin and Björklund 1989). EM studies have demonstrated that cortical afferents form "normal-looking" asymmetric synaptic contacts with neurons within the graft (Wictorin *et al.* 1989b, Xu *et al.* 1989) and these synapses are formed predominantly on dendritic spines (as in the normal striatum), although there is some debate over the exact percentages of synapses in appropriate and inappropriate position (compare Wictorin *et al.* 1989b and Xu *et al.* 1989). Electrophysiological studies both *in vivo* and on slice preparations have shown connectivity between the cortex and striatum (Rutherford *et al.* 1987, Xu *et al.* 1991b).

1.3.2.4.3.3. Thalamostriatal afferents

Thalamic neurons extending axons to striatal grafts have been labelled via retrograde tracers injected into the graft, however the number of labelled thalamic neurons only represents 5-30% of those normally observed (Wictorin *et al.* 1988, Wictorin and Björklund 1989). Anterograde labelling of thalamic afferents to the striatum has demonstrated that the majority of fibres are observed at the periphery of the graft (Wictorin *et al.* 1988), perhaps explaining the variable and low percentage of fibres seen following retrograde tracing, compared to that seen in the normal striatum. EM investigations have demonstrated that thalamic afferents form "normal-looking" asymmetric synaptic contacts with grafted striatal neurons, although not on the normal target area of the neuron (Xu *et al.* 1991a).

1.3.2.4.3.4. Afferents from mesencephalic raphe

Immunohistochemical studies have demonstrated the presence of 5-HT-containing fibres distributed throughout intrastriatal grafts of

foetal striatal tissue (Wictorin *et al.* 1988) while retrograde tracer applied to intrastriatal grafts of striatal tissue have labelled neurons within the mesencephalic raphe (Wictorin *et al.* 1988, Wictorin and Björklund 1989).

1.3.2.4.4. Efferent connections

As stated above, the major targets for striatal axons are the globus pallidus, entopeduncular nucleus and the substantia nigra pars reticulata. Of these, it is the globus pallidus that receives most fibres from grafted striatal neurons (Isacson *et al.* 1985, Pritzel *et al.* 1986, Wictorin *et al.* 1989a, 1989c), although there is some evidence of fibres entering the entopeduncular nucleus and the substantia nigra pars reticulata (Wictorin *et al.* 1989a). EM studies have demonstrated that graft-derived fibres form morphologically normal symmetric synaptic contacts on the dendritic shafts of the globus pallidus (Wictorin *et al.* 1990a) and using the push-pull perfusion technique (that constantly perfuses and collects from the globus pallidus), intrastriatal grafts of foetal rat striatum have been demonstrated to cause GABA release in the globus pallidus and substantia nigra pars reticulata to return to normal levels, following an ibotenic acid lesion (Sirinathsinghji *et al.* 1988).

Experiments using cross species transplants (foetal mouse or human to adult rat) have demonstrated that fibres grow to the globus pallidus via the myelinated fibre tracts of the internal capsule (Wictorin *et al.* 1990b, 1991); it is also evident that fibres from grafted human neurons are more capable of reaching the substantia nigra within the rat model, than grafted rodent neurons, perhaps reflecting the greater length of human striatonigral axons under normal circumstances (Wictorin *et al.* 1990b)

1.3.2.4.5. Functional/behavioural effects

The first reports of functional effects of intrastriatal grafts were seen in the mid-80's (Deckel *et al.* 1983, Isacson *et al.* 1984), and related to grafts ameliorating the spontaneous locomotor hyperactivity produced following excitotoxin lesions (see also Deckel *et al.* 1986a, 1986b, Isacson *et al.* 1986, Sanberg *et al.* 1986, Giordano *et al.* 1990). It is now apparent that intrastriatal grafts of foetal striatum have a positive effect on a number of other behavioural deficits (for review see Norman *et al.* 1989b, Wictorin 1992). For example:

- amphetamine-induced locomotor hyperactivity (following bilateral lesion: Sanberg *et al.* 1986)

- amphetamine/apomorphine-induced rotational behaviour (following an unilateral lesion; Dunnett *et al.* 1988, Norman *et al.* 1989a)

- deficits in skilled paw reaching (Dunnett *et al.* 1988, Montoya *et al.* 1990)

- deficits in spatial alternation learning (Deckel *et al.* 1986a, Isacson *et al.* 1986).

There are a number of possible explanations for these effects: for example the influence of a trophic factor produced by the graft, a diffuse release of neurotransmitter or the transplant becoming completely integrated with the lesioned host and replacing lost neurons (Wictorin 1992). Although all explanations have some merit, the well documented examples of grafted foetal striatal neurons extending some processes to the striatal targets and receiving innervation from structures normally innervating the striatum, and the presence of apparently normal/appropriate synaptic connections, makes it likely that it is the partial reconstruction of striatal

circuitry that allows grafts to have such a wide-spread positive actions.

1.3.2.4.6. Primate model

Studies using a primate model of HD have demonstrated that rodent foetal nigral neurons can survive, grow to normal size and develop striatal transmitters (Isacson *et al.* 1989) within the lesioned baboon caudate. Such grafts also had a positive effect on the behavioural deficits produced by the lesion, reducing apomorphine-induced turning and dyskinesia (Isacson *et al.* 1990). However a recent study of foetal Rhesus monkey neostriatal tissue implanted in the excitotoxin lesioned monkey striatum has found few surviving cells 8 months after implantation (Helm *et al.* 1993), perhaps suggesting that there may be some problems associated with implantation into higher mammals.

1.3.2.4.7. Clinical studies

The first report of a clinical trial of striatal grafts in patients suffering from HD does not categorically state the effect of such grafts (Sramka *et al.* 1992, cited in Dunnett and Svendesen 1993). More recently, Madrazo and colleagues (1993) have implanted human foetal striata into two Huntington's patients, with no apparent damaging effects, and claim that the disease is progressing less rapidly in one patient examined 20 months after surgery than was seen in an affected sibling. It is obviously premature to make judgments on the efficacy of such implants, and it has been suggested that further information is required regarding, the optimal donor age, dissection and surgical techniques, and the stage of the disease at which the results of grafting will be most effective, before full scale clinical trials should be attempted in patients.

1.4.Axonal regeneration.

For more than a century, axonal regeneration has been studied both in the PNS (Ramón Y Cajal 1928, Sunderland 1950, Guth 1956) and the CNS (Ramón Y Cajal 1928, Le Gros Clark 1942, 1943, Clemente 1964, Kiernan 1979, Berry 1984, 1989). The regenerative capacity of peripheral nerves is most well developed in the PNS of lower vertebrates (see Holder and Clarke 1988 for details), but is sufficient to enable the repair of injured nerve trunks in man.

The situation in the CNS is even more "polarized". CNS neurons in lower vertebrates exhibit a highly developed regenerative capacity (Hooker 1932, Koppanyi 1955, Hibbard 1963, Bernstein 1967) and regenerating axons reform appropriate synaptic contacts (Sperry 1944, Hibbard and Ornberg 1976). Mammalian CNS neurons, on the other hand, display a very limited regenerative capacity (Ramón Y Cajal 1928, Le Gros Clark 1942, 1943, Clemente 1964, Kiernan 1979, Berry 1984, 1989) and only under specific, rarely achieved circumstances can functional recovery be observed.

1.4.1.Neuronal response to injury and axonal regeneration in the PNS

As stated above, peripheral neurons are capable of regenerating their axons following axotomy (for review see Hall 1989, Lisney 1989, Fawcett and Keynes 1990), an ability that has been put down to both the regenerative capacity of injured neurons to grow new axons and to the ability of the injured peripheral nerve to support axonal growth (Ramón Y Cajal 1928, Aguayo and Bray 1984). The cell body of peripheral neurons (eg: sensory neurons) responds to axotomy by altering its metabolism, reducing synthesis of molecules associated with neurotransmission (such as the neuropeptides SP and CGRP), and increasing the synthesis of substances associated with axonal growth

(such as actin, tubulin and GAP-43: McQuarrie 1983, Aldskogius *et al.* 1992). There is also a paradoxical down-regulation in the synthesis and transport of neurofilament triplet protein (McQuarrie 1983). Although actin production will return to normal, the rate of synthesis of other cytoskeletal proteins only returns to normal once reinnervation occurs (McQuarrie 1983). This response of peripheral neurons to axotomy is thought to come about via a number of different ways, involving axonal transport (see Aldskogius *et al.* 1992 for details).

Soon after axotomy small axonal sprouts can be observed originating from myelinated (Morris *et al.* 1972, McQuarrie 1985) or unmyelinated (Bray *et al.* 1972, Bray and Aguayo 1974, Fawcett and Keynes 1990) axons. Regenerating PNS axonal sprouts have been described as closely packed profiles of 1-15 μm diameter, containing clear and dense-cores vesicles, neurofilaments, microtubules, smooth ER and mitochondria (Bray *et al.* 1972, Morris *et al.* 1972, Bray and Aguayo 1974, Duce and Keen 1976).

As sprouts form, axons proximal to the site of injury become swollen, forming terminal clubs packed with axoplasmic organelles (mitochondria, smooth ER, synaptic vesicles, neurofilaments and dense bodies: Morris *et al.* 1972, Kao *et al.* 1983). The terminal clubs appear 1-2mm from the site of injury, within the basal lamina, in contact with Schwann cells (Kao *et al.* 1983). Axonal sprouts form from the terminal clubs and from nodes proximal to the site of injury within one day of axotomy (Kao *et al.* 1983).

As the proximal stump of the lesioned PN reacts to injury, the distal stump undergoes Wallarian degeneration. Axonal and myelin debris is removed by Schwann cells and invading macrophages (Allt 1976, Sunderland 1978, Beuche and Friede 1984, 1985, Scheidt and

Friede 1987, Perry and Brown 1992) leaving "bands of Büngner", columns of Schwann cells inside the tubes of basal lamina which had surrounded the original nerve fibres (Sunderland 1978). Regenerating PNS axons cross from the proximal to the distal stump and become associated with the Schwann cells in the bands of Büngner (Anderson *et al.* 1983, Hall 1986a, 1986b)

1.4.2.Neuronal response to injury- mammalian CNS

CNS neurons respond to axotomy in a number of ways, ranging from cell death (the most extreme) to alterations in morphology (chromatolysis, nuclear eccentricity, changes in nucleolar and cell body size and in dendritic morphology: for reviews see Lieberman 1971, Barron 1983a, 1983b, 1989) or alterations in metabolic activity (eg: enzyme down-regulation or increases in synthesis of cytoskeletal and growth-associated proteins; see Lieberman 1971, Barron 1983a, 1983b, 1989, Skene 1989).

1.4.2.1 Cell death.

It has been known for many years that CNS neurons can die as a consequence of axotomy. For example, Betz cells in the cerebral cortex degenerate following lesions of their axons in the internal capsule (Marinesco 1901, reviewed by Lieberman 1974); cells in Clarke's nucleus in the L3 segment of the cat spinal cord die following lesions in the dorsolateral quadrant at the T12 or C1 level (Liu 1955, Loewy and Schader 1977); cells in the magnocellular portion of the red nucleus, in adult and newborn rats, degenerate after spinal cord hemisection at the mid-thoracic level (Prendergast and Stelzner 1976); cells in the paraventricular and supraoptic nuclei die in patients following hypophysectomy (Morton 1969). There are also examples of cells degenerating after loosing afferent

connections. For example, neurons in the ventral tegmental and medial mammillary nuclei are completely lost following limbic decortication in new born rabbits (Bleier 1969). In the past, there has been some confusion over the extent of cell death. Thus, it has been reported that cholinergic neurons in the medium septum disappear following lesions of the septohippocampal pathway (Gage *et al.* 1986), but addition of NGF causes these neurons to reappear, suggesting the cells shrink in response to axotomy and the loss of trophic support (Hagg *et al.* 1988).

1.4.2.2. Cytological changes in axotomized CNS neurons.

Chromatolysis is an early indication of neuronal reaction to axotomy and typically begins with dispersion and disintegration of Nissl material in the nuclear region adjacent to the axon hillock (see Lieberman 1971, Barron 1983a, 1983b, 1989 for review), which may form an axon "cap" (Egan *et al.* 1977, Barron 1989). At the EM level, affected neurons have disrupted rough ER, the rough ER cisternae becoming shorter, loosing the characteristic parallel arrangement and becoming dispersed throughout the cytoplasm (Lieberman 1971, Egan *et al.* 1977, Barron 1893a, 1983b).

CNS neurons that undergo cell death following axotomy exhibit other cytological alterations such as the dispersal of cytoplasmic polyribosomal clusters and increased amounts of smooth ER are also observed (eg: red nucleus, Barron *et al.* 1975, Egan *et al.* 1977; lateral geniculate nucleus, Wong-Riley 1972). The Golgi apparatus in affected neurons becomes vesiculated and vacuolated (Barron *et al.* 1975) and mitochondria are found to accumulate in some affected cells (Wong-Riley 1972), but not in others (Egan *et al.* 1977). Changes in neurofilament content in the perikarya of axotomized CNS neurons that go on to die are variable, with some cells displaying great increases

(eg: red nucleus, Barron *et al.* 1975, Egan *et al.* 1977), while no changes are observed in other degenerating neurons (eg: lateral geniculate nucleus, Barron *et al.* 1967). Alterations have also been reported in dendrites of axotomized CNS neurons, with the presence of enlarged mitochondria, glycogen accumulation within the dendrites and abnormalities in the electron-density of dendrites (Barron *et al.* 1975).

A number of differences have been observed in the response to axotomy between different types of CNS neurons. CNS neurons that extend outside the CNS (extrinsic) are more likely to regenerate following axotomy and behave like peripheral neurons (Lieberman 1974, Aldskogius *et al.* 1980, Barron 1983a, 1983b). Extrinsic CNS neurons exhibit enlarged cell bodies and nucleoli after axotomy, but revert to normal morphological characteristics 2-8 weeks after axotomy (Barron *et al.* 1977, Egan *et al.* 1977, Barron 1989). Intrinsic CNS neurons (those that do not extend beyond the CNS) may undergo rapid cell body atrophy, although even those that do not fully atrophy, do not completely recover their morphological characteristics (Barron *et al.* 1977, Egan *et al.* 1977, Barron 1989).

1.4.2.3. Metabolic changes in axotomized CNS neurons.

Axotomy of intrinsic CNS neurons, has been demonstrated to lead to a reduction in RNA and protein content within the neuron (Barron *et al.* 1977), although there are a number of reports of transient up-regulation of the proto-oncogene *c-jun* (Jenkins *et al.* 1993, Koistinaho *et al.* 1993), and the increased expression of proteins JUN D and KROX (Herdegen *et al.* 1993). Axotomy of extrinsic neurons, on the other hand, produces an increase in cytoplasmic RNA synthesis (Aldskogius *et al.* 1980), although the delay before the onset of

increased synthesis varies depending on the type of neuron and the distance between the site of injury and the cell body (Barron *et al.* 1982).

PNS and intrinsic CNS neurons responding to axotomy also display paradoxically elevated levels of phosphorylated neurofilament protein in the cell body (whereas it is usually found in axons; Rosenfeld *et al.* 1987, Oestreicher *et al.* 1988). This may be a result of abnormal phosphorylation of the proteins and blockage of axonal transport (Rosenfeld *et al.* 1987).

A number of studies have reported the reduction in enzymes associated with neurotransmitters in CNS neurons after axotomy (Waldron and Gwyn 1969, Gwyn 1971, Wooten *et al.* 1978). Some neurons respond to injury by reducing neurotransmitter receptor expression (eg: hypoglossal neurons: Rotter *et al.* 1977, 1979), while receptor expression is unaltered in other neurons (eg: pontocerebellar projection neurons: Rotter *et al.* 1979). It has been suggested that the differences in receptor expression are related to the metabolic reaction to axotomy, since axonal regeneration can occur in motor neurons (eg: hypoglossal neurons), but is not a feature of intrinsic CNS neurons (eg: pontocerebellar projection neurons: see Rotter *et al.* 1979 for discussion).

1.4.3. **Factors affecting the response of intrinsic CNS neurons.**

A variety of studies have demonstrated that the response of a CNS neuron to axotomy is affected by many different factors. For example, a number of studies have examined the fate of CNS neurons in different mammalian species, following axotomy and the results obtained suggest that CNS neurons in different species do react differently, although no overall theory can be put forward to explain the different responses (for review see Lieberman 1974, Barron

1983a).

1.4.3.1. Age.

It is also apparent that age is an important determinant of the response of CNS neurons to axotomy. Intrinsic CNS neurons that undergo axotomy at an early post-natal age are particularly "prone" to rapid cellular atrophy, necrosis and even cell death (reviewed by Bleier 1969, Lieberman 1974, Barron 1983a, 1983b). It is likely that neonatal CNS neurons are more vulnerable to axotomy because of their under-developed nature, a different vulnerability to excitotoxins or because they are under more "strict" requirements for target-derived trophic factors (see review by Barron 1983a, 1983b). There is also evidence to suggest that neonatal CNS extrinsic neurons are more vulnerable to axotomy than those in adult animals, perhaps for similar reasons (Lowrie *et al.* 1987).

1.4.3.2. Distance between lesion site and neuronal cell body.

The distance between the lesion site and neuronal cell body is also an important determinant of the neuronal response to injury. In general, the closer the injury is to the cell body, the more rapid is the retrograde response and the more extensive the neuronal degeneration (reviewed by Lieberman 1974, Barron 1983a, 1983b). It has been speculated that the signal responsible for the cell body response to axotomy is conveyed retrogradely to the cell body (as blocking retrograde axonal transport delays the onset of chromatolysis, see Singer *et al.* 1982, Bray and Aguayo 1989) or that the loss of a peripherally derived influence is involved in the retrograde degeneration of axotomized neurons (Bray and Aguayo 1989).

1.4.4. **Axonal regeneration of adult CNS neurons after axotomy.**

Although it is well established that intrinsic CNS neurons are

incapable of fully regenerating their axon after axotomy, there is evidence that some intrinsic CNS neurons mount a regenerative response following axotomy (Ramón Y Cajal 1928, Lampert and Cressman 1964, Borgens *et al.* 1986, Hall and Berry 1989, Zeng *et al.* 1991). This abortive sprouting response was investigated by Ramón Y Cajal (1928) in a number of different regions of the CNS, including the spinal cord, spinal roots and optic nerve. Within the spinal cord, growth cones are found emanating from the lesioned axons, which increased in diameter and ramified to form axonal sprouts (Ramón Y Cajal 1928). These axonal sprouts grew within the spinal cord, and some became associated with invaded Schwann cells and were myelinated (Lampert and Cressman 1964). Regenerating axonal sprouts did not grow far into the glial scar at the site of injury (Ramón Y Cajal 1928, Lampert and Cressman 1964) and were observed to have disappeared 1 month after spinal cord transection (Ramón Y Cajal 1928, Lampert and Cressman 1964).

A similar abortive sprouting response has been reported in the optic nerve following transection (Zeng *et al.* 1991). Axonal sprouts were identified 24 hours after transection and increased in number up to five days after transection. By three days after transection, the sprouts were associated with astrocytes and extended as far as the end of the optic nerve. This abortive sprouting response is also observed when the transected optic nerve is attached to an acellular peripheral nerve graft; axotomized retinal ganglion cells form regenerative axonal sprouts, which grow out from the transected optic nerve towards the acellular graft, but die back after 10-20 days (Hall and Berry 1989). This has led investigators to suggest that intrinsic CNS neurons are capable of mounting a regenerative response following axotomy, but that the response is ultimately abortive due

to the presence of inhibitory factors or the absence of factors necessary for the growth of CNS regenerative axons.

1.4.5.Growth of CNS regenerating axons into grafts, following axotomy

As stated above, the majority of CNS neurons do not regenerate their axons following axotomy, however extrinsic CNS neurons are capable of mounting a regenerative response after axotomy (Lieberman 1974, Barron 1983a, 1983b). In addition, however, some intrinsic CNS neurons have been shown to be capable of mounting a regenerative response into various types of grafts.

1.4.5.1. PN grafts.

Since axonal regeneration in PNS has long been known to be vigorous, the question was soon raised as to whether this regenerative capacity was the result of an inherent ability of peripheral neurons, or of the environment which the injured PN provided for regenerating axons. This was answered by implanting pieces of PN into the CNS (eg: Tello 1911, Ramón Y Cajal 1928, Le Gros Clark 1943). Although some success had been reported with this technique, (Ramón Y Cajal 1928), it was not until the advent of retrograde labelling techniques that workers were able to categorically demonstrate the CNS origin of axons invading PN grafts (Richardson *et al.* 1980, 1982, 1984, David and Aguayo 1981, Benfey and Aguayo 1982, Berry 1984, Berry *et al.* 1986a, 1986b, 1987, 1988, Hall and Berry 1989). However, some studies have indicated that not all CNS neurons are equally capable of regenerating axons into PN grafts. For example, the majority of axons invading a PN implanted in the thalamus come from neurons in the thalamic reticular nucleus (Benfey *et al.* 1985, Morrow *et al.* 1993), while in the cerebellum, neurons in the deep cerebellar nuclei are capable of regenerating

their axons within a PN graft, unlike Purkinje cells in the cerebellar cortex (Dooley and Aguayo 1982, Vaudano *et al.* 1993).

1.4.5.2. Grafted embryonic CNS tissue.

Experiments utilizing grafts of embryonic CNS tissue in lesioned animals have demonstrated that the grafts are innervated by CNS axons (see section 1.3.2.1 and 1.3.2.4). CNS axonal regeneration has been observed in a few areas of the CNS where it is been possible to axotomize all the neurons. For example, retinal ganglion cells have been demonstrated to regenerate axons into grafts of embryonic neural tissue (eg: tectum, thalamus, cerebral cortex and spinal cord) after optic nerve transection (Hausmann *et al.* 1988); axons within the spinal cord have been shown to regenerate into grafts of embryonic spinal cord (eg: Reier *et al.* 1986), and regenerating CNS axons in neonatal animals are able to grow through grafts of embryonic spinal cord and restore function (Iwashita *et al.* 1994). There are many other studies where CNS axons have been demonstrated to innervate grafts of embryonic neural tissue. For example embryonic striatal tissue is innervated by cortical and nigrostriatal afferents (eg: Wictorin and Björklund 1989 and see sections 1.3.2.4.3.1. and 1.3.2.4.3.2.), and grafts of embryonic nigral tissue is innervated by cortical afferents (Doucet *et al.* 1989 and see section 1.3.2.1.3.). However, in these experimental paradigms it is not possible to determine whether the CNS axons that invade the graft derive from lesioned CNS neurons (regenerative sprouting) or from unlesioned CNS neurons (collateral sprouting), since some of the afferents to the graft may not have been lesioned prior to growth.

Having said that, it is still evident that embryonic tissue constitutes a permissive environment for the growth of adult CNS axons. However, the environment within a particular area of embryonic

CNS tissue is not suitable for the growth of all CNS neurons, since studies comparing two types of grafted embryonic tissue have demonstrated that embryonic CNS tissue grafted into ectopic, non-target sites are not innervated as densely or in the same pattern as is observed in embryonic tissue implanted into its normal target site (Labandeira-Garcia *et al.* 1991).

1.4.5.3. Non-neural tissue grafts

Some of the first experiments conducted into the occurrence of CNS regenerative sprouting were performed using grafts of non-CNS tissue. Intracerebral implants of smooth muscle have been shown to be innervated by adrenergic and cholinergic CNS axons, the extent of innervation being in proportion to the normal autonomic innervation of the tissue (Björklund and Stenevi 1971, Björklund *et al.* 1975, Emson *et al.* 1977). This proportional response could be related to the number of Schwann cells in the grafted tissue, since Schwann cells will be present at the site of innervation and will be more numerous in densely innervated tissue. Similarly, grafts of skin, muscle and other peripheral tissues have been shown to become innervated after intracerebral implantation, the extent of innervation being apparently related to the presence of extravascular protein, and thus to the degree of BBB breakdown (Heinicke and Kiernan 1978, Heinicke 1980). Once again, it is not possible to determine whether the CNS axons that innervated these grafts of non-neural tissue were derived from axotomized neurons (regenerative sprouting) or from uninjured neurons (collateral sprouting).

1.4.6.**Factors that may play a part in graft-induced regeneration.**

1.4.6.1. Trophic factors

There is evidence that the distal stump of peripheral nerve contains high levels of trophic factors, produced by Schwann cells and potentially capable of promoting axonal regeneration. NGF is produced biphasically, the second peak being dependent on interleukin-1 (IL-1), produced by macrophages (Brown *et al.* 1991). *In situ* hybridization studies have shown a transient reduction in neurotrophin-3 (NT-3) mRNA in the distal stump following transection, while brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 mRNA's were increased (Funakoshi *et al.* 1993).

As detailed above (section 1.3.2.2.) grafts of adrenal medullary cells produce an ameliorating effect on the DA-denervated striatum, both in preclinical and clinical trials, although morphological examination has shown few surviving cells (for review see W.Freed *et al.* 1990 and above) and the beneficial action of adrenal medulla grafts has been ascribed to the variety of trophic factors produced by medullary cells (Unsicker 1993). Although a number of molecules have been suggested to be responsible for the ameliorating effect of adrenal medullary chromaffin cells in the DA-denervated striatum (including bFGF, transforming growth factor β, interleukins, neurotrophins and CNTF; see Unsicker 1993 for details), it is not yet clear which is responsible for the amelioration of symptoms in patients, and the sprouting of TH-immunoreactive axons in animal studies.

There is now a mass of data to indicate that certain types of CNS neuron are capable of regenerating their axons or of altering their response to injury following axotomy when given additional trophic support. For example, NGF released by polymer-encapsulated genetically engineered cells prevents the lesion-induced reduction in ChAT expression in axotomized septal cholinergic neurons (Hoffman *et*

al. 1993), and axotomized septal cholinergic neurons have been demonstrated to form regenerative axonal sprouts following the infusion of NGF or bFGF (Miyamoto *et al.* 1993). CNTF infusion has also been demonstrated to prevent the lesion-induced degeneration of dopaminergic nigral neurons (Hagg and Varon 1993).

There are many other examples where trophic factors released by implanted cells have been demonstrated to have a positive effect on axotomized CNS neurons, leading to the axotomized CNS neurons regenerating their axons or altering their response to injury. Although it is likely that the trophic factor is responsible for the response, the possibility of interactions between trophic factors and cell adhesion molecules and extracellular matrix (ECM) matrix molecules can not be ruled out. For example, medial septal neurons will regenerate their axons following fimbria-fornix transection if supplied with a PN graft (Hagg *et al.* 1990), NGF (Hagg *et al.* 1991, Kawaja *et al.* 1992, Gage *et al.* 1993) or BDNF (Morse *et al.* 1993), and the behavioural deficits produced by fimbria-fornix lesions are attenuated after the addition of such grafts (Eagle *et al.* 1993). BDNF has also been demonstrated to reduce the severity of Parkinsonian-symptoms in MPTP-lesioned primates (Tsukahara *et al.* 1993), possibly via the prevention of axotomy-induced degeneration or down-regulation of enzymes. Neonatal rat motor neurons have been shown to be protected from axotomy-induced degeneration by the addition of BDNF (Sendtner *et al.* 1992, Yan *et al.* 1992) and BDNF has been demonstrated to prevent the axotomy-induced down-regulation of ChAT in neonatal and adult rats (Friedman *et al.* 1993, Yan *et al.* 1993). Similarly, NGF and NT-3 increased the extent of regeneration in the rat corticospinal tract following transection (Schwab *et al.*

1992, Tuszynski *et al.* 1993, Schnell *et al.* 1994).

1.4.6.2. Cell adhesion molecules

Schwann cells in the distal stump of the severed PN become dedifferentiated, and myelin-forming Schwann cells re-express the cell adhesion molecules L1 and N-CAM, especially in central parts of the bands of Büngner (Martini and Schachner 1988, Martini *et al.* 1990). *In vitro* studies have demonstrated both molecules stimulate neurite outgrowth (Keilhauer *et al.* 1985, Kleitman *et al.* 1988, Wehrele and Chiquet 1990). Grafts of adrenal medullary chromaffin cells have been shown to express increased levels of L1 (Poltorak and Freed 1990) and preliminary studies suggest the pattern of expression of cell adhesion molecules within PN grafts in the CNS may be similar to those found in the distal stump (Zhang *et al.* 1993).

1.4.6.3. ECM molecules

As well as possible effects of trophic factors and adhesion molecules, studies have indicated that ECM molecules may also play a part in the "stimulatory" effect of PN grafts. Tenascin is only observed at those parts of the Schwann cell close to or abutting the basal lamina (Martini and Schachner 1988, Martini *et al.* 1990), while Schwann cell laminin is restricted to the surface of Schwann cells *in vivo* and *in vitro* (Cornbrooks *et al.* 1983), but can be detected intracellularly in the distal stump of injured PN (Bignami *et al.* 1984). However, Schwann cells have been demonstrated to produce laminin regardless of whether they are in contact with axons (Cornbrooks *et al.* 1983). *In vitro* studies have demonstrated tenascin can stimulate neurite outgrowth (Wehrele and Chiquet 1990), although there is evidence that it can also inhibit neurite outgrowth (Crossin *et al.* 1990). Laminin has a number of actions *in vitro*, including promoting the spread and neurite outgrowth of injured adult rat

retinal neurons (Ford-Holevinski *et al.* 1986), dissociated neurons from embryonic chick spinal cord, sympathetic ganglia and retina (Rogers *et al.* 1983) and dissociated cells from newborn rat brain (Liesi *et al.* 1984). *In vivo*, laminin may act to enhance axonal regeneration in crushed rat optic nerve and transected rat sciatic nerve (Poltis 1989), although the basal laminae of frozen grafts is incapable of supporting regeneration over long distances (Nadim *et al.* 1990) or over short distances in the absence of Schwann cells (Hall 1986b).

1.4.6.4. Lack of neurite-inhibitors.

There is now a wealth of evidence to suggest that area of the CNS contains neurite-inhibitory factors including myelin or myelin-breakdown products, which prevent the growth of CNS regenerative axonal sprouts. Some CNS neurons are capable of regenerating their axons following axotomy if these neurite-inhibitors are neutralized experimentally, or the axons are permitted to grow in a more permissive environment, such as a PN graft.

The first suggestion that axonal growth inhibitory factors were released by CNS myelin or myelin breakdown products was made by Berry in 1982. This suggestion was supported by the fact that non-myelinated or thinly-myelinated CNS axons (eg: noradrenergic fibres in the locus coeruleus, cholinergic fibres in the septohippocampal pathway, non-myelinated hypothalamohypophyseal tract axons) display a distinct capacity for regeneration after injury (Björklund and Stenevi 1979, Barron 1983a, Berry 1982). Oligodendrocytes and CNS myelin exert an inhibitory effect on regenerating neurites in culture (Schwab and Caroni 1988, Caroni and Schwab 1988a, 1988b). This non-permissive substratum/substance has been extracted and is associated

with two CNS myelin membrane protein fractions (NI-35, NI-250), which can not be found in PNS myelin or in a liver-derived membrane fraction (Caroni and Schwab 1988a). An antibody (IN-1) has been raised against the myelin inhibitory proteins, which is capable of neutralizing or reducing the inhibitory effects of myelin proteins (Caroni and Schwab 1988b). *In vivo*, CNS axonal sprouts extending for several mm are observed within the rat spinal cord following transection of the corticospinal tract and addition of IN-1 secreting cells (Schnell and Schwab 1990). Similarly, regenerating cholinergic septohippocampal fibres grew into the hippocampus 3-5 weeks after aspiration lesions of the rat fimbria-fornix, when the IN-1 antibody was present (Cadelli and Schwab 1991). The exact mechanism of myelin-inhibition is unknown, although it has been suggested that myelin-inhibitors may affect the function of N-CAM on the neurite surface (Chiquet 1989), or cause collapse of growth cones via changes in intracellular calcium (Bandtlow *et al.* 1993).

Astrocytes have also been put forward as potential inhibitors of regenerative neurite outgrowth, since there is evidence that the glial scar, observed following CNS damage, acts as a physical barrier to neurite growth (Ramón Y Cajal 1928).

The glial scar hypothesis is widely favoured. For example after crushing the dorsal root, afferents from the dorsal root ganglion exhibit a robust outgrowth in the peripheral part of the dorsal root, but do not extend any further than the dorsal root entry zone, (which is dominated by astrocytes) and so do not re-enter the spinal cord (Reier *et al.* 1983, Stensaas *et al.* 1987). This can be more obviously demonstrated when a piece of living optic nerve is sutured to the proximal and distal end of a transected PN (Reier *et al.* 1983, Anderson and Turmaine 1986, Hall and Kent 1987, Anderson *et al.*

1989). EM and immunohistochemical studies show that few fibres extend from the proximal stump into the optic nerve graft (Anderson *et al.* 1989). However, although many regenerating PNS axons cease growth when they are in contact with astrocytes it is difficult to exclude the possibility of inhibitory influences emanating from other cell types.

There has been some suggestion that astrocytes may block axonal regeneration by inducing the regrowing axon to form a presynaptic terminal (Richardson *et al.* 1982, Carlstedt 1985) or by the activity of proteolytic enzymes and the subsequent degradation of cytoskeletal proteins (Liuzzi and Lasek 1987). This suggests that astrocytes, like normal targets, are able to activate "physiological stop pathways" (Rees *et al.* 1976, Liuzzi and Lasek 1987, Carlstedt 1985).

It has also been suggested that the surface of mature astrocytes is inhospitable for neurite growth, since cortical lesions have been demonstrated to contain increased expression of both tenascin and chrondroitin sulphate proteoglycan on GFAP-positive astrocytes and factors released around such lesions inhibit neurite outgrowth *in vitro* (McKeon *et al.* 1991). A number of other studies, however, have demonstrated that astrocytes are capable of supporting the outgrowth of CNS neurons after injury. For example, following fimbria-fornix lesions, increased GFAP expression is observed in the areas along which regenerating septal fibres will grow, suggesting the GFAP-containing astrocytes act as a permissive substrate for regenerative growth (Gage *et al.* 1988). Similarly, axons emanating from the nucleus basalis extending into grafts of genetically modified fibroblasts in the striatum are accompanied by, but not necessarily led by, astrocytes (Kawaja and Gage 1991). Thus it can be

seen that there is still controversy regarding the role of astrocytes in CNS neuronal regeneration.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. General.

All experiments described in this thesis utilized Fischer rats, an inbred strain of albino rats supplied by Harlan-Olac (UK). Adult rats (250-400g) acted as recipients, while material for grafting was obtained from adult (chapter 3 and 4) and young (16-20 day old) rats (chapter 5, 6 and 7). All animals were housed in a 14 hour day, 10 hour night cycle and received food and water *ad libitum*.

2.2. Operative procedures.

Recipient rats were deeply anaesthetised with pentabarbitone (40mg/kg i.p., Rhône Merieux, UK; chapter 3) or an inhaled mixture of (1.5%) halothane (ICI, UK), (3%) nitrous oxide and (1.5%) oxygen (chapter 4-7). For lesioning and implantations, rats were placed in a modified Kopf stereotaxic small animal frame and the scalp opened. A 1.5mm hole was drilled in the right parietal bone 0.03cm anterior to bregma and 0.3cm lateral to the midline, and the underlying dura was opened. Grafts of muscularis externa (chapter 3 and 4) were then implanted at a depth of 0.4cm using a pulled pipette, while grafts of myenteric plexus (chapter 5 and 6) and smooth muscle (chapter 7) were implanted via a glass cannula placed on the end of a Hamilton syringe, at a depth of 0.49cm, corresponding to the centre of the striatum (see Paxinos and Watson 1982). The scalp was then sutured closed using mersilk 3/0 sutures (Ethicon, UK) and all recipient animals received buprenorphine (0.2mg/kg/i.m., Reckitt and Colman, UK) as a post-operative anaesthetic.

2.3. Surgical removal of the superior cervical ganglia.

Approximately a third of the recipient animals in chapter 4 received bilateral superior cervical ganglionectomies 3 days prior to

perfusion, to discount the possible ingrowth of peripheral sympathetic fibres. These animals were deeply anaesthetised with an inhaled mixture of halothane (1.5%, ICI, UK), nitrous oxide (3%) and oxygen (1.5%) and placed on their back with the neck area visible. An incision was made in the skin around the "throat", and the paratoid glands were dissected away and clamped. On either side of the trachea, the sympathetic chain was found and followed up to the superior cervical ganglion, which was excised. After removal of both superior cervical ganglia, the skin around the throat was sutured. Buprenorphine (0.2mg/kg/i.m., Reckitt and Colman, UK) was administered as a post-operative analgesic and the animal allowed to recover.

2.4. **Quinolinic acid lesions.**

In chapters 5 and 6, grafts were implanted in the striata of animals that had been lesioned with the excitotoxin quinolinic acid (QA: Sigma, UK), 7-14 days prior to implantation. Under anaesthesia, rats were placed in a modified Kopf small animal stereotaxic frame. A 1.5 mm hole was drilled in the right parietal bone 0.03 cm anterior to bregma and 0.3 cm lateral to the midline, and the underlying dura was opened. A 10 µl Hamilton syringe containing 60 nanomolar QA (Sigma, UK) dissolved in sterile water (pH 7.4) was lowered 0.49 cm below the pial surface such that the point of the needle was in the centre of the right striatum. One microlitre of QA was injected over 5 minutes and the syringe was left in place for a further 5 minutes to allow the excitotoxin to diffuse into the surrounding striatal tissue. The syringe was removed and the scalp sutured. Buprenorphine (0.2mg/kg/i.m., Reckitt and Colman, UK) was administered as a post-

operative analgesic, and animals were allowed to recover.

2.5. Preparation and implantation of muscularis externa grafts.

Donor rats were killed by an overdose of ether, and small segments of distal ileum were excised. Intestinal contents were removed and the muscularis externa was peeled away from the underlying layers. This was then cut into 0.5 mm^2 pieces which were washed four times in Hanks Balanced Salt Solution (Gibco, UK) containing antibiotic and antimycotic agents. Freeze-killed grafts were prepared by placing the tissue on aluminium foil which was then plunged into liquid nitrogen. Freezing and thawing were repeated four times. Recipient rats were anaesthetised using Sagatal (40 mg/kg/i.p., Rhône Merieux, UK) and a hole was drilled in the skull 3 mm lateral to bregma. Two pieces of live or freeze-killed muscularis externa were then inserted at a depth of 4 mm into the brain.

2.6. Preparation and implantation of myenteric plexus grafts.

Segments of the myenteric plexus from donor Fischer rats (15-19 days old) were obtained as previously described (Jessen *et al.* 1983a). Animals were killed by dislocation of the neck. The proximal colon was then removed aseptically, washed in culture medium 199 (Life Sciences, UK) supplemented with 5 mg/ml glucose, 1000 units/ml penicillin, 50 µg/ml metronidazole and 200 µg/ml gentamycin. Segments of the colon were placed in collagenase (1 mg/ml Boehringer-Mannheim, Germany) for 2 hours at 4°C and then incubated in the same solution at 37°C for 30 minutes. Pieces of the myenteric plexus (consisting of 10-30 connected ganglia) were then freed from the surrounding smooth muscle by microdissection and placed in cold medium 199 supplemented with 5 mg/ml glucose and 1000 units/ml penicillin. The colonic

myenteric plexus dissected from two donor animals was pooled and approximately one half of the pool was implanted into each host. Thus 15-18 pieces were drawn up into a glass cannula on the end of a 10 µl Hamilton syringe such that the final volume was 2 µl. This was then implanted at a depth of 0.49cm, the tissue being slowly forced from the cannula over 3 minutes. The cannula was left in place for a further 5 minutes before being slowly removed from the brain.

2.7. **Preparation and implantation of smooth muscle grafts.**

Donor Fischer rats (15-19 days old) were killed by dislocation of the neck. The proximal colon was then removed aseptically, washed in culture medium 199 (Life Sciences, UK) supplemented with 5 mg/ml glucose, 1000 units/ml penicillin, 50 µg/ml metronidazole and 200 µg/ml gentamycin. Segments of the colon were placed in collagenase (1 mg/ml Boehringer-Mannheim, Germany) for 2 hours at 4°C and then incubated in the same solution at 37°C for 30 minutes. Pieces of myenteric plexus were excised from the surrounding longitudinal and circular smooth muscle by microdissection and the pieces of smooth muscle were placed in cold medium 199 supplemented with 5 mg/ml glucose and 1000 units/ml penicillin. The smooth muscle pieces were further checked to ascertain that all ganglia had been removed and then transferred to fresh cold medium. Grafts of freeze-killed smooth muscle were obtained by freezing and thawing the freshly dissected smooth muscle tissue four times in liquid nitrogen, to cause cellular lysis and produce tissue devoid of living cells. Pieces of longitudinal and circular muscle dissected from two donor animals were pooled and approximately one third of the pool was implanted into each host. Thus approximately 10-12 pieces were drawn up into a

glass cannula on the end of a 10 µl Hamilton syringe such that the final volume was 2 µl. Grafts of smooth muscle were implanted in the right striatum of 10 hosts, using the co-ordinates AP = 0.3mm, ML = 3mm, DV = 4.9mm with respect to bregma (Paxinos and Watson 1982) as has been previously described for the implantation of myenteric ganglia (see section 2.6). Briefly, the tissue was slowly forced from the cannula over 3 minutes, then the cannula was left in place for a further 5 minutes before being slowly removed from the brain.

2.8. Tissue preparation for electron microscopy.

At the end of the experimental period, 10 days to 6 weeks after implantation, animals were given a lethal dose of pentabarbitone and then perfused transcardially with fixative (4% paraformaldehyde (Taab, UK), 0.5% glutaraldehyde (BDH, UK) and 0.5% D-glucose (BDH, UK) in 0.1M Millonig's buffered phosphate- see below). The brains were removed and kept overnight in fixative, at 4°C. Vibrotome sections of 175µm were taken through the striatum, osmicated then block stained in 2% uranyl acetate (Agar Scientific, UK), before being processed into Araldite resin (see below). The desired piece of brain was cut from the vibrotome section using razor blades and glued on to an araldite stub. Semi-thin (0.5µm) and ultrathin (70-100nm) sections were cut on an Ultracut E microtome. Semithin sections were stained with Toluidine blue for observation at the light level, ultrathin sections were collected on copper coated grids (Agar Scientific, UK) or carbon coated slot grids (Agar Scientific, UK) and stained with 2% lead citrate (in distilled water), then examined in a Jeol 1010 electron microscope.

2.8.1. EM processing procedure

Take vibrotome sections and place in the following solutions

1x5 min 0.1M Millonig's buffer at room temperature (RT).

1x60 min 1% osmium tetraoxide (Johnson Matthey Chemicals, UK) made up in 0.1M Millonig's buffer at 4°C.

1x5 min 0.1M Millonig's buffer at RT

1x5 " 0.1M sodium acetate buffer at RT

1x45 min 2% uranyl acetate (Agar scientific, UK)- made up in 0.1M sodium acetate buffer at 4°C.

1x5 min 0.1M sodium acetate buffer at RT

1x5 min distilled water at RT

1x5 min 25% ethanol at RT

1x5 min 50% " "

1x5 min 70% " "

1x5 min 80% " "

1x5 min 90% " "

5x10 min Absolute ethanol (BDH, UK) at RT

4x10 min Propylene oxide (BDH, UK) "

1x45 min 1:1 Araldite resin: propylene oxide at RT

2x24 hrs Araldite resin at 4°C.

Place processed sections on acetate sheets, between glass slides, weighed down to produce flat sections and remove excess resin, in an oven at 60°C for 16hrs.

2.8.2. **EM solutions**

a) 0.1M Millonig's buffer

0.1M $NaH_2PO_4.H_2O$ (BDH, UK)

titrate with 1M NaOH to pH 7.4.

eg: for 1 litre, 13.7 g $NaH_2PO_4.H_2O$

approx. 160ml 1M NaOH

approx. 840ml distilled water.

b) Araldite resin

For 20 ml resin

10 ml Dodecenyl succinic anhydride (Agar Scientific, UK)

10 ml Araldyte CY212 (Taab, UK)

0.8 ml dibutyrl phthalate (Agar Scientific, UK)

Heat and stir at 60°C for 5 min then add 0.4 ml benzyldimethylamine (Agar Scientific, UK) and continue to heat and stir for 2 mins.

2.9. Tissue preparation for NADPH-diaphorase histochemistry.

Animals were given a lethal dose of pentabarbitone and perfused transcardially with 100 ml 0.1M phosphate buffer (pH 7.4-see below) followed by 400 ml 4% paraformaldehyde (BDH, UK) in 0.1M phosphate buffer (adjusted to pH 7.4). Brains were removed and placed in 30% phosphate buffered sucrose (30% sucrose in 0.1M phosphate buffer) overnight at 4°C. Sections of striatum were cut at 50 µm on a freezing microtome and stained using a modified NADPH-diaphorase method (Thomas and Pearse 1964). Briefly, sections were placed in a solution containing 0.01% NADPH (Sigma, UK), 0.02% nitroblue tetrazoleum (Sigma, UK), 0.27% L-malic acid (Sigma, UK) in a 0.1M Tris buffer (pH 7.6; with 0.1% Triton-X 100 added to aid penetration) for 90-100 minutes at room temperature. At the end of that time, sections were rinsed in 0.1M Tris buffer and mounted on gelatin subbed slides. After air drying the sections were dehydrated, cleared (see below) and coverslipped with DPX mountant (BDH, UK). Sections were examined using a Zeiss Axioplan microscope.

2.9.1. Procedure for dehydrating and clearing

few sec.	70% ethanol (in distilled water)
" "	95% " "
" "	absolute alcohol (BDH, UK)

1x15 min.	Histoclear (National Diagnostics, USA)
1x5 min.	95% alcohol in distilled water
" min.	absolute alcohol
1x10 min	" "
" "	Histoclear

2.9.2. **NADPH-diaphorase-solutions**

0.1M phosphate buffer

For 1 litre 2.62g $NaH_2PO_4.H_2O$ (BDH, UK)

11.5g Na_2HPO_4 (BDH, UK)

Add @700ml distilled water, and alter pH to 7.4 with HCL/NaOH, then make up to 1 litre.

0.1M Tris Buffer

For 1 litre 12.1g Tris(hydroxymethyl)methylamine

Add @700ml distilled water, and alter pH to 7.4 with HCL/NaOH, then make up to 1 litre.

2.10. <u>Tissue preparation for immunohistochemistry.</u>

Three and six weeks after implantation, rats were deeply anaesthetised with pentobarbitone and perfused transcardially with 0.1 M phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed and placed in 30% sucrose in 0.1M phosphate buffer (pH 7.4) at 4°C overnight. The middle cerebral and basilar arteries of both ganglionectomized animals and those with intact superior cervical ganglia, were dissected away from the base of the brain, and kept in 0.1M phosphate buffer (pH 7.4) at 4°C overnight.

50µm sections through striatum were cut on a freezing microtome

and placed in blocking serum (2% goat serum (Seralab, UK), 0.5% bovine serum albumin (Sigma, UK), 0.3% Triton-X-100 in 0.1M phosphate buffer, pH 7.4) for 1 hour at RT. Cerebral vessels were permeabilized by dehydrating then rehydrating in a series of alcohols (see below). Vessels were then washed in 0.1M phosphate buffer and incubated in blocking serum (1hr at RT). All vessels and most of the sections were then incubated with a polyclonal antibody against TH (1:800, Affiniti, UK, made up in blocking serum; 36 hrs at 4°C). Sections to act as controls were incubated in normal rabbit serum (1:800, Sigma, UK, made up in blocking serum; 36 hrs at 4°C) instead of the primary antibody. All sections and vessels were then incubated in the biotinylated secondary antibody (biotinylated-goat-anti-rabbit, 1:250, Vector Laboratories, UK, made up in 0.1M phosphate buffer; 2hrs at RT) before being incubated in an avidin-biotin complex in 0.1M phosphate buffer (1:1:100, Elite kit, Vector Laboratories, UK; 2hrs at RT). The reaction product was then visualised using 0.05% diaminobenzidine tetrahydrochloride (Sigma, UK) and 0.03% hydrogen peroxide (Sigma, UK) in 0.1M phosphate buffer. Sections and vessels were mounted on slides, airdried and osmium intensified before being dehydrated, cleared and coverslipped. Sections were then examined under bright field illumination using a Zeiss Axioplan microscope.

2.10.1. **Procedure for dehydrating and rehydrating vessels**

3x10 min. distilled water

" 25% ethanol (made up in distilled water)

" 40% " "

" 50% " "

" 60% " "

" 50% " "

" 40% " "

" 25% " "

" distilled water

2.10.2. **Procedure for osmium intensification of ABC/DAB reaction product**

1x2 min. 70% ethanol (made up in distilled water)

" 50% "

" distilled water

" 0.1% osmium tetraoxide (Johnson Matthey chemicals, UK, in distilled water)

" distilled water

" 50% ethanol (made up in distilled water)

Dehydrate and clear as above (2.9.1)

2.11. Data analysis

The majority of the work covered in this thesis is qualitative, however it has been possible to examine certain aspects of the information more quantitatively. The size of NADPH-diaphorase-containing neuronal grafts (chapter 5) was estimated using a Seescan image analysis system, where the cross-sectional area of grafts was determined in order that the total graft volume could be estimated. The extent to which grafted enteric ganglia were in direct connection with the surrounding striatum (chapter 6) was also estimated using a Seescan image analysis system.

CHAPTER 3.

IMPLANTATION OF THE MYENTERIC PLEXUS INTO THE CORPUS STRIATUM OF ADULT RATS: SURVIVAL OF THE NEURONS AND GLIA AND INTERACTIONS WITH HOST BRAIN.

3.1. Summary.

Live or freeze-killed syngeneic adult muscularis externa, comprising myenteric plexus sandwiched between two layers of smooth muscle, was implanted into the corpus striatum of adult Fischer rats and examined electron microscopically 10 days to 6 weeks after operation.

Living grafts contained healthy neurons and glial cells at all time periods examined, although some areas of necrosis were observed. After 10 days, the glia limitans around the grafts were poorly developed and the adjacent brain tissue contained only a small number of small non-myelinated axons. After 3 and 6 weeks, the brain surrounding the living grafts contained many clusters of small non-myelinated axons. Bundles of putative central nervous system (CNS) axonal sprouts had invaded the grafts, making contact with enteric glia, despite the presence of a well-developed glia limitans at the interface with the brain. In the longer term grafts, some CNS axonal sprouts in the myenteric plexus enlarged and became myelinated. A few astrocyte processes but no axons were found in the freeze-killed grafts. The brain surrounding the freeze-killed grafts appeared to contain fewer axonal sprouts than were present around the living grafts.

The possibility that the living grafts may promote both the sprouting and the elongation of CNS axons is discussed.

3.2. Introduction.

The transplantation of neural tissue into the adult brain has become an experimental technique used in the treatment of neurodegenerative diseases (see Introduction 1.3.2). However, there are several problems inherent to the use of the different types of graft. For example, although alterations in cell isolation procedures for adrenal medullary chromaffin cells may produce increased cell survival and facilitate the production of tyrosine-hydroxylase (TH: Schueler *et al.* 1993), the restricted range of transmitters these cells synthesize limits the clinical use of these grafts to Parkinson's disease. Clinical trials in Parkinsonian patients have demonstrated a moderate improvement in motor function, but not as startling as was first reported (see Introduction 1.3.2.1.7). Foetal CNS tissue has great potential as a source of material for implantation into areas of neurodegeneration (see Introduction 1.3.2) but there are also disadvantages associated with its use, since the tissue may be subject to ethical restrictions (Hoffer and Olson 1991), or problems of immune rejection (Sloan *et al.* 1991).

Theoretically the enteric nervous system has many qualities making it worth investigation as a source of neurons for grafting into the CNS. In contrast to sympathetic and parasympathetic ganglia, enteric ganglia show both a morphological and a histochemical similarity to the CNS (Jessen and Burnstock 1982, and see Introduction 1.1). They contain neurons which utilise a wide variety of neurotransmitters, and enteric glial cells that have a number of properties in common with astrocytes but can be distinguished from Schwann cells by morphological and immunohistochemical criteria (Gabella 1981a, Gershon and Rothman 1991). It may even be possible to perform autografts using enteric neurons, thus ensuring no

immunological rejection occurs. Furthermore, adult peripheral ganglia survive transplantation into the CNS better than neonatal ganglia (Stenevi *et al.* 1976, Rosenstein and Brightman 1979), in contrast to CNS neurons which only survive transplantation during their foetal period. Recently, it has been demonstrated that neonatal enteric neurons and glial cells can survive for 1 month after implantation into the cholinergically denervated adult hippocampus (Lawrence *et al.* 1991).

In the present study the primary aim was to determine whether adult enteric neurons and glial cells could survive transplantation into the adult rat brain, and to observe whether any interactions took place between graft and host brain. In addition, freeze-killed grafts were used to investigate whether the effects on the host brain were a function of the living cells in the transplanted tissue.

3.3. Materials and Methods.

Grafts of living or freeze-killed muscularis externa were obtained as described in chapter 2.5, and operative procedures were performed as described in chapter 2.2. After 10 days, 3 or 6 weeks, animals were anaesthetised and transcardially perfused (see section 2.8), and vibrotome sections were processed from electron microscopy (see section 2.8).

3.4. Results.

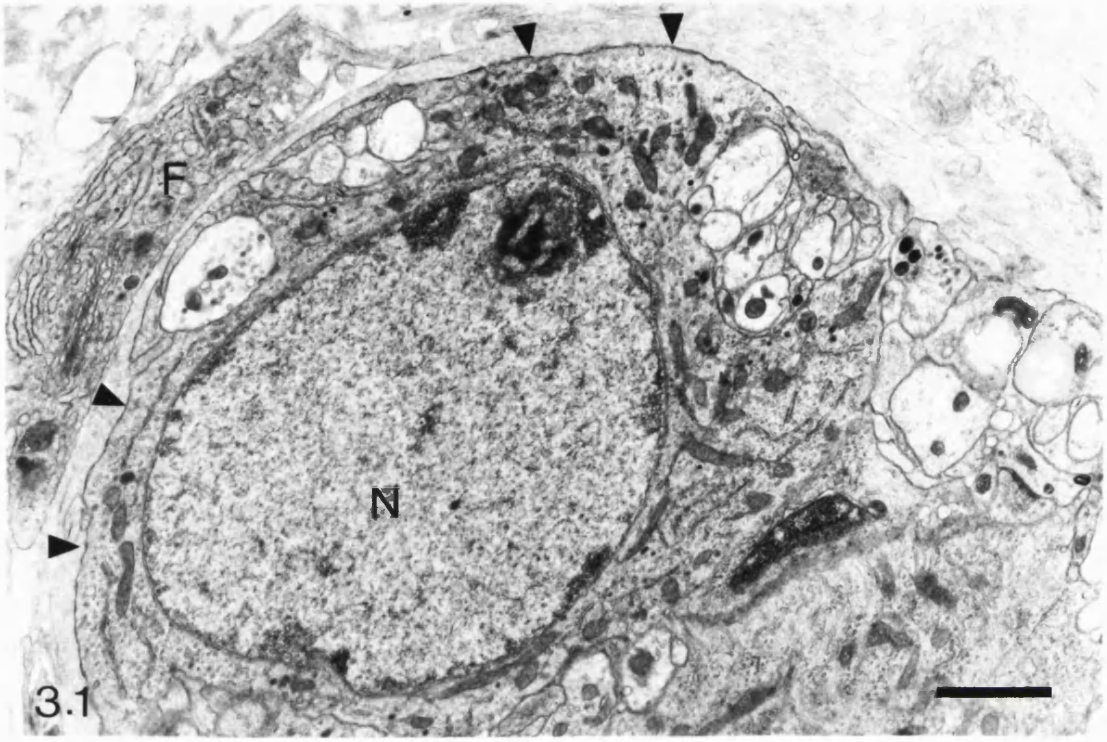
3.4.1. Survival of living grafts.

In all cases the living grafts contained smooth muscle cells, fibroblasts and collagen as well as enteric neurons and glial cells and cellular debris, which enabled the grafts to be clearly differentiated from the surrounding brain. There was little evidence of mitotic activity within cells of the graft. The grafts were always well vascularized. The enteric ganglia contained neurons, glial cells and neuropil in close apposition, surrounded by a basal lamina. As in the gut they were not penetrated by blood vessels. Enteric neurons (**Figs.** 3.1 and 3.2) could be identified by their large centrally located nuclei with little heterochromatin, surrounded by a large area of cytoplasm containing mitochondria, much rough endoplasmic reticulum, a well-developed Golgi apparatus and many free ribosomes. In some ganglia, axosomatic synapses were observed.

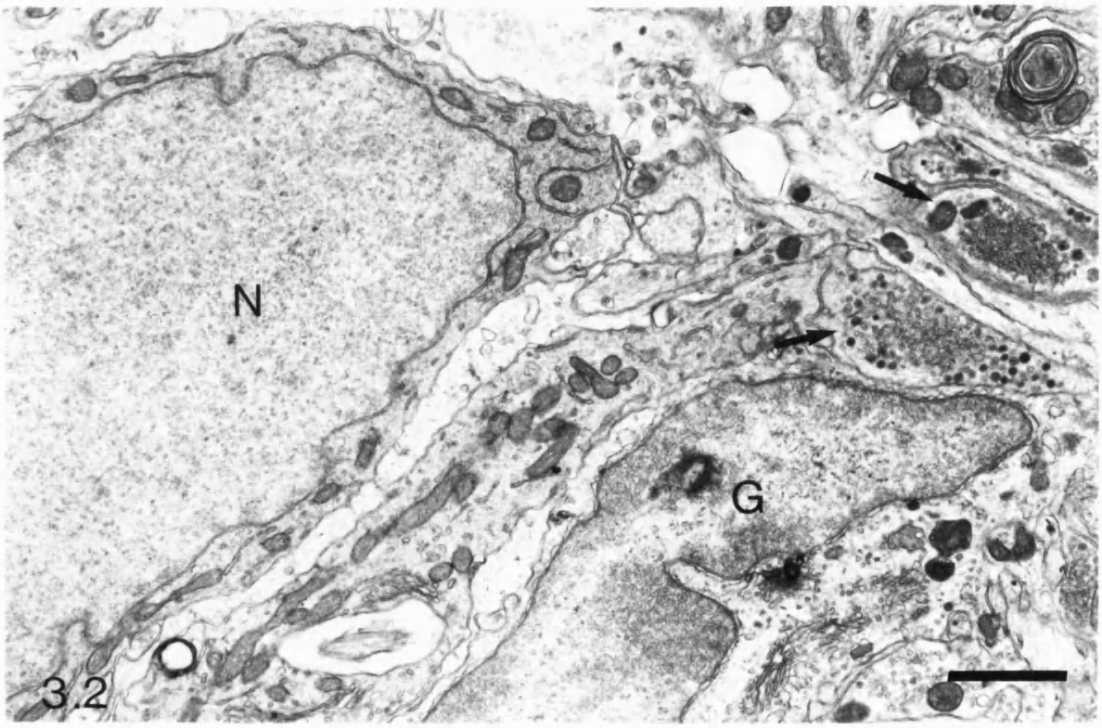
Enteric glia (**Figs.** 3.2 and 3.3) had smaller cell bodies than neurons. Their nuclei were more electron dense, irregularly shaped and often highly indented. A thin palisaded layer of heterochromatin was always present lining the nuclear envelope (**Fig.** 3.3a). This layer was disrupted only at nuclear pores. The cytoplasm was generally less extensive than that of neurons, containing mitochondria, sometimes a Golgi apparatus, and less rough endoplasmic reticulum than neurons. Intermediate filaments were observed both in the cytoplasm around the nucleus, and more prominently in the glial processes between and around the neurites of the neuropil. In the majority of enteric glia, no caveolae were observed at the cell surface. However, a small proportion of enteric glia were observed to contain many caveolae. It was possible to distinguish enteric glia from reactive astrocytes near the graft because of the latter's less

Fig. 3.1. Electron micrograph of a rat ileal enteric ganglion, 10 days after implantation into the striatum. A neuronal cell body (N) and tightly packed ganglionic neuropil surrounded by a basal lamina is shown. Parts of the neuronal cell body are in direct contact with the basal lamina (arrowheads). Collagen, cellular debris and a fibroblast (F) are seen outside the ganglion. Scale bar = 1 μm

Fig. 3.2. Electron micrograph of a rat ileal enteric ganglion 3 weeks after implantation into the corpus striatum. A neuron is depicted which has a large, regularly shaped nucleus (N), while the nucleus of the glial cell (G) is smaller, highly indented and contains more electron-dense heterochromatin. Axonal profiles containing dense-cored and small agranular vesicles can be seen in the neuropil (arrows). Scale bar = 1 μm



3.1



3.2

indented, spherical nuclei (**Fig.** 3.6). Astrocytes deeper into the striatum had ovoid nuclei with heterochromatin clumped at the nuclear envelope.

The ganglia appeared to contain fewer neuronal cell bodies than seen *in situ*. The ganglionic neuropil was heterogeneous, exhibiting many of the vesicle-containing varicosities (**Fig.** 3.2) described by Cook and Burnstock (1976). However, it seemed that there were proportionally fewer of these vesicle-containing varicosities than were present *in situ*. Profiles of degenerating neurites and neurites containing myelin figures were also observed (**Fig.** 3.2). A covering of basal lamina surrounded, but never penetrated, the ganglia. In many instances neuronal cell bodies were observed to have parts of their surface in direct contact with the basal lamina, as they do in the gut (**Fig.** 3.1).

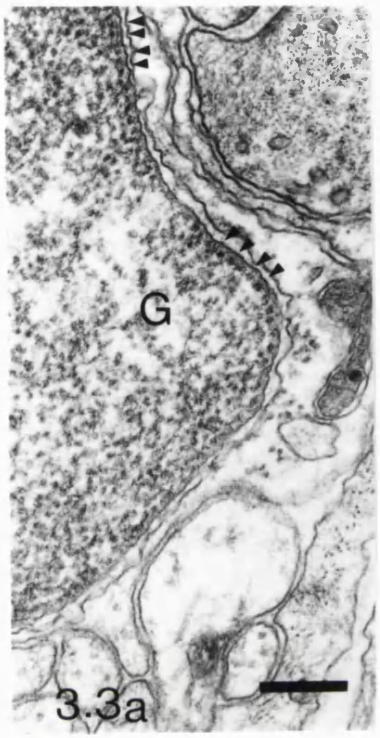
Throughout the graft, interconnecting strands of myenteric plexus comprising discrete bundles of axons and glial cells or their processes could be seen. Profiles of vesicle-containing varicosities, sometimes containing more than one type of vesicle, and narrower diameter intervaricose fibres containing neurofilaments and microtubules were also identifiable in these strands (**Fig.** 3.3b).

The grafts contained many smooth muscle cells corresponding to the longitudinal and circular muscle layers of the muscularis externa. These cells appeared healthy, and some were observed to be connected via gap junctions. Fibroblast-like cells were seen throughout the graft. These cells could have been derived from the pial surface and brought into the brain by the transplantation process, or they may have been interstitial cells of the enteric plexus, which many have described as being morphologically similar to fibroblasts (Rogers and Burnstock 1966, Komoru 1982).

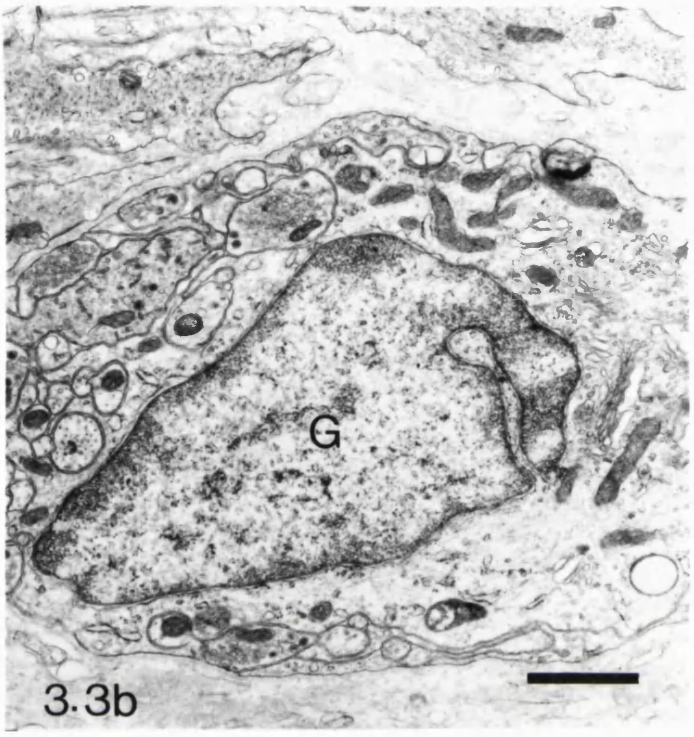
Fig. 3.3a. A high-power electron micrograph of a rat ileal enteric glial cell nucleus (G), 6 weeks after implantation into the striatum. With the fixative used (see chapter 2.8), the nuclei of enteric glia were characterised by the thin very regularly arranged layer of heterochromatin found at the inner border of the nuclear envelope (small arrowheads). Scale bar = 0.1 μm

Fig. 3.3b. Electron micrograph of a transverse section through an interconnecting strand of myenteric plexus, 6 weeks after implantation into the brain. At the periphery of the enteric glial cell (G), axonal profiles can be seen, some of which contain granular and agranular vesicles. Scale bar = 1 μm

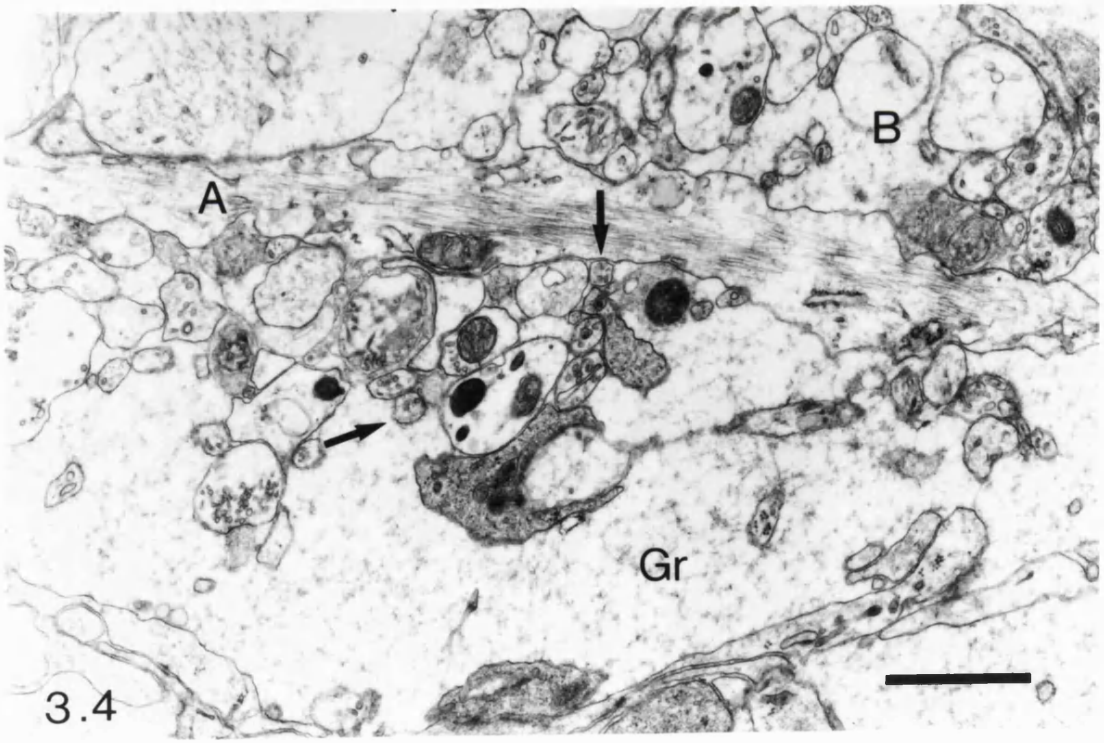
Fig. 3.4. An electron micrograph of the interface between brain (B) and graft (Gr) 10 days after implantation. An astrocyte process (A) is seen near the interface, but is not covered by basal lamina. Small non-myelinated axons (arrows) may be seen extending into the graft intermingled with processes of unidentified non-neuronal cells. Scale bar = 1 μm



3.3a



3.3b



3.4

Not all cells survived equally well. At 10 days after implantation, occasional necrotic regions containing cellular debris and macrophages were seen in the grafts. Necrotic regions were less obvious three and six weeks post-implantation and degenerating neurons and other cells were rarely seen. However, the grafts still contained some cellular debris and macrophages as well as collagen and amorphous EC matrix. Even after 6 weeks, enteric neurons and glia and smooth muscle cells of essentially normal appearance (**Fig. 3.3b.**) were still found in the graft.

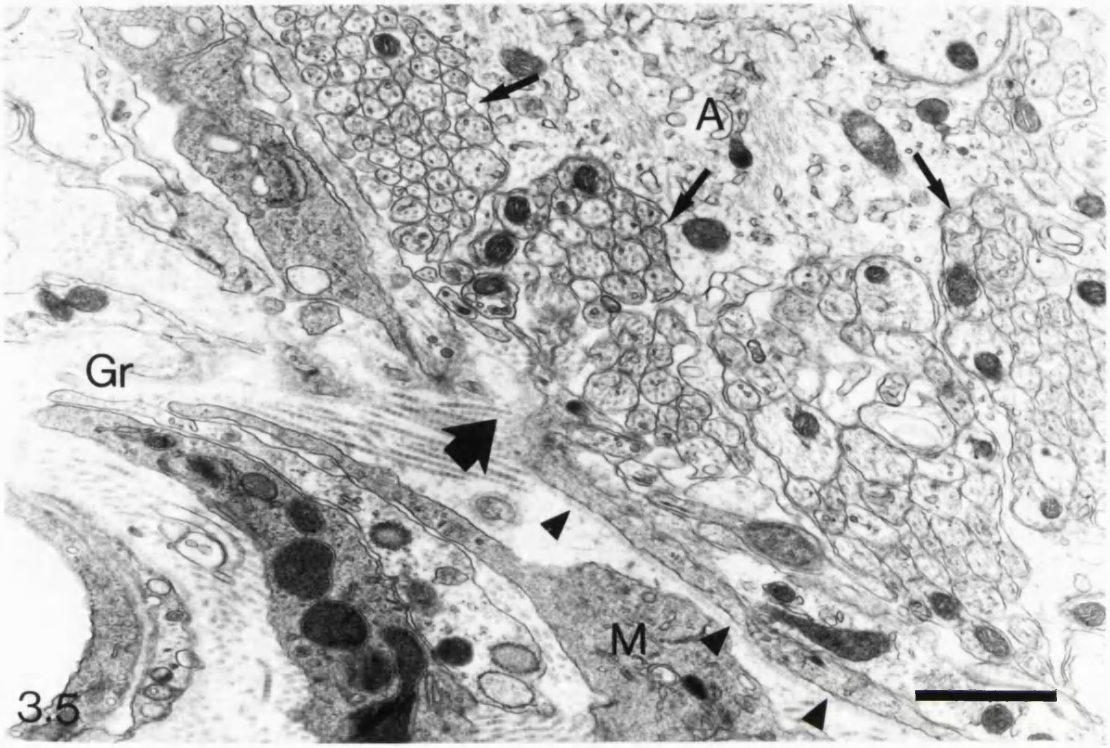
3.4.2. **Interface region.**

Ten days after implantation, the glial limitans was poorly developed. The interface between graft and brain was characterised by astrocyte processes and cell bodies near the surface of the brain bordering the graft. In some places, a single thin layer of astrocyte processes was present. In others, a thick layer of eight to ten closely aligned processes separated the brain from the graft. In some areas the astrocytes were covered by patches of basal lamina, but at most of the interface, no basal lamina could be seen (**Fig. 3.4**). In many areas small finger-like astrocyte processes extended into the grafts. A few small non-myelinated axons were seen scattered along the forming glial limitans (**Fig. 3.4**).

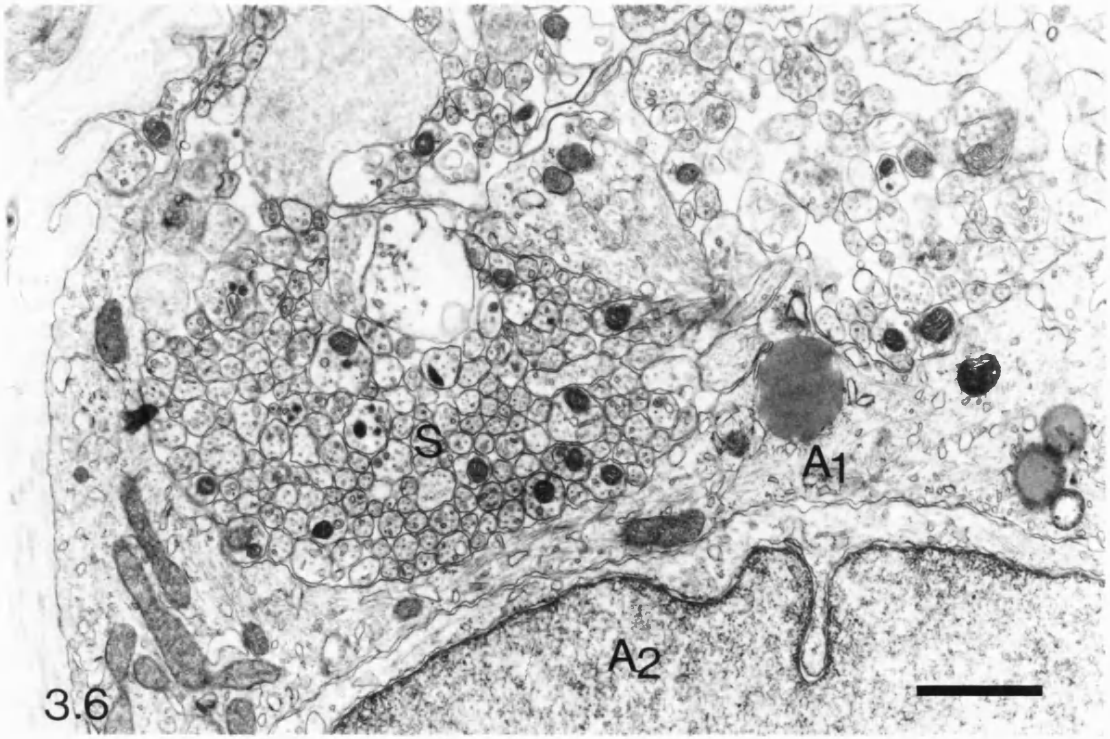
Three and 6 weeks after grafting, most of the interface region exhibited a well-developed glial limitans (**Figs. 3.5** and **3.6**). However, there were still areas where either the layer of astrocyte processes or the basal lamina was incomplete (**Fig. 3.5**). Regions where the interface was extremely complex were observed, with astrocyte processes extending into the graft in contact with fibroblasts, axons or other cells, so that no clear demarcation

Fig. 3.5. An electron micrograph of the interface between graft (Gr) and brain 3 weeks after implantation of myenteric plexus into the striatum. The glia limitans (arrowheads) is incomplete at one point (large arrow) where collagen can be seen in close proximity to small non-myelinated axons (arrows) in the brain. These axons are arranged in clusters, surrounded by astrocyte processes (A). (M =macrophage process within the graft) Scale bar = 1 μm

Fig. 3.6. An electron micrograph of the interface between brain and graft, 3 weeks after implantation of myenteric plexus. The glia limitans is complete in this region. A large cluster of presumptive CNS axonal sprouts (S) can be seen partially surrounded by processes of an astrocyte (A_1). Below is the nucleus of a second astrocyte (A_2) exhibiting different nuclear characteristics to that seen in enteric glia (see **Figs. 3.3** and 3.4) Scale bar = 1 μm



3.5



3.6

between graft and brain could be made.

In the brain surrounding the graft 3 and 6 weeks after implantation, many small non-myelinated axons were seen (**Figs.** 3.5 and 3.6). Near the glial limitans these axons were mostly found in large clusters in astrocyte pockets (**Fig.** 3.6). Further into the brain they were also found associated with astrocyte processes and less frequently with oligodendrocytes. These clusters were of variable size but in all cases the axons were remarkably similar, being of small diameter with a round profile. This often gave the cluster the appearance of a loosely packed geometrical pattern. Although some small-diameter axons were seen in the contralateral striatum, bundles of small non-myelinated axons were not observed.

There was some evidence that these small non-myelinated axons grew into the graft. Three and 6 weeks after implantation, bundles of small non-myelinated axons extended between the brain and the graft (**Figs.** 3.7 and 3.8). Enteric glia were associated with some of these bundles (**Fig.** 3.7). Astrocyte processes were seen in or around many bundles of axons near the brain-graft interface. In larger bundles of ingrowing small non-myelinated axons a few axons of larger diameter were observed, some of which were undergoing myelination (**Fig.** 3.9). The myelin surrounding such axons was morphologically typical of the CNS. Large bundles of putative invading axons were mainly found close to the brain-graft interface but smaller groups of similar axons were identified deeper into the grafts, associated with a variety of types of cell.

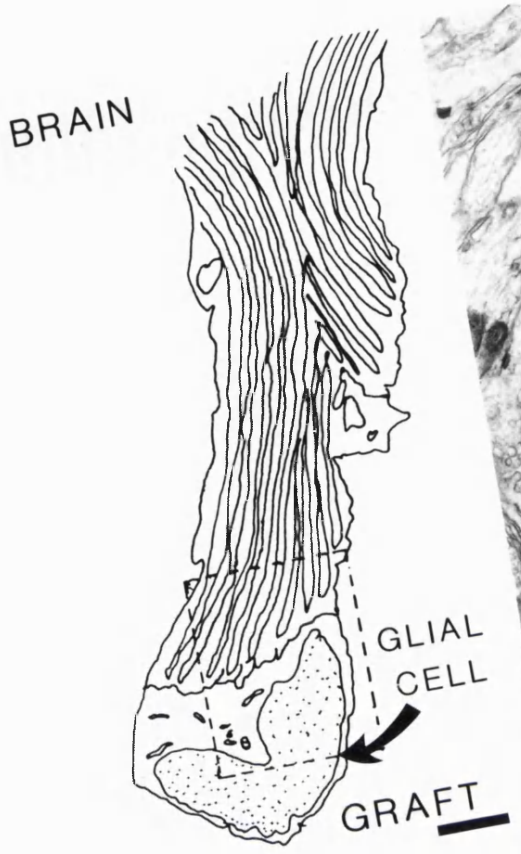
3.4.3. **Freeze-killed grafts.**

Three and 6 weeks after implanting, the freeze-killed grafts comprised large amounts of collagen and cellular debris. A small

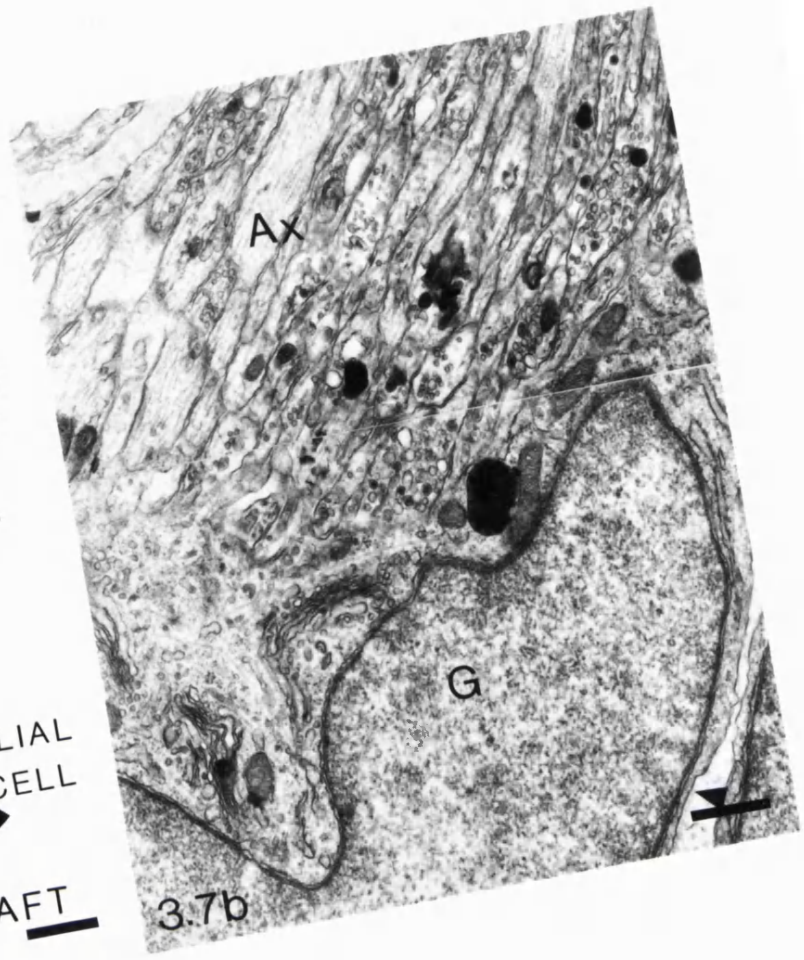
Fig. 3.7a. *Camera lucida* drawing of a bundle of small non-myelinated axons growing into the graft, 3 weeks after implantation of myenteric plexus into striatum. Scale bar = 2 μm.

Fig. 3.7b. An electron micrograph of the indicated area. The ingrowing axons (Ax) are in close apposition to an enteric glial cell (G) within the graft. Note the characteristic indented nucleus and regular layer of heterochromatin at the nuclear envelope. The enteric glial cell is partially surrounded by basal lamina (arrowhead). Scale bar = 1 μm

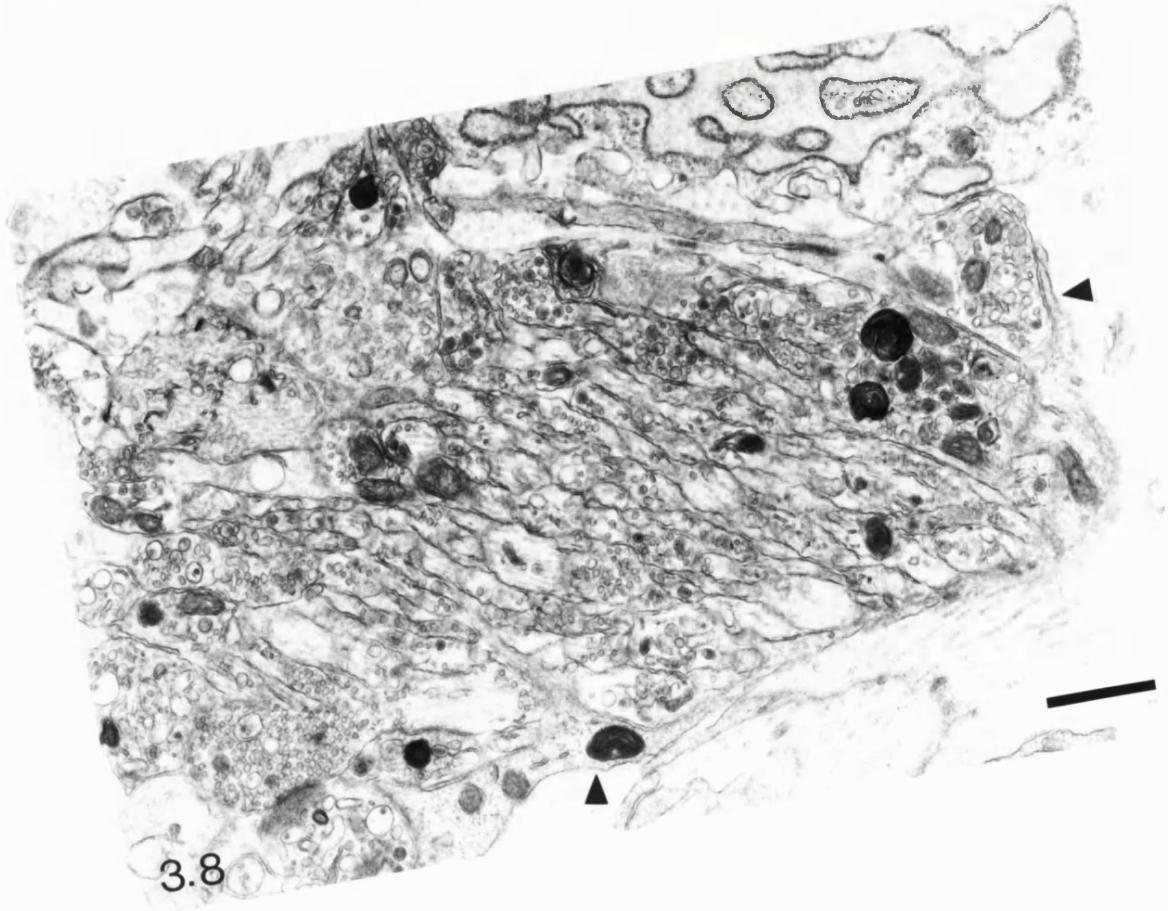
Fig. 3.8. An electron micrograph of an area inside the graft, close to the interface with the brain, 3 weeks after implantation. A bundle of small diameter axons, similar to those in **Fig. 3.7b.** is present. The bundle is partially enclosed by glial cell cytoplasm covered by basal lamina in places (arrowheads). Scale bar = 0.5 μm



3.7a



3.7b

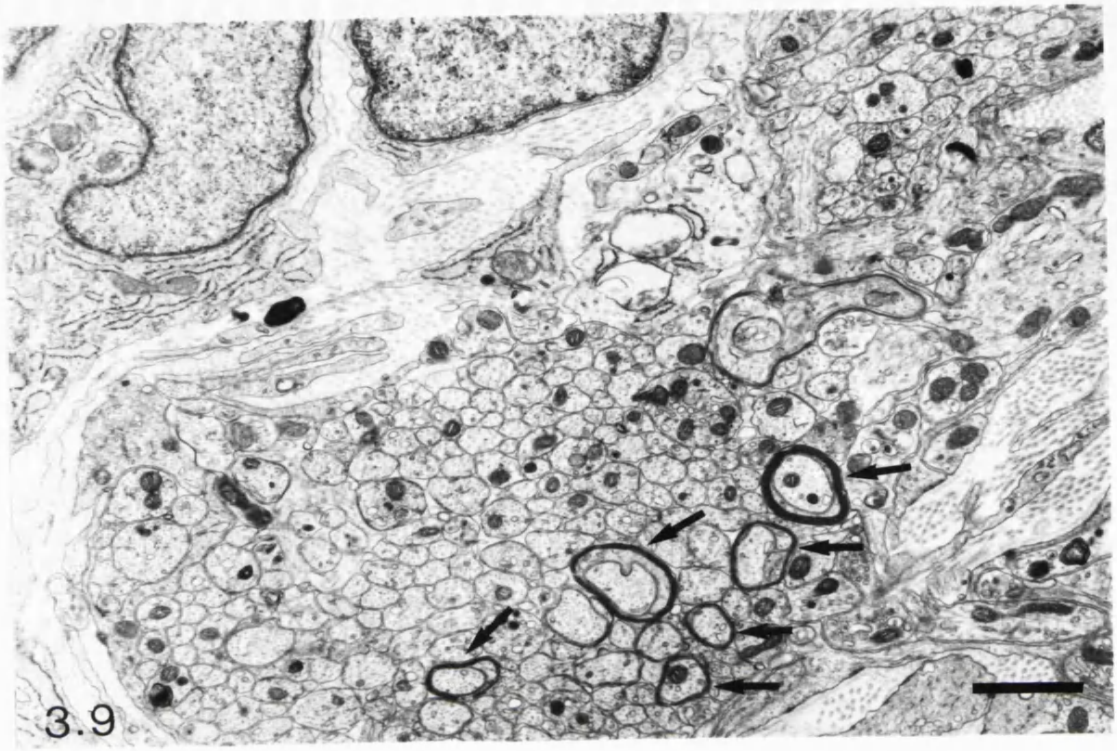


3.8

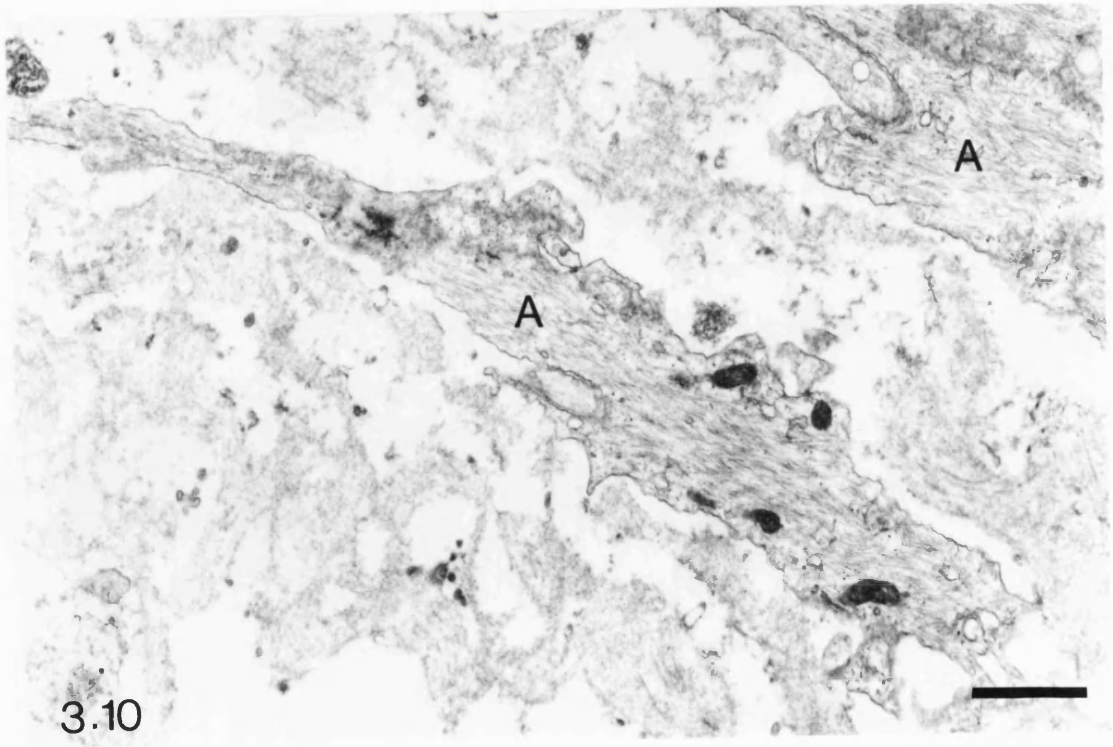
number of living macrophages, fibroblasts and astrocyte processes were found in the grafts but no axons were present (**Fig. 3.10**). The number of axonal sprouts in the brain adjacent to the grafts appeared to be much lower than in similar experiments with living grafts.

Fig. 3.9 An electron micrograph of an area within the graft 3 weeks after implantation. A large bundle of ingrowing CNS axons can be seen. Some are of larger diameter than those in **Figs. 3.7** and **3.8**, while others are surrounded by CNS myelin (arrows). Scale bar = 1 μm

Fig. 3.10 An electron micrograph of a graft of freeze-killed muscularis externa 6 weeks after implantation in the striatum. Astrocyte processes (A) can be seen amongst collagen and cellular debris. Scale bar = 1 μm.



3.9



3.10

3.5. Discussion.

In these experiments we have demonstrated that adult enteric neurons and glia can withstand transplantation into the brain and survive for up to 6 weeks. There was a profound regenerative response by the striatum to living muscularis externa grafts, and many putative CNS axons invaded the transplanted myenteric plexus. Lawrence *et al.* (1991) have shown that some neonatal enteric neurons and glial cells survived in the cholinergically denervated hippocampus for 1 month after transplantation. However, the cholinergic neurons of the graft failed to reinnervate the surrounding brain, and there was no report of any ingrowth of CNS axonal sprouts into the neonatal grafts.

Undoubtedly a number of neurons died during or following the transplantation procedure, as shown by the somewhat reduced size of the ganglia within the graft as compared with those seen in the gut and by the identification of a few necrotic neurons. It is possible that there is some preferential survival of some specific types of neuron in the grafts, although this requires immunohistochemical confirmation. Apart from neuronal cell death, much of the reduction in the neuropil of the ganglia observed 10 days after transplantation may have been due to loss of axons derived from extrinsic sources, including sympathetic, parasympathetic and sensory fibres, or due to loss of afferents from the plexus outside the excised segment. It is also possible that some neurite retraction had occurred, perhaps reflecting a reorganisation of the plexus within the grafts.

Enteric glial cells also survived transplantation. There was little morphological difference between the majority of grafted cells and the enteric glia seen in the gut. Caveolae are not a common feature of enteric glia in the gut (Gabella 1981a), although they

have been reported to be present in grafted neonatal enteric glia (Lawrence *et al.* 1991). Thus the small proportion of enteric glia within the graft exhibiting caveolae may represent enteric glia in a different physiological state induced by transplantation. The association between enteric glia and the bundles of host axons growing into the graft raises the possibility that enteric glia may provide trophic support for such axons. However, astrocyte and presumably oligodendrocyte processes (some axons were surrounded by CNS-type myelin) were also present among the host axons as they crossed the interface into the grafts. While it would be advantageous to identify enteric glial cells with an immunohistochemical marker, this is made difficult by the fact that enteric glia and astrocytes both contain S-100 and glial fibrillary acidic protein (Jessen and Mirsky 1980, Ferri *et al.* 1982).

The presence of many bundles of small non-myelinated axons in the host striatum around the graft was not entirely unexpected. We believe these axons are regenerating axonal sprouts as they resemble developing CNS axons (Henrikson and Vaughn 1974) and regenerating axonal sprouts seen in the optic nerve after injury (Hall and Berry 1989, Zeng *et al.* 1991), and thalamus after implantation of a peripheral nerve (G.Campbell *et al.* 1990, 1992). The putative sprouts in the striatum were morphologically unlike peripheral nervous system fibres regenerating into central nervous tissue (Anderson *et al.* 1989). Furthermore, although the uninjured striatum contains some small-diameter axons, these form a part of the heterogeneous neuropil and are not found in the vast numbers or in the characteristic bundles observed around the muscularis externa grafts. Although the sprouting response has been well documented both in the optic nerve

and the thalamus, it is our clear impression that even more sprouts were seen around the living muscularis externa grafts in the striatum than were seen around grafts of peripheral nerve into thalamus. This may be due to the muscularis externa graft possessing a more potent stimulus for sprouting than the peripheral nerve grafts, or may be due to the striatum being intrinsically capable of a more vigorous regenerative sprouting than the dorsal thalamus. In this regard it may be relevant that GAP-43 has been identified in neuronal processes in the intact striatum (DiFiglia *et al.* 1990).

The presence of bundles of fibres traversing between graft and brain may be due to fibres growing from the brain into the graft or from graft into brain. The glia limitans is incomplete 10 days after implantation and so would not act as a barrier to such growth. While we have no unequivocal evidence that any individual axon originated in the host brain, we believe that most of these fibres were growing into the grafts as there was a comparative paucity of axons deep within the graft and a great abundance of axonal sprouts observed within the host striatum adjacent to the grafts. Living myenteric plexus grafts have previously been reported to support substantial axonal regeneration from mouse sciatic nerves (Anderson and Turmaine 1987). The axons traversing the interface were identical to those in the surrounding brain. It is entirely possible that some axons from enteric neurons grew into the host brain, but as such fibres would be morphologically indistinguishable from the large numbers of axons growing from brain into graft, they could not have been identified in the present study.

The enlargement, maturation and subsequent myelination of regenerating CNS axons inside a peripheral nerve graft has been previously reported (Hall and Berry 1989, G.Campbell *et al.* 1990,

1992). In our case similar events occurred in the muscularis externa. The myelin that formed around axons in the graft was morphologically typical of the CNS. This suggests that myelination had been performed by oligodendrocyte processes. The myenteric plexus of the rat normally contains no myelinated axons, and neurons in the rat myenteric plexus are believed to lack the signals for myelination. Consequently these myelinated axons were probably of CNS origin.

The absence of axons in the freeze-killed grafts suggests that it was the living cells of the muscularis externa, presumably the enteric neurons, glia and smooth muscle cells rather than extracellular matrix components, which were supporting CNS axonal regeneration. Furthermore, it seems unlikely that the invasion of astrocyte processes alone could support the ingrowth of CNS axons since we found no evidence that axons followed astrocyte processes into the freeze-killed grafts. The apparently reduced numbers of axonal sprouts in the brain surrounding killed grafts may also indicate that the living cells of the muscularis externa can stimulate sprout formation. This contrasts with the situation in the injured optic nerve where large numbers of axonal sprouts are formed even in the absence of a graft (Hall and Berry 1989, Zeng *et al.* 1991).

It appears that CNS axons require neurotrophic support for prolonged growth through implanted peripheral nervous tissue. CNS axons do not grow into freeze-killed peripheral nerve grafts (Hall and Berry 1989) unless they are also supplied with neurotrophic factors specific for the regenerating axons (Hagg *et al.* 1991). We cannot yet be sure which of the components of the muscularis externa grafts, enteric neurons, glia or smooth muscle cells were most

important in supporting the ingrowth of regenerating CNS axons. However, the close association of enteric glial cells with the ingrowing CNS axons raises the possibility that enteric glia produce trophic factors capable of supporting the regeneration of at least some types of axons found in the corpus striatum.

CHAPTER 4.

TYROSINE-HYDROXYLASE-CONTAINING FIBRES EXTEND FROM THE CORPUS STRIATUM INTO GRAFTS OF MUSCULARIS EXTERNA AND MYENTERIC PLEXUS.

4.1. Summary.

Intrastriatal grafts of myenteric plexus produced a vigorous sprouting response in the surrounding rat brain. Since the striatum receives a profuse dopaminergic innervation from the substantia nigra, we have investigated whether central catecholaminergic neurons participated in the sprouting response and grew into grafts of adult myenteric plexus (surrounded by smooth muscle) implanted in the adult corpus striatum. Three and six weeks after implantation, tyrosine hydroxylase-containing fibres were observed to have grown into, and ramified within, the grafts. The extent of innervation was increased six weeks after implantation, and was not diminished if the animal received a superior cervical ganglionectomy three days prior to perfusion to eliminate the possibility that the fibres were of sympathetic origin.

4.2. Introduction.

Previous studies of grafts of dissected myenteric plexus from young donors, or myenteric plexus and surrounding smooth muscle (the muscularis externa) from adult donors, have demonstrated that the neurons and glia of the myenteric plexus, taken from young or adult rats, are capable of surviving in the adult brain and spinal cord for at least eight weeks (Lawrence *et al.* 1991, Jaeger 1993, Jaeger *et al.* 1993, also see chapter 3). Grafted enteric neurons and glial cells were morphologically unaltered as compared to those seen in the gut wall (Jaeger 1993, Jaeger *et al.* 1993, see chapter 3). The presence of the myenteric plexus and its surrounding smooth muscle produced a vigorous sprouting response in the striatum at the borders of the graft, and some large bundles of putative CNS axons passed between the brain and the graft (chapter 3). However, in the previous electron microscopic study, it was not possible to categorically identify individual CNS fibres extending into the grafts (chapter 3).

It is well documented that nigrostriatal dopaminergic fibres innervate grafts of embryonic striatal tissue implanted into the excitotoxin lesioned striatum (where the postsynaptic targets of nigrostriatal dopaminergic axons have been destroyed: Clarke *et al.* 1988, Wictorin *et al.* 1988, 1989c, Liu *et al.* 1990, Sanberg *et al.* 1990, Labandeira-Garcia *et al.* 1991, Defontaines *et al.* 1992). Grafted foetal striatal tissue is also innervated by dopaminergic fibres, when implanted into striata which have not received a prior excitotoxin lesion (although there is debate over the extent of innervation in this situation; Zhou *et al.* 1989, Labandeira-Garcia 1991).

In the present investigation, we have utilized an antibody raised against tyrosine hydroxylase (TH) in order to

immunohistochemically identify catecholamine-containing axons growing into small pieces of adult rat muscularis externa implanted in the unlesioned adult rat corpus striatum. Although catecholamine-containing neurons have been described in various species and sections of the gut (eg: TH-containing neurons in the human oesophagus, Wakabayshi *et al.* 1989; dopamine-β-hydroxylase-containing neurons in the rat colon, Schultzberg *et al.* 1980), catecholamine-containing neurons have not been reported in the rat ileum, thus any TH-containing axons found in the graft must be of host origin. Superior cervical ganglionectomies were performed on a number of recipient rats prior to examination of the graft to eliminate any peripheral sympathetic fibres which may have entered the graft.

4.3. Materials and Methods.

Grafts of muscularis externa were obtained as described in section 2.5), and were implanted into the striata of adult rats as described in section 2.2. Of the nine animals examined, three underwent bilateral superior cervical ganglionectomies three days prior to perfusion, six weeks after implantation (see section 2.3 for details). Animals were anaesthetised and transcardially perfused, then sections of the brain containing grafts and cerebral vessels underwent immunohistochemistry to visualise TH (see 2.10).

4.4. Results.

The grafts were located in the dorsal and medial regions of the corpus striatum and could be identified as a paler stained area, which contained no myelinated fibre bundles, surrounded by a number of haemosiderin-containing macrophages (**Fig. 4.1a** and **4.1b**). No TH-containing neuronal cell bodies were identified within any of the grafts examined. In control sections, incubated in normal rabbit serum rather than TH antibody, grafts could still be distinguished by their lack of pale staining myelinated fibre bundles but no identifiable fibres stained with reaction product were detected, either in the striatum or in the graft itself.

Three weeks after implantation, the striatal neuropil surrounding the grafts contained many fine diameter TH-containing axons with varicosities along their length. The interface between graft and the surrounding striatum could be identified as an area of increased immunoreactivity (**Fig. 4.1b**). TH-containing axons could be identified both crossing the interface between the striatum and the graft and within the graft (**Fig. 4.1c, 4.1d**).

Most of the TH-containing fibres within the graft were thicker/coarser than those seen in the surrounding striatum, had few branches and could often be followed for comparatively long distances within the graft (**Fig. 4.1d**), although finer diameter axons could also be distinguished which were highly branched and contained many varicosities of variable sizes along their length (**Fig. 4.1d**).

Six weeks after implantation, grafts were invaded by TH-containing fibres to a greater extent than at three weeks after implantation (**Fig. 4.2a**) although the density of innervation remained lower than that present in the surrounding brain (**Fig. 4.2a**). In many grafts, fibres were not uniformly distributed in the graft but

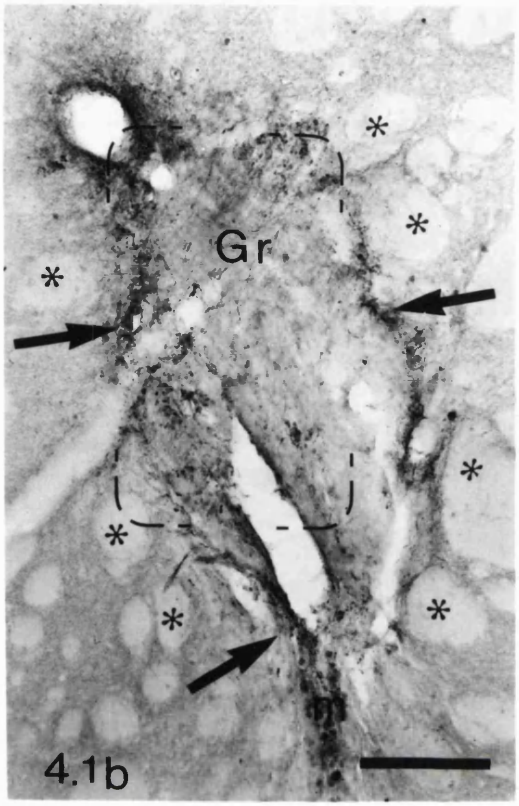
Fig. 4.1a. Camera lucida drawing of a section of brain with a muscularis externa graft (dotted area), examined 3 weeks after implantation for TH. Scale bar = 2mm.

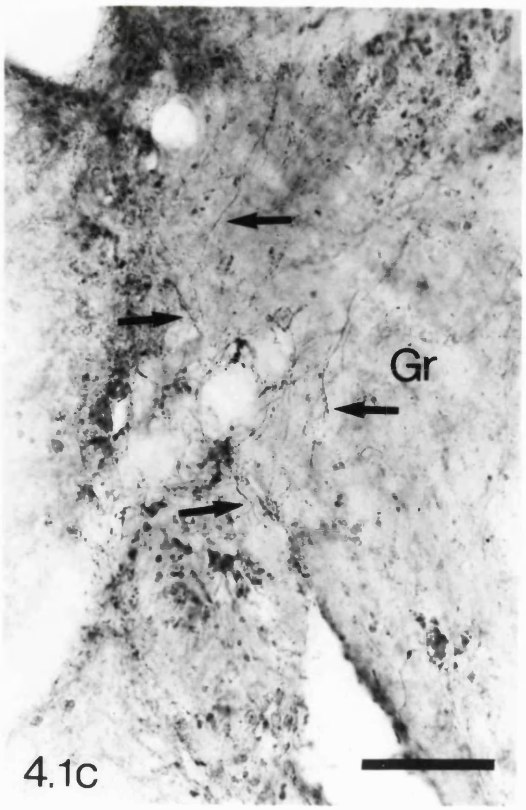
Fig. 4.1b. Photomicrograph of graft and surrounding striatum as depicted in **Fig. 4.1a.** The graft (Gr) can be distinguished from the surrounding striatum as it lacks pale staining bundles of myelinated fibres (asterisks). The interface between graft and brain is more heavily stained (large arrows) and haemosiderin-containing macrophages can be observed around the graft (m). Scale bar = 200μm.

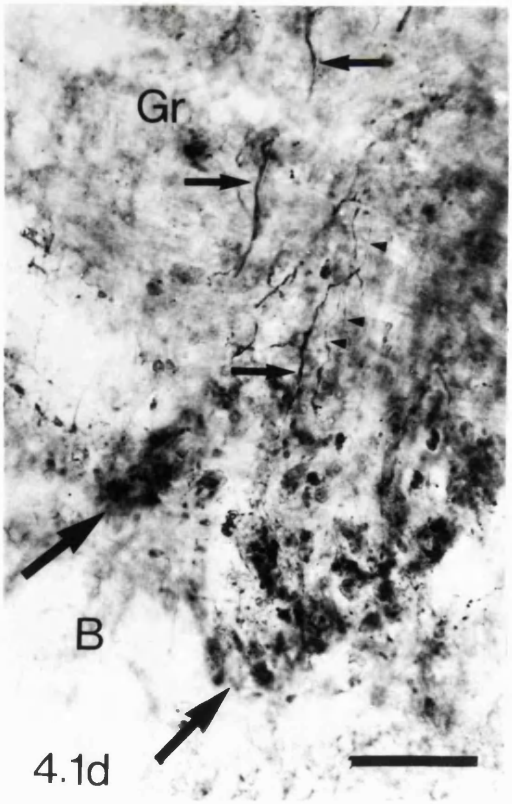
Fig. 4.1c. Higher power photomicrograph of the indicated area in **Fig. 4.1b.** TH-containing fibres (small arrows) can be observed within the graft (Gr). Scale bar = 100μm.

Fig. 4.1d. High power photomicrograph of muscularis externa graft in unlesioned striatum. TH-containing fibres can be observed within the graft, near the interface between graft (Gr) and surrounding brain (B). The majority of TH-containing fibres within the graft are coarse (arrows), although finer fibres can be observed (arrowheads). The increased staining at the interface between graft and brain (large arrows) makes it difficult to see fibres crossing between the brain and graft. Scale bar = 50μm.

4.1a







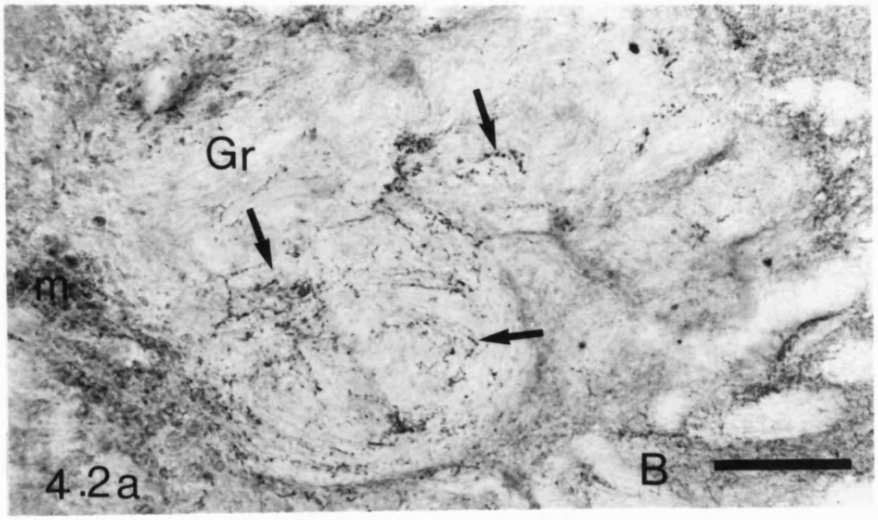
appeared to be concentrated in discrete areas of the graft (**Fig. 4.2a**). The majority of TH-containing fibres within the graft examined six weeks after implantation were fine with a variable number of varicosities along their length (**Fig. 4.2b** and **4.2c**), although some thicker, more coarse fibres were identified. Haemosiderin containing macrophages were less evident around the graft than at 3 weeks after implantation (**Fig. 4.2a**) and the interface between the graft and brain was less heavily stained (**Fig. 4.2a**).

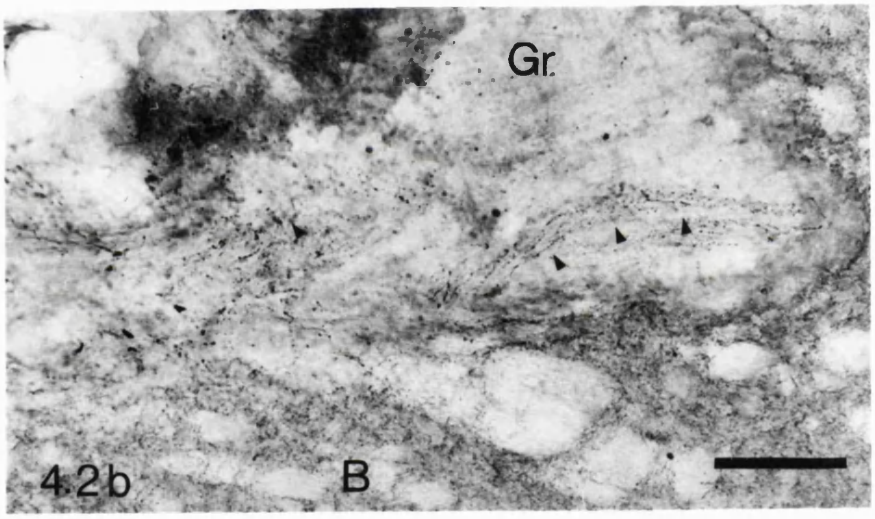
In all four experiments of six week duration in which the host was subject to a superior cervical ganglionectomy three days prior to perfusion to eliminate the possibility of peripheral sympathetic fibres being present in the grafts, the efficacy of the ganglionectomies was demonstrated by the absence of TH-containing fibres on the middle cerebral arteries (**Fig. 4.3a**) as well as by the animal exhibiting pupillary constriction and persistent ptosis. None the less, there was no apparent reduction in the TH-containing fibres present in the grafts in ganglionectomized hosts compared to grafts in the four, six week experiments in which the superior cervical ganglia were left intact (compare **Fig. 4.3b** and **4.2a**). Once again, grafts contained fine diameter fibres with varicosities along their length (**Fig. 4.3b** and **4.3c**), although fibres were not uniformly distributed throughout the graft (compare **Fig. 4.3b** and **4.3c**).

Fig. 4.2a. Photomicrograph of muscularis externa graft in the corpus striatum, examined for TH-immunoreactivity, 6 weeks after implantation. TH-containing fibres (small arrows) can be observed within the graft (Gr), although not to the same extent as in the surrounding striatum (B). Haemosiderin-containing macrophages (m) can be identified around the graft. Scale bar = 100μm.

Fig. 4.2b. High power photomicrograph of interface between muscularis externa graft (Gr) and surrounding striatum (B), examined for TH-immunoreactivity 6 weeks after implantation. The graft contains fine diameter TH-containing fibres, with periodic varicosities (arrowheads). Scale bar = 100μm.

Fig. 4.2c. High power photomicrograph of interface between muscularis externa graft (Gr) and surrounding striatum (B), examined for TH-immunoreactivity 6 weeks after implantation. The graft contains both fine diameter TH-containing fibres with periodic varicosities (arrowheads), and coarser fibres lacking periodic varicosities (arrow). Scale bar = 100μm.


Gr
m
4.2a
B


Gr
4.2b
B

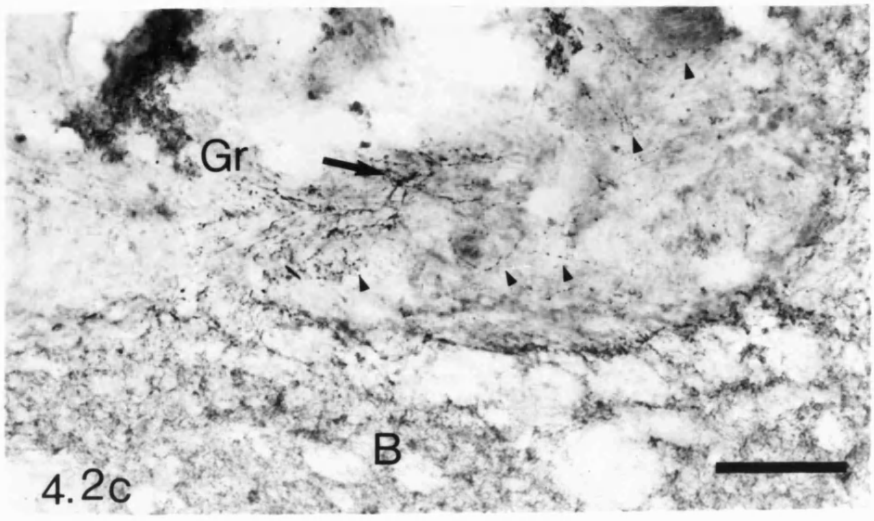
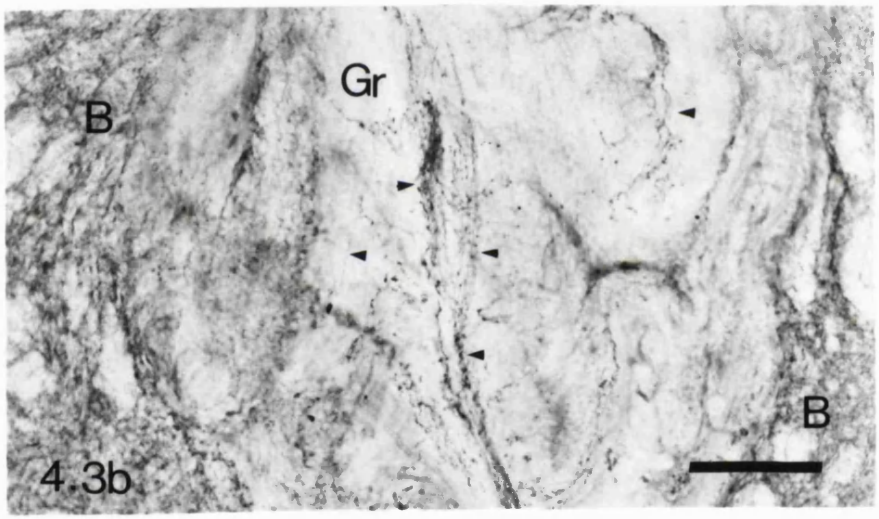

Gr
B
4.2c

Fig. 4.3a. Photomicrograph of middle cerebral artery, excised from a recipient rat that had a bilateral superior cervical ganglionectomy 3 days prior to perfusion and examination with TH-immunohistochemistry. No TH-containing fibres can be observed on the vessel. Scale bar = 200μm.

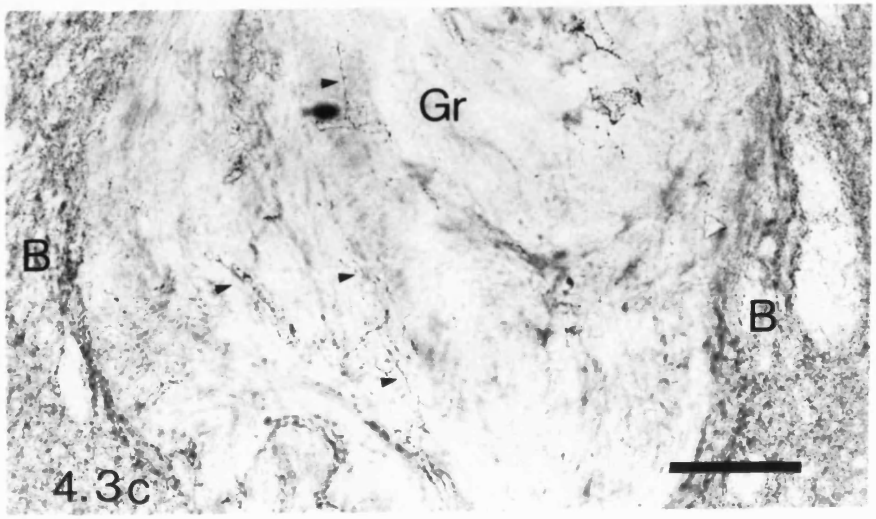
Fig. 4.3b. Photomicrograph of a muscularis externa graft (Gr) in the unlesioned corpus striatum (B) of a recipient rat that had received a bilateral superior cervical ganglionectomy 3 days prior to perfusion and examination for TH-immunoreactivity. TH-containing fibres mostly of fine diameter with periodic varicosities (arrowheads), can be seen in discrete areas within the graft. Scale bar = 100μm.

Fig. 4.3c. Photomicrograph of another section through a muscularis externa graft (Gr) in the unlesioned corpus striatum (B) of a recipient rat that had received a bilateral superior cervical ganglionectomy 3 days prior to perfusion and examination for TH-immunoreactivity. Although TH-containing fibres can be seen (arrowheads) within the graft, they are much less prevalent than in **3b**. Scale bar = 100μm.

4.3a



4.3b



4.3c

4.5. Discussion.

The present study demonstrates that catecholamine-containing fibres originating in the host brain are capable of growing into grafts of adult muscularis externa. This result supports one conclusion of the previous electron microscopic study of muscularis externa grafts implanted into the corpus striatum (chapter 3), that at least some of the large number of small unmyelinated, regenerating axonal sprouts which formed bundles extending between the brain and the grafts were of CNS origin. From the present study, it would appear that at least some of the fibres which grow into such grafts are catecholaminergic. However, as no large bundles of TH-containing fibres were seen growing into or within the graft, we must conclude that TH-containing fibres are not the predominant constituent of the axonal bundles observed in our previous study (chapter 3).

Bilateral removal of the superior cervical ganglia has been shown to result in complete loss of sympathetic fibres in the anterior cerebral circulation and the basilar artery, at least during the first seven days after ganglionectomy (Nielsen and Owman 1967). This is borne out in our study, where no TH-containing fibres were seen on the basilar or middle cerebral arteries three days after ganglionectomy. Since there was no evidence of a reduction in the density of innervation in grafts following bilateral superior cervical ganglionectomy, it is apparent that the majority of ingrowing TH-containing fibres were of central origin. Given that the striatum is host to numerous afferent dopaminergic fibres derived from the substantia nigra pars compacta, it would seem probable that the majority of ingrowing TH-containing fibres were also derived from that source, although ingrowth of noradrenergic fibres from the locus coeruleus cannot be ruled out.

Previous studies of intrastriatal grafts of embryonic striatum have demonstrated that the first catecholaminergic fibres to enter the graft are coarse and densely immunoreactive with "enlarged varicosities" (Labandeira-Garcia *et al.* 1991, Defontaines *et al.* 1992). These fibres then ramify within the graft and appear to be of fine diameter by three weeks after implantation. This process occurs in both lesioned and unlesioned hosts with the same time course (Labandeira-Garcia *et al.* 1991). In the present investigation the extensive ramification of fine fibres was not observed until six weeks after implantation. This difference in the time course of TH-containing fibres growing into the grafts of muscularis externa may be due to the nature of the substratum for axonal elongation provided by the graft.

TH-containing fibres within the grafts did not reach the same density of innervation as seen in the surrounding striatum. Nevertheless, in grafts examined six weeks after implantation, TH-containing fibres appeared to be concentrated into discrete areas of the graft, although it was not possible to discern whether these areas corresponded to enteric ganglia. It has previously been demonstrated that CNS fibres do not extend into lesioned areas that do not contain living grafted cells (Zhou *et al.* 1988, and see chapter 3). However, only intrastriatal grafts of embryonic striatal tissue are innervated to the same extent as the surrounding striatum (Clarke *et al.* 1988, Wictorin *et al.* 1989c, Labandeira-Garcia *et al.* 1991). Grafts of embryonic CNS non-striatal tissue or adult peripheral tissue (eg: iris, uterus or diaphragm) receive a random and low density of TH innervation (Björklund and Stenevi 1971, Emson *et al.* 1977, Labandeira-Garcia *et al.* 1991). It has been suggested

that ingrowth of TH-containing fibres into grafts is governed by the presence of neurotrophic factors or extracellular matrix molecules. Both brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) have been shown to enhance the survival of cultured dopaminergic substantia nigra neurons (Knüsel *et al.* 1990, Hyman *et al.* 1991, Spina *et al.* 1992) although it has been demonstrated that BDNF can not prevent degeneration of nigral neurons following axotomy (Knüsel *et al.* 1992). There have been no extensive studies to investigate the possibility that these molecules may be produced by the ENS. It is also feasible that the cell adhesion molecule L1 may play a part in the ingrowth of fibres, since L1 has been shown to increase the outgrowth of neurites from nigral neurons in culture (Poltorak *et al.* 1992) and enteric glia have been demonstrated to express L1 (Mirsky *et al.* 1986).

This study has demonstrated that intrastriatal grafts of myenteric plexus and surrounding smooth muscle are invaded by TH-containing CNS axons. There is an increase in the extent of innervation seen between three and six weeks after implantation, although the density of fibres never reaches that seen in the surrounding striatum. The numbers of TH-containing axons which enter the grafts suggest that they may be only a minor constituent of the large bundles of axons found passing between brain and graft in our previous EM study (chapter 3).

CHAPTER 5.

POSTNATAL RAT NADPH-DIAPHORASE-CONTAINING MYENTERIC NEURONS EXTEND PROCESSES WHEN TRANSPLANTED INTO ADULT RAT CORPUS STRIATUM.

5.1. Summary.

Pieces of isolated myenteric plexus, freshly dissected from the colon of young inbred Fischer rats, were implanted unilaterally into the corpus striatum of adult Fischer rats which had received a prior quinolinic acid lesion. Similar implants were made into the unlesioned striatum of a second group of Fischer rats.

The survival of a subpopulation of grafted neurons, and the incidence of fibre outgrowth from these neurons into the host striatum, was examined after 3 and 6 weeks using a histochemical technique for the demonstration of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-diaphorase) activity. This enzyme is found both in some enteric neurons and a subpopulation of striatal neurons.

Grafted enteric neurons expressing NADPH-diaphorase were easily distinguished in both the lesioned and unlesioned host striata at 3 and 6 weeks post implantation. Fibres could be found extending from NADPH-diaphorase-containing enteric neurons into both the lesioned and unlesioned corpus striatum, although fewer graft-derived fibres were seen in the unlesioned striatum. This is the first demonstration that enteric neurons transplanted into the adult mammalian brain are capable of extending processes into the surrounding CNS tissue.

5.2. Introduction.

As has already been discussed (see section 1.1), the similarities between the ENS and CNS make the myenteric plexus a particularly interesting source of material for transplantation into the brain. Previous studies have demonstrated that neurons and glia of the myenteric plexus are able to survive in the CNS for at least 2 months after implantation into the brain of adult animals. Lawrence and colleagues (1991) implanted isolated myenteric plexus from rats into the cholinergically denervated hippocampus and demonstrated the survival of both neurons and glial cells, but, using acetylcholinesterase histochemistry, found no evidence for the outgrowth of acetylcholinesterase-containing processes from grafted enteric neurons into the host tissue.

In a previous electron microscopical study (chapter 3) we have shown that the adult myenteric plexus transplanted with its surrounding circular and longitudinal muscle layers into the corpus striatum of adult host rats was able to survive well and appeared to stimulate the production of axonal sprouts in the surrounding brain, many of which invaded the graft. However, electron microscopy was unsuitable for studying the pattern and extent of outgrowth of myenteric axons into the host brain.

NADPH-diaphorase has been described in CNS neurons (Thomas and Pearse 1964, Vincent and Kimura 1992) and in neurons of the myenteric plexus of the guinea pig (Furness *et al.* 1991, Nichols *et al.* 1992, Saffrey *et al.* 1992, Young *et al.* 1992) and rat intestine (Bredt *et al.* 1990, Dawson *et al.* 1991a, Belai *et al.* 1992), and there is a substantial population of NADPH-diaphorase-containing neurons in the rat colon (Belai *et al.* 1992). Evidence suggests that NADPH-diaphorase is a neuronal form of nitric oxide synthase (NOS; Bredt *et*

al. 1991, Dawson *et al.* 1991a, Hope *et al.* 1991) and that nitric oxide produced by NOS may act as a neurotransmitter or neuromodulator in various parts of the nervous system (see Garthwaite 1991, Rand 1992, Snyder 1992 for reviews). The enzyme has also been localized within a subpopulation of striatal interneurons: the medium aspiny neurons that contain somatostatin and neuropeptide Y (Vincent *et al.* 1983a).

Evidence suggests that neuronal grafts into areas that have received a prior lesion exhibit enhanced survival (Gage and Björklund 1986) and display a greater fibre outgrowth (Schmidt *et al.* 1981, Wright *et al.* 1991) when compared with similar grafts in an unlesioned environment. The quinolinic acid (QA) lesioned striatum has been used as animal rodent model of Huntington's disease since the neurochemistry of the lesioned striatum is remarkably similar to the corpus striatum of patients with Huntington's disease (Beal *et al.* 1986, Davies and Roberts 1987), and rodents lesioned in this way display behavioural abnormalities similar to those seen in patients with the disease (Sanberg *et al.* 1989). The central zone of the QA lesioned striatum is devoid of NADPH-diaphorase-containing interneurons as well as the striatal GABA-containing projection neurons, though there is some debate over the loss of NADPH-diaphorase-containing neurons at the periphery of the lesion (see for example Beal *et al.* 1986, Davies and Roberts 1987, Boegman and Parent 1988, Koh and Choi 1988, Roberts and DiFiglia 1988, Sanberg *et al.* 1992). The use of the lesioned striatum as a site for graft implantation in the present study thus allowed both the examination of the graft in a lesioned environment and also aided the identification of graft derived axons invading the host brain.

In the present study we have used NADPH-diaphorase histochemistry to investigate whether neurons from isolated myenteric ganglia survive transplantation into the striatum (in the absence of surrounding smooth muscle). We have also examined the extent to which such cells send out processes into the host brain and the pattern of such outgrowths.

5.3. Materials and Methods.

Grafts of dissected myenteric plexus were obtained as described in section 2.6 and implanted into the striata of adult Fischer rats (see section 2.2) some of which had received a QA lesion 7-14 days prior to implantation (see section 2.4). After 3 or 6 weeks survival, animals were anaesthetised and transcardially perfused (see section 2.9) and sections of brain containing grafts were reacted for NADPH-diaphorase (see section 2.9). The cross-sectional area of individual sections was measured using a Seescan image analysis system, and this allowed the volume of the grafts to be estimated.

5.4. Results.

Grafts consisted of approximately 15-18 segments of myenteric plexus, each segment comprising some 10-30 connected ganglia. Myenteric ganglia vary considerably in size and shape, and contain highly variable numbers of neurons (see Furness and Costa 1987). Thus it was not possible to determine with accuracy the numbers of neurons implanted in the present study. However, extrapolating from what quantitative data is available (Furness and Costa 1987, Gabella 1971b) each graft probably contained some 2000-5000 neurons, of which approximately a third are likely to contain NADPH-diaphorase (Saffrey *et al.* 1992, Belai-personal communication).

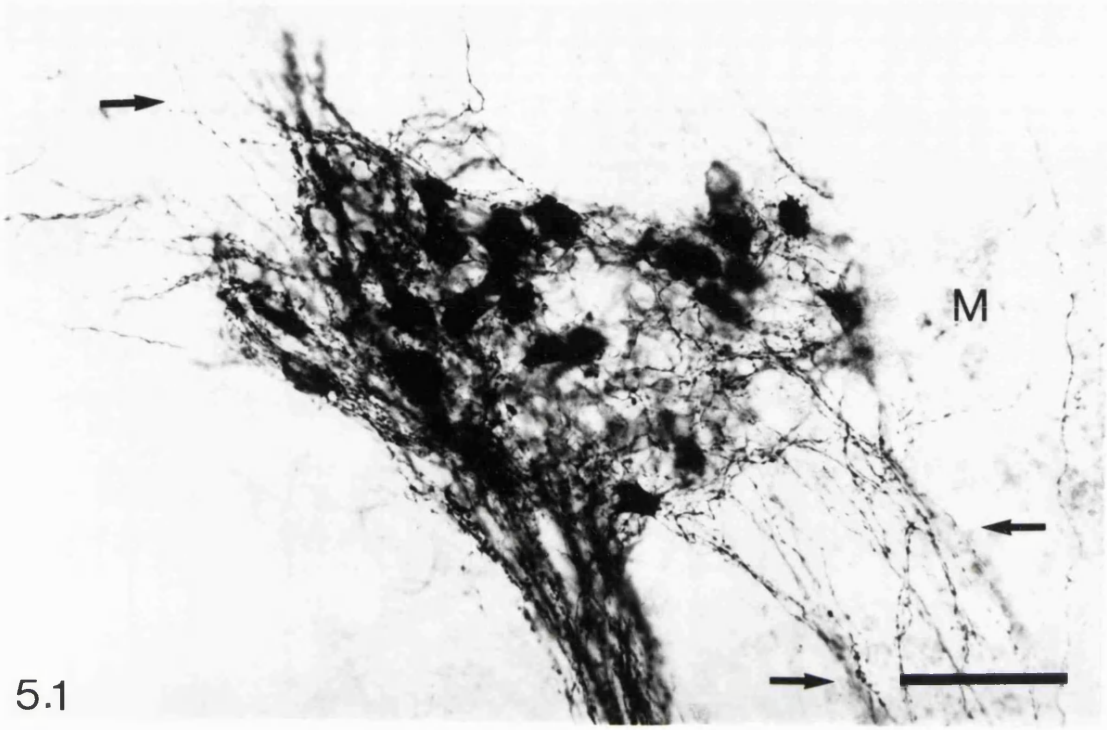
5.4.1. General observations.

Of the 23 grafted striata examined at both 3 and 6 weeks after implantation, 16 contained NADPH-diaphorase stained enteric neurons. In the remaining brains, grafted cells were found in the corpus callosum or cerebral cortex, probably as a result of tissue being pulled back up the needle tract during implantation. The volume of the grafts varied between $0.22mm^3$ and $6.51mm^3$. At all sites, grafted cells occurred singly or in clusters. In order to fully visualise NADPH-diaphorase-containing processes, sections were incubated for long periods (90-100 mins). This had the effect of making cellular borders difficult to discern in those grafts where the myenteric neurons were tightly packed within the ganglia. However in less tightly packed grafts, the morphology of the perikarya of implanted cells could be easily seen.

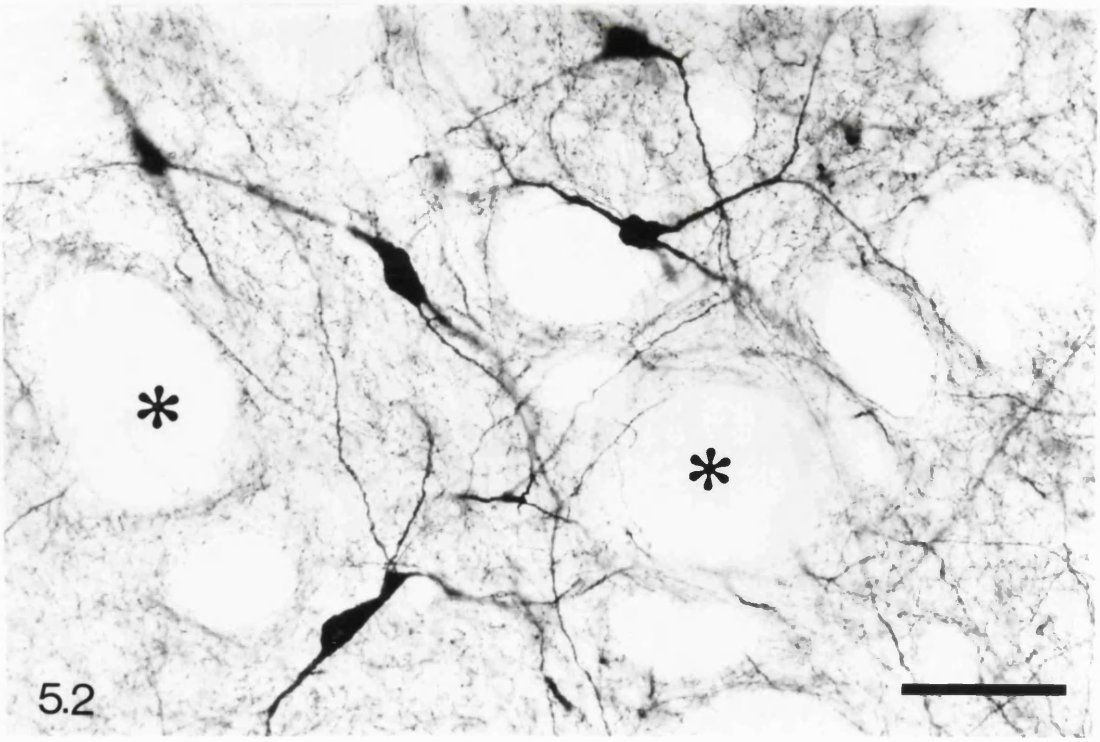
NADPH-diaphorase-containing neurons of the transplanted enteric ganglia had somewhat rounded cell bodies (**Fig. 5.1**). The processes extending from them and forming part of the graft neuropil were thin with periodic varicosities and tended to fasciculate into larger

Fig. 5.1. Section through grafted rat enteric ganglia, 3 weeks after implantation into the quinolinic acid lesioned adult rat corpus striatum, stained for NADPH-diaphorase. Rounded enteric neuronal cell bodies are visible. The graft neuropil includes thin diaphorase positive fibres with periodic varicosities. Fibres can be identified extending from the graft (small arrows) into the quinolinic acid lesioned striatum (where the striatal NADPH-diaphorase-containing cells have been killed). (M= haemosiderin containing macrophages) Scale bar = 500 μm.

Fig. 5.2. Section through the contralateral, unoperated corpus striatum of an adult rat, stained for NADPH-diaphorase. Striatal medium aspiny neurons can be identified with spindle shaped cell bodies and prominent dendrites. The striatal neuropil includes a felt of NADPH-diaphorase-containing fine fibres which do not invade the pale, myelinated fibre bundles (asterisk). Scale bar = 500μm.



5.1



5.2

bundles in some areas of the graft. In contrast, host striatal NADPH-diaphorase-containing neurons were more spindle shaped with prominent long, thick, sparsely branching dendrites (**Fig.** 5.2). The striatal neuropil in unlesioned animals contained a felt of fine NADPH-diaphorase positive axons which were largely excluded from the pale, myelinated, fibre bundles (**Fig.** 5.2).

5.4.2. **Grafts in lesioned corpus striatum.**

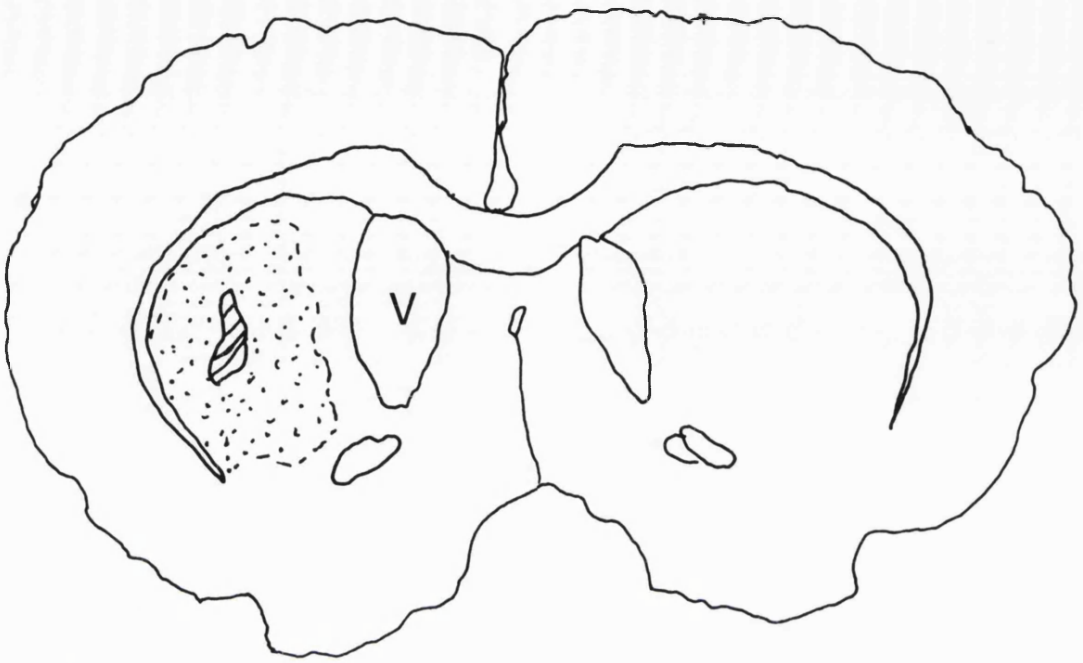
Three weeks after implantation, the QA lesioned striatum was characterised by a loss of NADPH-diaphorase-containing neuronal perikarya and a great reduction of staining of the neuropil within the lesioned zone (compare **Fig.** 5.1 and **Fig.** 5.2). The lesioned striatum was shrunken, as demonstrated by the enlarged lateral ventricle on the operated side (**Fig.** 5.3a). In two brains of animals that had received lesions prior to implantation and were examined 3 weeks after implantation, an area of increased staining was observed at the outer edge of the lesion zone. The NADPH-diaphorase-containing cells appeared to be astrocytes.

Grafts of myenteric ganglia contained clusters of cell bodies and a darkly stained neuropil, thus allowing them to be clearly differentiated from the surrounding lesioned striatum (**Fig.** 5.1 and 5.3b). At 3 weeks after implantation, many of the fibres of the neuropil of the grafts appeared to be coarse and run in bundles (**Fig.** 5.3b), though thin fibres with periodic varicosities could also be detected (**Fig.** 5.1).

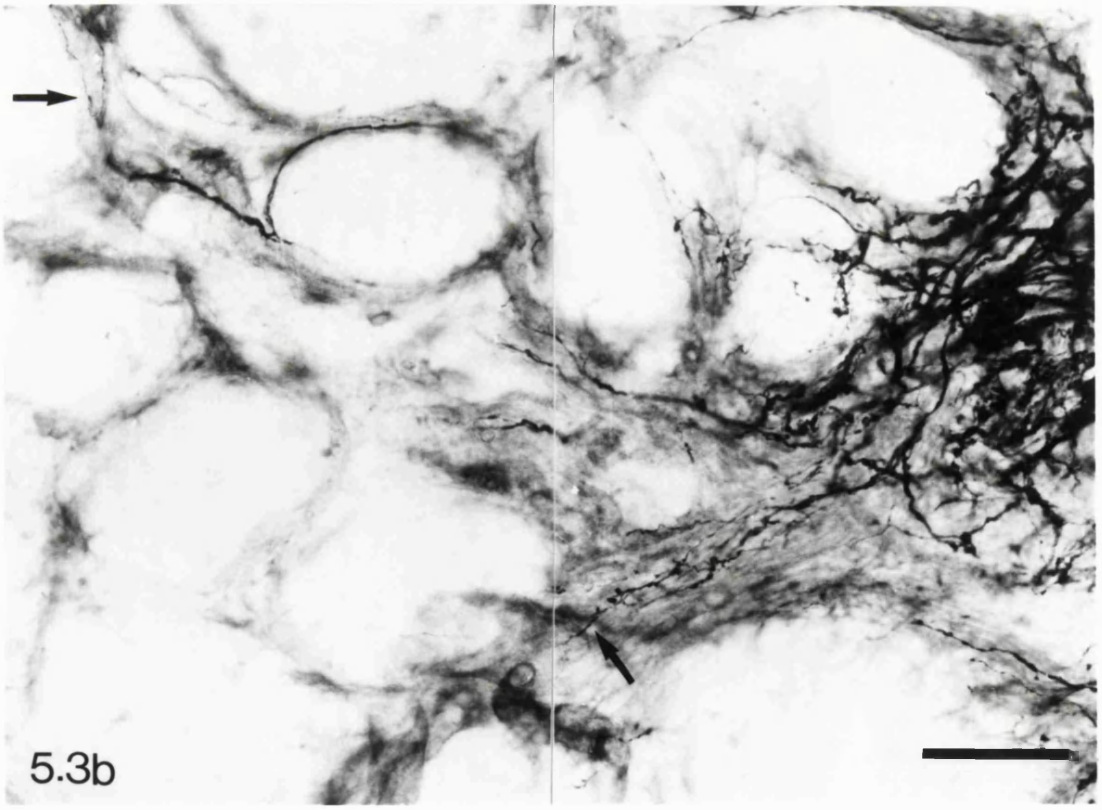
Although there was a clear demarcation between graft and brain, there was also evidence of the outgrowth of NADPH-diaphorase positive fibres from the grafted neurons into the lesioned striatum. Fibres were found to have grown away from the grafted perikarya, both in the

Fig. 5.3a. Camera lucida drawing of the position of a myenteric plexus graft (indicated by the hatched area) implanted into the quinolinic acid lesioned striatum, 3 weeks after implantation and stained for NADPH-diaphorase. The stippled area indicates the extent of the quinolinic acid lesion. The ventricle (V) of the operated side is enlarged indicating the reduction in striatal volume. Scale bar = 2 mm.

Fig. 5.3b. Light micrograph of the grafted NADPH-diaphorase-containing ganglia seen in **Fig. 5.3a.** Bundles of fibres can be seen extending from the graft, circling myelinated fibre bundles, and separating in places to reveal individual thin fibres with periodic varicosities (small arrows). Scale bar = 500 μm.



5.3a



5.3b

needle tract and running for considerable distances in the striatum outside the graft, where they circumnavigated myelinated fibre bundles (**Fig. 5.3b**). Some of the fibres growing into the lesioned striatum were fasciculated to form bundles which split at certain points revealing the individual fibres to be fine with periodic varicosities (**Fig. 5.3b**). Bundles of fibres which appeared to be of graft origin were found in sections anterior and posterior to the graft.

Six weeks after implantation, grafts in lesioned striatum contained many neurons expressing NADPH-diaphorase. The neuropil contained a greater proportion of thinner fibres with periodic varicosities and fewer thick bundles of fibres. NADPH-diaphorase positive fibres had again extended from the neurons in the graft into the lesioned striatum or along the needle tract. Clusters of fibres, which appeared to be of graft origin, were again observed in sections anterior and posterior to the graft.

5.4.3. **Grafts in unlesioned corpus striatum.**

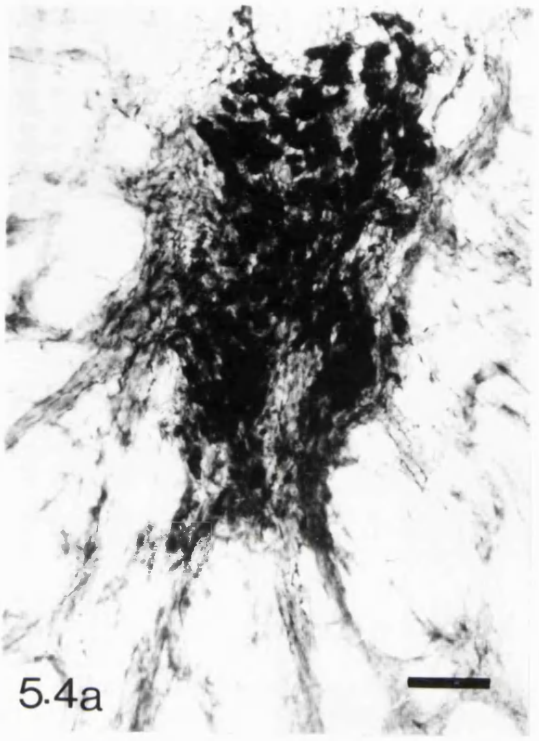
Three and 6 weeks after implantation, NADPH-diaphorase-containing enteric neuronal cell bodies were readily identifiable in the unlesioned striatum (**Fig. 5.4a** and **5.4b**). The grafts could be distinguished by the increased density of packing of the grafted cell bodies, which exhibited a different morphology and distribution compared to NADPH-diaphorase stained striatal neurons (see above).

In some animals, it was difficult to ascertain whether fibres had grown from the grafts into the lesioned host striata. However, on examining the interface zone in sections through the graft that contained fewer cell bodies and where the "background" NADPH-diaphorase fibre staining was low, thin fibres with periodic

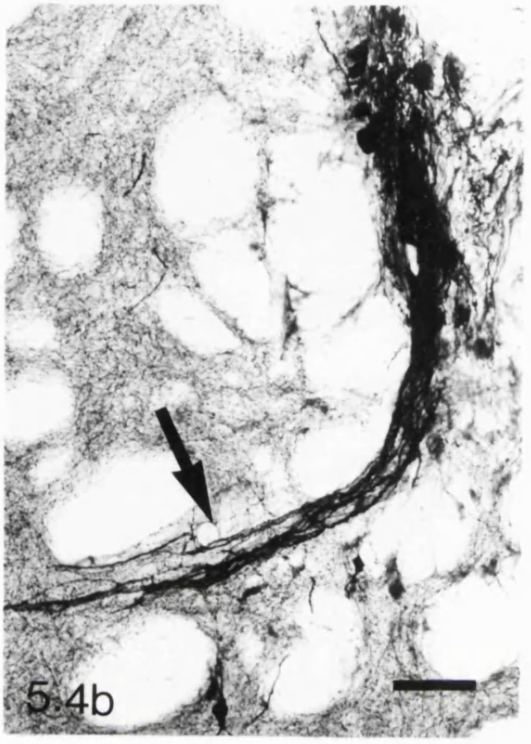
Fig. 5.4a. Rat enteric ganglia 3 weeks after implantation into the unlesioned adult rat corpus striatum. The graft contains densely packed NADPH-diaphorase-containing neuronal cell bodies, making it easily distinguishable from the surrounding striatum. There is some reduction in the NADPH-diaphorase staining of the striatal neuropil around the graft. Scale bar = 500 μm.

Fig. 5.4b. A micrograph of a similar graft of enteric ganglia in the unlesioned striatum, examined 6 weeks after implantation and stained for NADPH-diaphorase. A large bundle of neuronal processes containing NADPH-diaphorase can be seen extending from the graft (large arrow) into the unlesioned striatum. Scale bar = 500 μm.

Fig. 5.4c. The same section as **Fig. 5.4b.** examined at higher power. Some fine fibres can be seen extending from the graft into the host striatum (small arrows). Fine fibres, whose origin cannot be determined, are also observed (arrowheads). Scale bar = 500 μm.



5.4a



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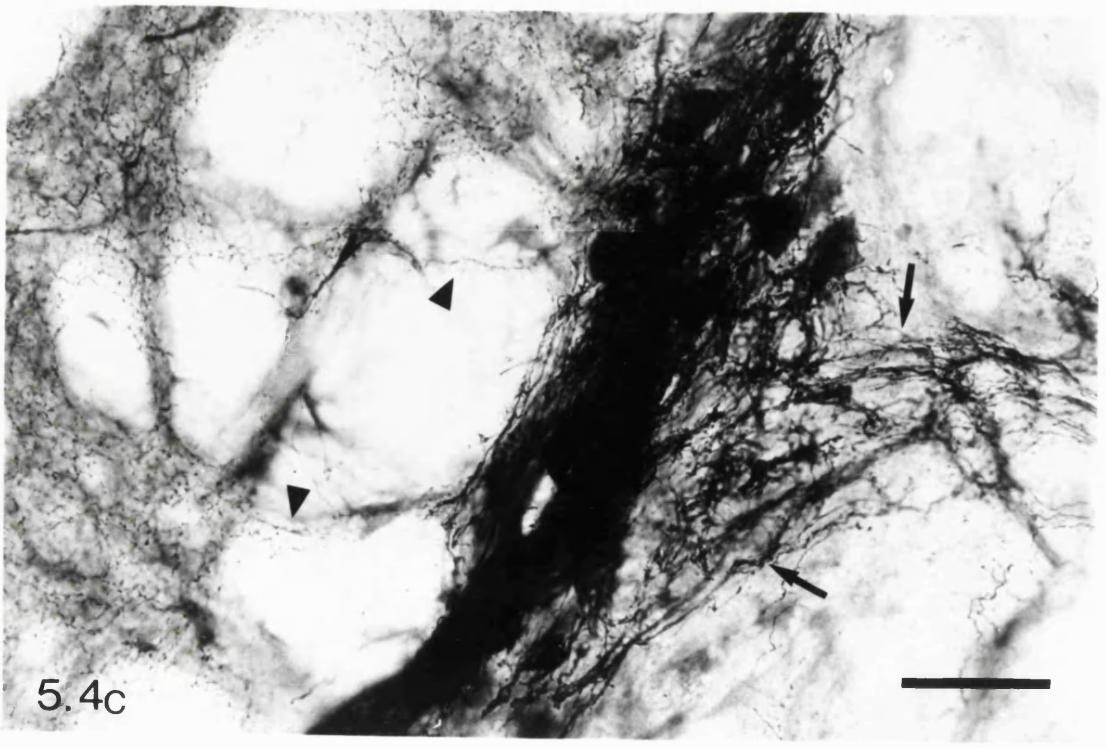


5.4c

varicosities could be seen emerging from the graft into striatal tissue, at both 3 and 6 weeks (**Fig. 5.4a** and **5.4b**). Because of the small diameter of the fibres leaving the grafts, it was difficult to determine how far they extended into the brain. A small number of grafts examined 6 weeks after implantation demonstrated large bundles of NADPH-diaphorase-containing fibres which extended from the body of the graft and penetrated undamaged areas of the unlesioned striatum (**Fig. 5.4c**). In most grafts in unlesioned striatum, there were a greater proportion of graft-derived fibres in the needle tract than in the surrounding striatum. Other NADPH-diaphorase fibres found within the graft damaged zone could have been of striatal or graft origin (**Fig. 5.4c**). In sections through unlesioned striatum anterior and posterior to the graft, it was possible to discern small clusters of coarse fibres which appeared to be of graft origin, providing further evidence that axons from the graft grew into the unlesioned striatum.

5.5. Discussion

In these experiments, we have demonstrated that a subpopulation of neurons from freshly isolated rat myenteric plexus implanted into the adult rat corpus striatum are capable of surviving for at least 6 weeks. Further, these neurons send processes out into the surrounding brain irrespective of whether the implantation site had been previously subjected to a QA lesion.

In a previous study myenteric plexus taken from young rats survived for 2 months in the cholinergically denervated hippocampus of adult rats (Lawrence *et al.* 1991). NADPH-diaphorase-containing neurons were not investigated, but the grafted neurons which contained acetylcholinesterase did not appear to innervate the surrounding brain. It is possible that the differences in neurite outgrowth between the present study and that of Lawrence *et al.* (1991) may be due to the striatum constituting a more permissive environment for neurite elongation than the hippocampus or to the NADPH-diaphorase-containing enteric neurons being intrinsically more capable of regenerating axons than those containing acetylcholinesterase.

Both hippocampus and striatum are capable of being reinnervated, whether lesioned or unlesioned, by grafts of embryonic neurons that normally project to the site of implantation (for example; nigral cells in striatum, septal cells in hippocampus: Dunnett *et al.* 1985, Gage *et al.* 1987a, Zhou *et al.* 1988, S.Lu *et al.* 1991). It would appear that even when such tissue is implanted into an unlesioned environment, the subsequent hyperinnervation of the striatum or hippocampus is functional (Lu *et al.* 1990, Buzsáki *et al.* 1991). However the lesioned hippocampus is incapable of being adequately reinnervated by central cholinergic neurons that do not

normally innervate the hippocampus (Nilsson *et al.* 1988, Clarke *et al.* 1991), perhaps suggesting that the hippocampus is not conducive to the outgrowth of "foreign" neurons. Experiments implanting tissue into the lesioned striatum have shown that embryonic CNS neurons that project to the striatum (eg: cortex, substantia nigra) are capable of extending fibres into the surrounding brain (Wictorin *et al.* 1990b, 1991 and see section **1.3.2.1.**). The neurite extension of embryonic CNS neurons which are neither found in nor project to the striatum when implanted in the striatum is varied; cerebellar neurons do not extend fibres into the surrounding brain (Labandeira-Garcia *et al.* 1990), while neurons within spinal cord grafts are reported to be able to extend fibres into the surrounding brain (Wictorin 1992), perhaps suggesting that the striatum constitutes a permissive environment for regenerative axonal growth of some types of neurons.

The alternative explanation for the difference in the results of the present study and that of Lawrence *et al.* (1991) is that the subpopulation of NADPH-diaphorase-containing neurons in the rat colon may be intrinsically more able to mount a vigorous regenerative response when transplanted into the CNS than those containing acetylcholinesterase. This presumes that NADPH-diaphorase is not colocalized with acetylcholinesterase in enteric neurons. While this point has not been extensively investigated, studies in the myenteric plexus of the rat oesophagus have demonstrated that the two enzymes were not colocalized in neurons (Aimi *et al.* 1993) and, since acetylcholine is an excitatory transmitter in the ENS of most species (Furness and Costa 1987) while nitric oxide (produced by NOS/NADPH-diaphorase) has been shown to act as a non-adrenergic, non-cholinergic inhibitory "transmitter" in many parts of the ENS (Rand

1992), their extensive colocalization would seem unlikely. When grown in culture, neurons-containing either of the enzymes are capable of a strong regenerative response (Jessen *et al.* 1983b, Saffrey *et al.* 1992). It is not known if NADPH-diaphorase-containing neurons have special regenerative properties, although the neurons of the ENS normally express high levels of the growth associated protein GAP-43 (Sharkey *et al.* 1990, Stewart *et al.* 1992). The ENS appears to have inherent plasticity. For example, enteric neurons are capable of withstanding periodic compression (Gabella and Trigg 1984), of fibre regeneration across a transection lesion (Galligan *et al.* 1989, Kobayashi *et al.* 1989). Consequently it would seem plausible that enteric neurons should be capable of sending out long processes into an alien environment.

It is interesting to note that when embryonic CNS tissue is implanted into the hippocampus, the greatest outgrowth is observed from neurons that are normally afferents to the site of the graft. CNS neurons whose axons are not afferent to the hippocampus have been shown to be incapable of growing axons into the alien environment and their axons have been described as spiralling around the inside of the graft (Nilsson *et al.* 1988, Clarke *et al.* 1991). On the other hand, grafts of neonatal and adult peripheral ganglia transplanted into artificially hypervascularized beds in the dorsal striatum and hippocampus have been observed to extend processes into the striatum or hippocampus (Stenevi *et al.* 1976). Peripheral ganglia implanted into the choroid fissure or lateral ventricle have also been shown to extend processes into the striatum and hippocampus (Stenevi *et al.* 1976, Rosenstein and Brightman 1979). Thus peripheral autonomic ganglia may be intrinsically capable of mounting a more vigorous response than embryonic central neurons or they may be less sensitive

to whatever limits CNS regeneration (eg: an absence of trophic factors or the presence of inhibitory factors). The present study demonstrates that the ability to extend neurites into the CNS is well developed in enteric neurons.

In the present study, NADPH-diaphorase-containing axons from the graft grew more extensively into the lesioned brain than into the unlesioned brain, whether that be the chemically lesioned zone produced by QA or the mechanically lesioned zone formed by the needle tract. It has been postulated that lesion zones contain higher levels of neurotrophic and neurite-promoting factors than intact brain (Nieto-Sampedro *et al.* 1982). These factors appear to enhance neuronal survival (Nieto-Sampedro *et al.* 1984) and stimulate both an increase in graft volume (Gage and Björklund 1986) and axonal outgrowth (Neuberger *et al.* 1992). The observation that NADPH-diaphorase-containing axons in the unlesioned environment grew extensively in the needle tract may also be due to the fact that the needle tract contains an increased number of fibroblasts and macrophages (Maxwell *et al.* 1990), which may secrete trophic factors or extracellular matrix molecules which support regenerating processes. Nonetheless, enteric neurons grafted into an unlesioned environment also extended processes into the surrounding striatum, but to a lesser extent than such grafts in the quinolinic acid striatum.

Although NADPH-diaphorase is known to be a neuronal form of NOS (Bredt *et al.* 1991, Dawson *et al.* 1991a, Hope *et al.* 1991), it has also been reported to be found in activated astrocytes observed after intense neuronal activity or mechanical damage (Wallace and Fredens 1992, Murphy *et al.* 1993). This suggests that the NADPH-diaphorase-

containing cells found near the outer edge of the lesion zone may be astrocytes activated by the quinolinic acid lesion. The observation that putative NADPH-diaphorase-containing astrocytes were observed only in 2 animals may be explained by the form of the enzyme found in astrocytes being more easily lost during fixation or requiring more stringent conditions for its detection than those necessary for the visualisation of neuronal NADPH-diaphorase.

This is the first demonstration that grafts of enteric ganglia can successfully extend processes into the central nervous system. Although the present study was limited to NADPH-diaphorase-containing neurons, it remains to be determined if other subpopulations of enteric neurons for example those containing the enzyme acetylcholinesterase and various neuropeptides, are also capable of this response. Furthermore, it would be of great interest to discover whether the outgrowth of neurites from grafted enteric neurons has any functional consequences.

CHAPTER 6.

TRANSPLANTATION OF THE POSTNATAL RAT MYENTERIC PLEXUS INTO THE ADULT RAT CORPUS STRIATUM: AN ELECTRON MICROSCOPIC STUDY.

6.1 Summary.

Grafts of freshly dissected myenteric plexus taken from young inbred Fischer rats were implanted in the corpus striatum of adult Fischer rats, some of which had received a quinolinic acid lesion one to two weeks prior to implantation. Brain sections from the grafted animals were examined electron microscopically three and six weeks after implantation. Grafts contained many enteric neurons and glial cells in ganglia morphologically similar to those seen within the intestine. Although a glia limitans could be observed in parts of the interface between graft and brain, ganglia at the surface of the graft were observed to be closely apposed to the surrounding striatum, with no intervening basal lamina. CNS axonal sprouts were identified in the corpus striatum near the interface with the grafted enteric ganglia and bundles of similar axons, some containing fibres that had enlarged and become myelinated, could be seen within the grafts. CNS axonal sprouts were also observed around the grafts in the quinolinic acid lesioned striata. This study confirms that even in the absence of surrounding layers of smooth muscle, enteric ganglia grafted into the corpus striatum survive, and shows that such grafted ganglia are invaded by CNS axons.

6.2. Introduction.

In previous studies, it has been demonstrated that enteric neurons and glial cells from young postnatal donors can survive in the hippocampus (Lawrence *et al.* 1991) and corpus striatum (see chapter 5). Neurons and glia from older animals, when accompanied by surrounding smooth muscle, are also capable of surviving in regions of the brain and spinal cord (Jaeger *et al.* 1993, chapter 3 and 4). We have demonstrated that, when adult myenteric plexus with its surrounding smooth muscle is implanted into the striatum, a sprouting response occurs in the brain adjacent to the graft (chapter 3). Some of these axonal sprouts contain tyrosine-hydroxylase, suggesting they may be from dopaminergic afferents to the striatum (chapter 4).

In the present electron microscopic study, grafts of enteric ganglia (without the surrounding smooth muscle) were taken from young rats and implanted into the corpus striatum of adult syngeneic recipients. Of principle interest was whether these grafts provoked a similar axonal sprouting response in the brain to that produced by grafts containing both ganglia and smooth muscle, and whether enteric glial cells under these circumstances exhibited an altered morphology.

A number of grafts were made in the Quinolinic acid (QA) lesioned corpus striatum, a rodent model of Huntington's disease (Beal *et al.* 1986, Sanberg *et al.* 1989 and see section 1.2.3.1), to ascertain whether there was any alteration in survival of the graft in the environment provided by the lesioned brain and whether the CNS axonal sprouts found around grafts were derived mainly from intrinsic striatal neurons.

6.3. Materials and Methods.

Grafts of dissected myenteric plexus were obtained as outlined in section 2.6, and were implanted into the striata of rats (see section 2.2), some of which had received a QA lesion 7-14 days prior to implantation (see section 2.4). Two animals acted as controls and received QA lesions but myenteric plexus grafts were not subsequently implanted. Animals were anaesthetized and transcardially perfused (see section 2.8) and vibrosliced sections of the brain were processed for electron microscopic investigation (see section 2.8).

6.4. Results.

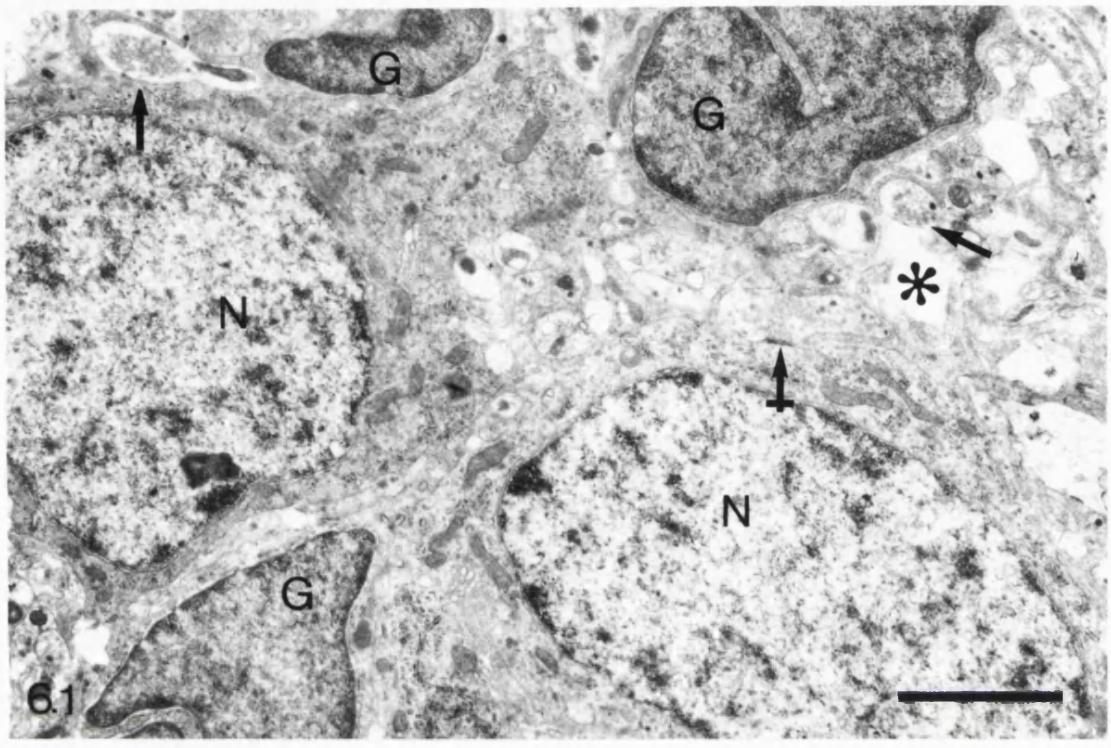
Eleven of the 21 brains examined three and six weeks after implantation contained living grafts of myenteric neurons and glia in the corpus striatum. The remaining brains mainly contained grafts located in the corpus callosum or cerebral cortex overlying the corpus striatum. However in some brains, no grafted cells were found.

6.4.1. Grafts in the unlesioned corpus striatum.

The grafts were found to contain large ganglia, composed of many neurons and glial cells. The cellular morphology of the enteric neurons was similar to that found *in situ* (Cook and Burnstock 1976, Gabella 1972: **Fig. 6.1**). The enteric neurons had large, somewhat rounded, eccentrically placed, pale staining nuclei, with 1 or 2 nucleoli. The perinuclear cytoplasm contained mitochondria, rough and smooth endoplasmic reticulum, free ribosomes and some golgi apparatus. Enteric glial cells within the ganglia (**Fig. 6.1**) had irregular shaped, indented, electron-dense nuclei, with a characteristic palisaded layer of heterochromatin at the nuclear envelope (see **Fig. 3.3a**). The ganglionic neuropil contained varicosities exhibiting small agranular vesicles and dense cored vesicles in variable proportions (**Fig. 6.1**). Axosomatic and axodendritic synapses could also be observed (**Fig. 6.1**). Profiles of neurites lacking vesicles and degenerating processes were also seen within the ganglionic neuropil. Unlike myenteric ganglia examined *in situ*, the neuropil of the grafted ganglia often contained "ghosts" of neurite profiles that had presumably degenerated (**Fig. 6.1**). Grafted enteric ganglia were largely surrounded by a layer of basal lamina, as are ganglia observed in the gut wall. In many cases, enteric neuronal cell bodies were observed at the surface of the ganglia,

Fig. 6.1. Electron micrograph of part of a rat enteric ganglion, examined 3 weeks after implantation into unlesioned corpus striatum of the adult rat, showing cell bodies of enteric neurons (N) and enteric glial cells (G). The ganglionic neuropil contains axonal profiles, some of which contain small agranular and dense cored vesicles (arrows). One profile is involved in a synaptic contact with the neuronal cell body (crossed arrow). The ganglionic neuropil also contains spaces (asterisk), possibly indicating the degeneration of neuronal processes. Scale bar = 2μm.

Fig. 6.2. Electron micrograph depicting part of an enteric ganglion at the interface with the unlesioned striatum, 3 weeks after implantation into adult rat corpus striatum. An enteric neuron (N) and part of an enteric glial cell (G) are shown. Much of the enteric neuronal cell body is surrounded by an astrocyte process (A) with only a thin layer of enteric glial cytoplasm intervening. No intervening basal lamina is visible. Scale bar = 2μm.



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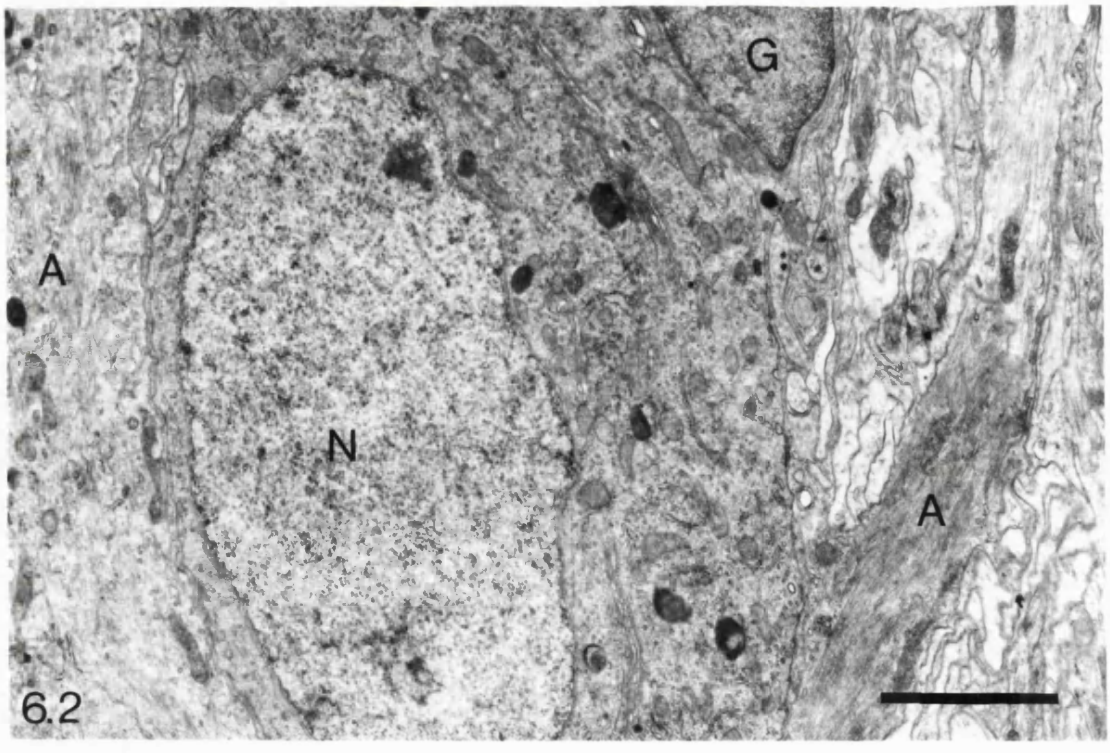


6.2

with no covering of enteric glial cytoplasm and in direct contact with the outer basal lamina, mimicking the situation seen in the gastrointestinal tract. There was no difference between grafts examined three and six weeks after implantation.

Although the grafts of enteric ganglia were dissected free of the circular and longitudinal muscle coats, a small number of smooth muscle cells (presumably left adhering to the plexus on dissection) were sometimes observed. Grafts also contained fibroblasts and macrophages. Bundles of neuronal processes, resembling the interganglionic strands of the enteric nervous system, were observed in the grafts. They were composed of tightly packed, irregularly shaped neuronal profiles, many of which contained small agranular or dense cored vesicles. All such bundles were partially or completely wrapped by glial processes surrounded by a layer of basal lamina (see **Fig. 6.4a** and **6.4b**).

Myenteric plexus grafts were well vascularized. The blood vessels were surrounded by a variety of cells; fibroblast processes were most often observed around the blood vessels, although smooth muscle cells, neuronal processes and enteric glial cells associated with axons were also observed. Enteric glial cells did not exhibit caveolae at the cell membrane or form perivascular end feet (compare with Lawrence *et al.* 1991).

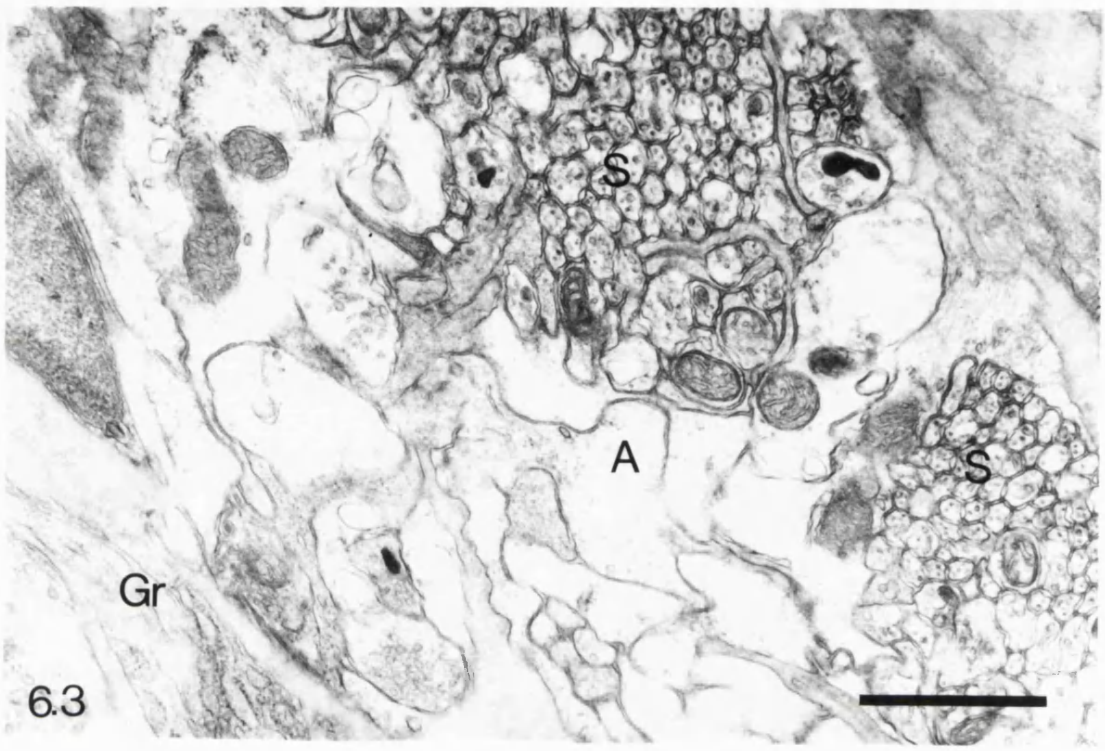
By 3 weeks after implantation, the glia limitans had developed to form an incomplete boundary between the graft and the brain. In some areas, the glia limitans was well formed and comprised up to 10 layers of glial processes covered by a layer of basal lamina which formed the final barrier between the brain and the graft. In other areas, the interface was more complex and composed of fewer glial

Fig. 6.3. Electron micrograph of the interface between grafted enteric plexus and normal adult rat corpus striatum, examined 6 weeks after implantation. Two bundles of small non-myelinated axons (S) can be seen, close to the astrocyte processes (A) which form the interface with the graft (Gr). Scale bar = 1μm.

Fig. 6.4a. A low power electron micrograph of part of a myenteric plexus graft, examined 3 weeks after implantation into unlesioned adult rat corpus striatum. A large bundle of axonal profiles can be seen; the left side (b) displays typical ENS morphology, while the right side (c) appears to contain CNS regenerating sprouts. Scale bar = 2μm.

Fig. 6.4b. High power electron micrograph of the area indicated in **Fig. 6.4a.** Many of the neurite profiles are irregularly shaped, containing aggregates of small agranular and dense-cored vesicles (arrows). The bundle is covered by basal lamina (arrowhead). Scale bar = 500nm.

Fig. 6.4c. High power electron micrograph of the area indicated in **Fig. 6.4a.** The majority of axonal profiles (S) are small, rounded and contain microtubules, neurofilaments and mitochondria, although one contains synaptic vesicles (arrow). This side of the bundle is also covered in basal lamina (arrowhead). Scale bar = 500nm.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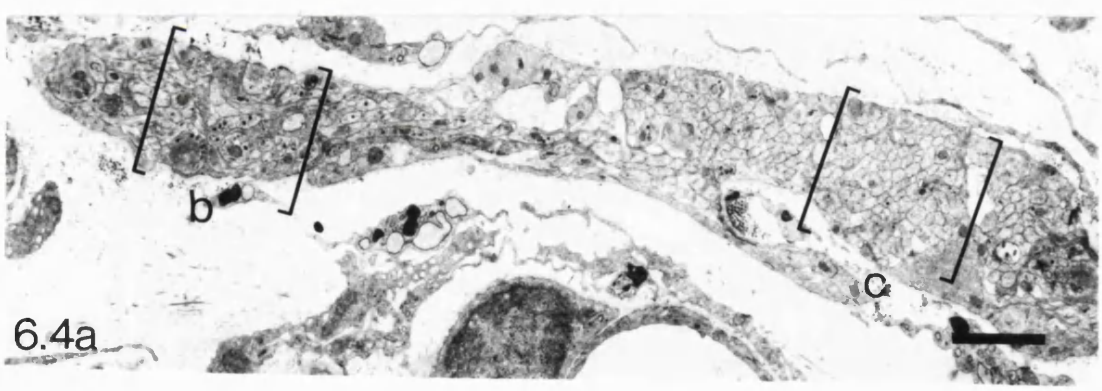
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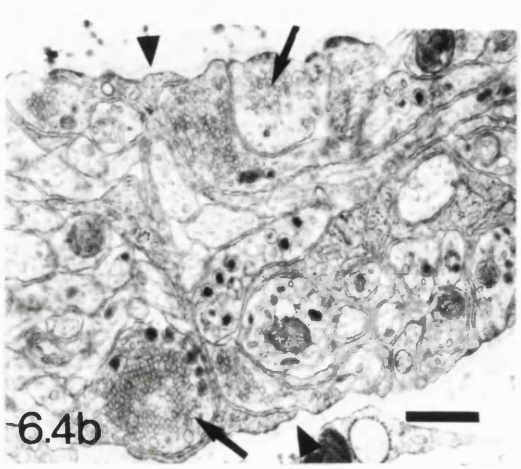
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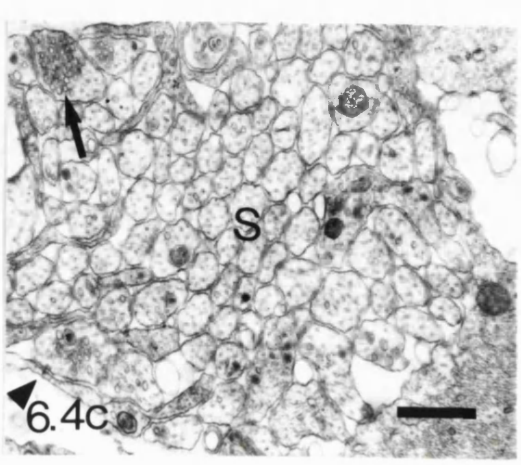
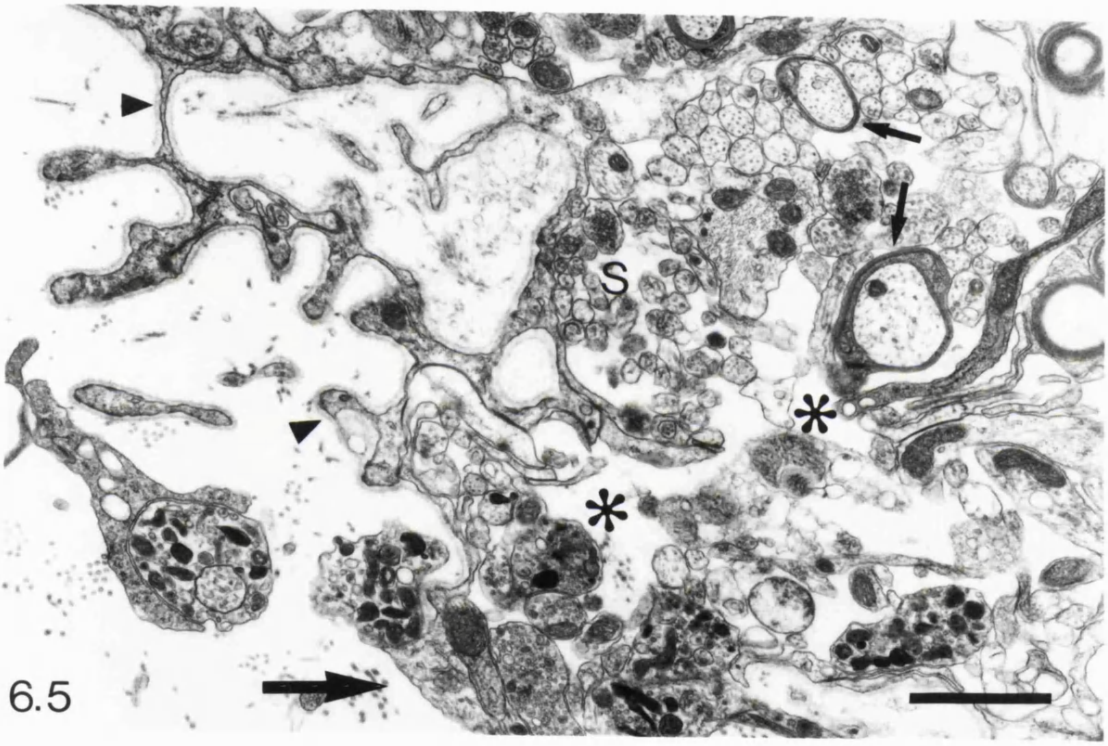

S
6.4c

processes often with no covering layer of basal lamina. In most of the grafts, some enteric ganglia were observed very close to or adjoining the CNS with no intervening non-neural cells or collagen fibrils (**Fig. 6.2**). Where this occurred, the basal lamina covering the enteric ganglia at the area of contact became patchy and although astrocyte processes were present, they also lacked a basal lamina (**Fig. 6.2**). When quantitatively assessed (using Seescan image analysis equipment), basal lamina was found to be absent in 63% to 76% of these closely apposed interface regions (see table 1), suggesting that in these areas there is no barrier between the CNS and the enteric neuronal grafts.

The corpus striatum surrounding the grafts of myenteric plexus contained bundles of regularly arranged small diameter axons, often devoid of organelles other than one or two microtubules (**Fig. 6.3**). These regularly arranged, small non-myelinated axons are thought to be CNS axonal regenerating sprouts (G.Campbell *et al.* 1992, see also chapter 3). Putative CNS axonal regenerating sprouts were observed within the brain, both at the glia limitans and deeper in the surrounding striatum, within 100μm of the interface (**Fig. 6.3**).

Bundles of CNS sprouts were also observed within the graft. These occurred both as simple bundles of similar small diameter fibres partially enclosed by glial processes and basal lamina, and as components of more complex bundles (**Fig. 6.4a** and **6.4c**) which also contained axons displaying the attributes of typical ENS fibres, namely a variety of differently shaped neuronal processes containing small agranular and dense cored vesicles in variable amounts (**Fig. 6.4b**). In some animals (examined both 3 and 6 weeks after implantation) oligodendrocyte-myelinated axons were observed inside some bundles.

Fig. 6.5. Electron micrograph of the interface between an enteric plexus graft and the quinolinic acid lesioned striatum, examined 3 weeks after implantation. The interface is composed of fine astrocyte processes covered in basal lamina (arrowheads), although in one area of the interface, CNS axonal profiles can be seen protruding into the graft (large arrow) without any glial cell or basal lamina covering. A number of small diameter unmyelinated axons (S) can be seen within the brain near the interface. The striatal neuropil near the graft is very loosely packed, with large extracellular spaces (asterisks) and contains some thinly myelinated axons (arrows). Scale bar = 1μm.



6.5

6.4.2. **Grafts in QA lesioned corpus striatum.**

There was little difference in the distribution or morphology of enteric ganglia and associated cells implanted in QA lesioned striata compared to those implanted in unlesioned striata. However, there was a major difference in the appearance of the surrounding striatum.

The QA lesioned striata of animals that did not receive a subsequent myenteric plexus graft were characterized by a distinct lack of neuronal cell bodies as has been previously reported (Schwarcz *et al.* 1983, DiFiglia 1990). Thus, the lesioned zone was composed of large bundles of oligodendrocyte-myelinated fibres, some of which contained disrupted myelin sheaths and areas of tightly packed neuropil which was composed of neurite and glial profiles and contained few synapses. Oligodendrocyte and astrocyte cell bodies were also observed.

In animals where myenteric ganglia had been implanted in QA lesioned striatum the area immediately surrounding the grafts and up to 150μm away from the interface, was composed of small neurites and oligodendrocyte myelinated axons, many of which were enwrapped by comparatively few layers of myelin, suggesting that they may have been newly myelinated (**Fig. 6.5**). In the area close to the interface, glial processes were less common than on the unlesioned striata (**Fig. 6.5**) and there was an increase in the volume of extracellular space. A number of small non-myelinated axons resembling CNS axonal sprouts were observed at and near the interface, although comparatively fewer than were seen in the unlesioned striatum surrounding myenteric plexus grafts (compare **Fig. 6.5** and **6.3**). A fine layer of glial processes covered in basal lamina was often observed at the interface between graft and brain, although enteric ganglia were observed in

direct apposition with CNS glia in some regions of the interface (See above).

TABLE ONE.

Ganglia abutting onto CNS parenchyma: percentage of interface covered by basal lamina (ie: regions of direct cellular contact between enteric ganglia and CNS tissue).

	LT161	LT180	LT160	LT57
Length of interface examined (μM).	128	85	89	33
Length of interface lacking basal lamina (μM).	85	54	58	25
	66%	63%	65%	76%

6.5. Discussion.

In this study, we have demonstrated that the neurons and glia of the enteric ganglia taken from young donors and dissected free of smooth muscle, are capable of surviving for at least 6 weeks in the adult corpus striatum. There was little apparent difference in the morphology of grafts implanted in the excitotoxin-lesioned or unlesioned environment. CNS sprouts were observed in and around the grafts in both lesioned and unlesioned striata, although sprouts appeared to be less abundant in the lesioned striata. While sprouts were observed in tightly packed bundles enclosed by astrocyte processes in the unlesioned striata, this was not their appearance in the lesioned striata, perhaps because of the increased volume of extracellular space and the reduction in the number of glial cells observed in the lesioned striatum and near the interface between graft and surrounding brain.

The grafted cells maintained many of the morphological features identified in enteric ganglia in the gut, but it was noticeable that, unlike in the gut, the grafted enteric ganglionic neuropil contained some degenerating profiles and empty spaces presumably representing completely degenerated neurites. This may have been the result of degeneration of axons from cell bodies outside the area dissected, or of the retraction of axons that had been remodelled following grafting. Similar spaces in the ganglionic neuropil have been described in the neuropil of cultured myenteric plexus (Baluk *et al.* 1983) and in the enteric ganglia of muscularis externa grafts implanted in the CNS (chapter 3).

Blood vessels were found throughout the grafts, but were not contacted by enteric glia cells. This is in contrast to previous studies in which similar enteric ganglia were implanted into the

denervated hippocampus (Lawrence *et al.* 1991). One explanation for this apparent discrepancy may be that the environments within the hippocampus and striatum are different. Alternatively, since the growth of axons into and out from the grafts was not reported by Lawrence *et al.* 1991), but was evident in the present study and in our previous investigations of enteric ganglia grafted into the striatum (see chapter 3 and chapter 5), it might be envisaged that many of the transplanted enteric glial cells in our experiments were involved in providing support for axons and were thus unavailable to carry out an astrocyte-like role around blood vessels. It is interesting to note that in the present study, enteric glial cells were not observed to have caveolae at the cell surface, a characteristic of perivascular enteric glia in grafts of myenteric plexus in the hippocampus (Lawrence *et al.* 1991). It is possible that caveolae may be a characteristic of enteric glia lacking axonal contact.

The extent to which enteric ganglia fused with the CNS tissue varied, such that in some areas of the grafts it was difficult to define a clear boundary between the graft and brain but in others there were regions where a well defined glia limitans had developed around the grafts. Young and adult enteric ganglia and foetal CNS tissue grafted into the striatum and other CNS areas also cause the production, to a variable extent, of glial limiting membranes marking the interface between graft and brain (Sievers *et al.* 1985, Krüger *et al.* 1986, Lawrence *et al.* 1991, Jaeger *et al.* 1993, and chapter 3). In a detailed study of foetal cerebellum and cortex implanted into the adult superior colliculus (Krüger *et al.* 1986), three types of interface were observed, the most intimate (no glial limiting

membrane and elements of the CNS and graft being "connected") occurring at the site of initial graft placement. As the presence of a glial limiting membrane is thought to prevent the penetration of axons and non-neural cells into CNS tissue (Anderson and Turmaine 1986, Anderson *et al.* 1989, Blakemore 1980), it is probable that the partial fusion of the enteric plexus with the CNS aids the outgrowth of axons from grafts into the brain (as described in chapter 5) and the ingrowth of CNS axonal sprouts into the grafts (as described in chapter 3).

The absence of basal lamina in areas of direct apposition is interesting since a basal lamina covering is a characteristic of both the surface of enteric ganglia (Gabella 1972) and astrocytes at the glia limitans (Peters *et al.* 1976). Immunohistochemical studies in the gut have demonstrated that basal lamina surrounding enteric ganglia is composed of laminin, type IV collagen, nidogen, heparan sulphate proteoglycan and probably fibronectin (Bannerman *et al.* 1986). These molecules are not normally observed within the ganglia (Bannerman *et al.* 1986). However, both laminin and type IV collagen are expressed by enteric glia when ganglia are grown in culture (Bannerman *et al.* 1988a) or implanted into the hippocampus (Lawrence *et al.* 1991). This would suggest that enteric glia produce at least some of the molecules which make up basal lamina, but for the production of a complete basal lamina, neural cells appear to require interactions with mesenchymal cells such as fibroblasts (Bunge *et al.* 1982, Obremski *et al.* 1993). Such interactions are obviously absent where enteric glia are in direct contact with CNS glia.

The striatum surrounding the grafts of enteric ganglia contained a number of bundles of small non-myelinated axons which were not observed in sections of striatum taken from the

contralateral side. The axons in these bundles appeared identical to profiles, identified as CNS sprouts, observed in the adult thalamus after implantation of a peripheral nerve (G.Campbell *et al.* 1992) and in the striatum after implantation of muscularis externa grafts (see chapter 3). There appeared to be fewer sprouts around the grafts of freshly dissected myenteric plexus than around grafts of muscularis externa (myenteric plexus and surrounding smooth muscle) in the study described in chapter 3. This may have been due to a number of factors. 1. Muscularis externa grafts were larger and thus axotomized more striatal neurons when implanted; this seems unlikely to be a complete explanation of the degree of sprouting, since grafts of freeze-killed muscularis externa produced relatively little axonal sprouting in the striatum (see chapter 3). 2. The muscularis externa grafts were taken from older donors and the myenteric plexus they contained may have different properties from the plexus implanted in the present study. While much of the development of the myenteric plexus appears to take place before birth, morphological studies have shown that between birth and adulthood (6 months), there is an increase in the number and size of myenteric neurons within the rat gut (Gabella 1971b). It is impossible to estimate at what age the plexus becomes mature, and thus whether enteric neurons in the present study (16-20 day) had different properties to those in the adult gut. 3. The muscularis externa grafts contained smooth muscle which may have produced sprout-inducing factors: this last possibility will be addressed in chapter 7 using intrastriatal grafts of colonic smooth muscle.

The QA lesioned striatum surrounding the grafts also contained a small proportion of similar axons. Following a QA lesion, the rat

striatal neuropil has been demonstrated to contain clusters of very small spines which receive asymmetric synapses (Roberts and DiFiglia 1990b), and have been described as regenerating sprouts. However, the putative regenerating sprouts observed in the lesioned striata surrounding grafts of enteric ganglia were far more frequent, were not associated with asymmetric synapses and were morphologically similar to putative CNS axonal sprouts observed in the unlesioned striatum surrounding grafts of myenteric plexus and muscularis externa (chapter 3). The observation that more sprouts were observed in the unlesioned striatum surrounding a myenteric plexus graft than in the QA lesioned striatum would suggest that both intrinsic striatal neurons and afferents to the striatum are capable of a regenerative sprouting response to the local injury caused by graft placement, but without immunohistochemical investigations or axonal tracing studies, it is impossible to determine which neurons/axons are involved.

As in chapter 3, bundles of putative CNS sprouts were found to have grown into grafts of enteric plexus, and some contained larger myelinated axons. The myelin was of CNS type, demonstrating that the axons had been myelinated by oligodendrocytes. Axons of rat enteric neurons are not myelinated (Cook and Burnstock 1976, Gabella 1979) and thus the oligodendrocyte myelinated axons are believed to represent sprouts of CNS myelinated axons which have matured within the grafts. The ingrowth of myelinated fibres has also been reported in grafts of adult myenteric plexus with surrounding smooth muscle implanted into the spinal cord (Jaeger *et al.* 1993) or adjacent to a severed peripheral nerve in the leg (Anderson and Turmaine 1987). In these grafts, the axons were all Schwann cell myelinated, while in present report, only oligodendrocyte-myelinated axons were observed.

This difference in the type of myelination may be due to the different sites of implantation; it is known that Schwann cells are able to invade the spinal cord if the glia limitans is not intact (as occurs after graft placement; Blakemore 1979). The absence of Schwann cell myelinated axons in myenteric plexus grafted into the CNS striatum may indicate that Schwann cells have more difficulty in penetrating the striatum. Alternatively, the method of implantation into the spinal cord may have caused damage to the oligodendrocytes, so reducing the number capable of remyelinating ingrowing axons.

This investigation has demonstrated that grafts of freshly dissected myenteric plexus are capable of surviving in the QA lesioned and unlesioned striatum for at least 6 weeks and fusing with the surrounding brain tissue. By using isolated ganglia, we have been able to implant many more neurons than were implanted in our previous study utilizing the muscularis externa containing the myenteric plexus (chapter 3). The extent to which the enteric neurons make functional connections with the host brain remains unclear, however the presence of enteric transplants appears to promote growth of both unmyelinated and myelinated axons in the adjacent regions of the CNS.

CHAPTER 7.

INTRASTRIATAL IMPLANTATION OF RAT COLONIC SMOOTH MUSCLE.

7.1. Summary.

Grafts of living or freeze-killed freshly dissected colonic smooth muscle from young inbred Fischer rats were implanted into the corpus striatum of adult Fischer rats. Sections of brain were examined electron microscopically three and six weeks after implantation. At both times, living grafts were vascularized and contained healthy differentiated smooth muscle cells, fibroblasts and some macrophages. Large bundles of CNS axonal sprouts could be observed in the brain at and near the interface between the living smooth muscle and the CNS tissue and bundles of regenerating CNS axons, often associated with astrocyte processes, had grown into the grafts. Some axons within the grafts had matured, enlarged and become myelinated by oligodendrocyte processes or Schwann cells. In some cases, smooth muscle cells were observed in intricate glial-like associations with axons.

In contrast to the living grafts, grafts of freeze-killed smooth muscle, examined three and six weeks after implantation, contained macrophages, fibroblasts, collagen and large amounts of cellular debris, but no living muscle cells, astrocytes or Schwann cells. The striatal neuropil around freeze-killed grafts did not contain large bundles of CNS axonal sprouts and bundles of axons were not observed within the freeze-killed graft.

This study demonstrates that non-neural material is capable of stimulating a vigorous regenerative response from CNS axons, after implantation in the corpus striatum. This response involves CNS glia and Schwann cells and it is unclear whether the grafts of living smooth muscle affect injured axons directly or via interactions with these cells.

7.2. Introduction.

Although the regenerative capacity of axotomized adult mammalian CNS neurons is very limited, injuries to the brain, spinal cord or optic nerve can stimulate the formation of variable numbers of axonal sprouts (Ramón Y Cajal 1928, Povlishock and Becker 1985, Harvey *et al.* 1986, Hall and Berry 1989). In the absence of further intervention, regeneration is abortive and the axonal sprouts may disappear (Zeng *et al.* 1991), however if a piece of peripheral nerve is implanted into the CNS, they are capable of considerable elongation through the Schwann cell columns where the regenerating CNS axons increase in diameter and become myelinated (Aguayo 1985, G.Campbell *et al.* 1992).

In the preceding experiments, grafts of myenteric plexus implanted into the corpus striatum of adult rats, have been examined in order to examine the enteric nervous system as a source of tissue for intracerebral implantation in models of neurodegenerative diseases (see chapters 3 to 6). From these investigations, it is evident that enteric neurons implanted together with the surrounding smooth muscle are able to survive in the corpus striatum for at least six weeks and stimulate a vigorous sprouting response in the surrounding striatum (see chapter 3). These CNS sprouts invaded the myenteric plexus and surrounding smooth muscle and some became enlarged and myelinated by oligodendrocyte processes. Similar grafts of myenteric ganglia separated from the surrounding muscle layers survived for at least six weeks in the corpus striatum and stimulated the production of a smaller number of CNS sprouts, some of which extended into the graft, matured and became myelinated (see chapter 6).

Therefore, it is of great interest to determine the effects of

grafting freshly dissected intestinal smooth muscle into the corpus striatum of the adult rat. The grafts and surrounding brain were examined electron microscopically to determine whether the isolated muscle survived and observe the reaction of the surrounding striatum. Of particular interest was whether the muscle would provoke a sprouting response from the injured CNS axons, and whether axonal sprouts would grow into the grafts.

7.3. Materials and Methods.

Grafts of living and freeze-killed smooth muscle were obtained as described in section 2.7 and operative procedures were performed as described in section 2.2. After three and six weeks, animals were anaesthetised and transcardially perfused (see section 2.8), and vibrotome sections were processed for electron microscopy (EM: see section 2.8)

7.4. Results.

All grafts were located in the corpus striatum. All 0.5μm toludine blue-stained sections and ultrathin sections of the living grafts were examined for the presence of any enteric ganglia left during the dissection process. Of the 10 living grafts examined at both three and six weeks after implantation, three were found to contain a small fragment of enteric plexus and so were not included in the recorded observations.

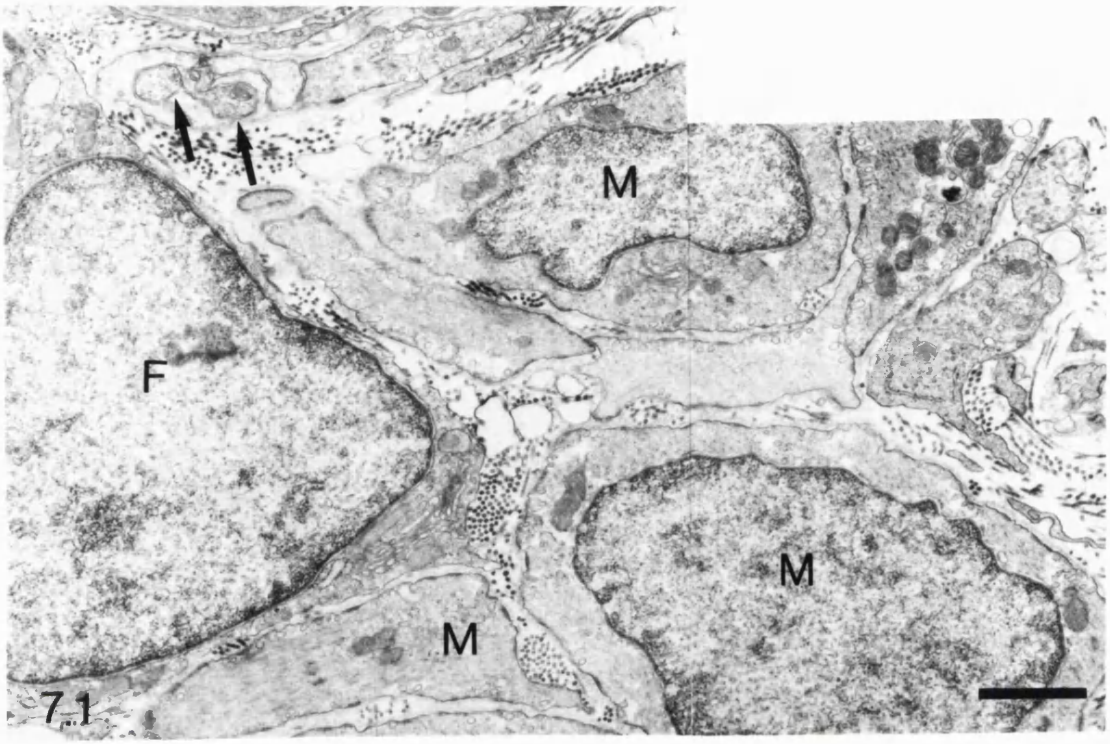
7.4.1. Grafts of living tissue.

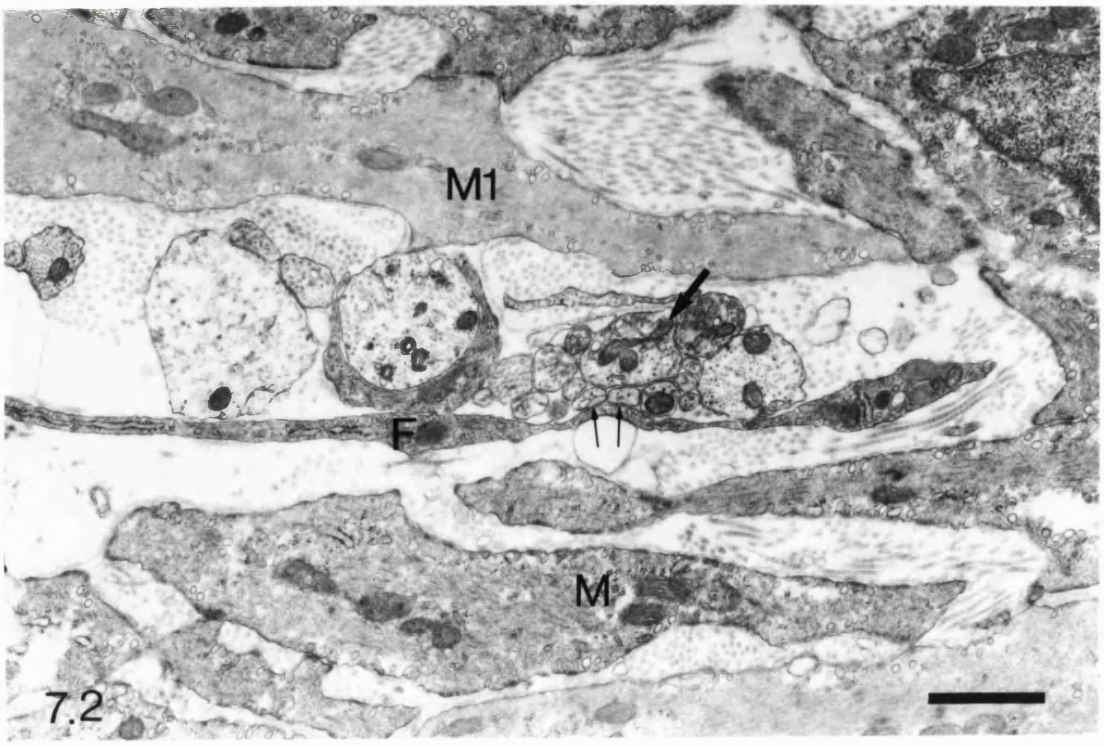
There was little difference between the grafts examined three weeks and six weeks after implantation. The major constituent of the grafts was smooth muscle. The muscle cells displayed all the characteristic morphological features which they exhibit in the gut wall (Gabella 1981b), including actin filaments and dense bodies within the cytoplasm, caveolae at the cell surface with dense bodies evenly spaced in between them (**Fig. 7.1**). Within the grafts there were also a small percentage of muscle cells that contained fewer cytoplasmic filaments and dense bodies, but these cells were not found in any specific area of the graft and were often observed close to healthy muscle cells (**Fig. 7.2**). Grafts also contained fibroblasts, macrophages, collagen and some cellular debris. All the grafts examined were well vascularized, the blood vessels being surrounded by smooth muscle cells, collagen and fibroblast processes.

The glia limitans between graft and brain was well formed at both three and six weeks after implantation, being composed of well defined astrocyte processes, covered in a basal lamina in most areas (**Fig. 7.3a** and **7.3b**). However areas of the interface where grafted smooth muscle cells were closely apposed to astrocyte processes without intervening basal lamina were also observed.

Fig. 7.1. Electron micrograph of an intrastriatal graft of colonic smooth muscle, examined 6 weeks after implantation. Collagen fibrils, a fibroblast (F) and some axons (arrows) associated with glial cells can be identified between the myocytes (M), which appear healthy. Scale bar = 1μm .

Fig. 7.2. Electron micrograph of a smooth muscle graft 6 weeks after implantation into the striatum. A pale muscle cell (M1), with less distinct cytoplasmic filaments, can be identified beside the more characteristic cells packed with filaments (M). A bundle of axons can be seen, some of which (small arrows) are in contact with a fibroblast processes (F) while others (arrows) are in contact with the inner aspect of the basal lamina of the pale muscle cell. Scale bar = 1μm.


M
F
M
M
7.1


M1
F
M
7.2

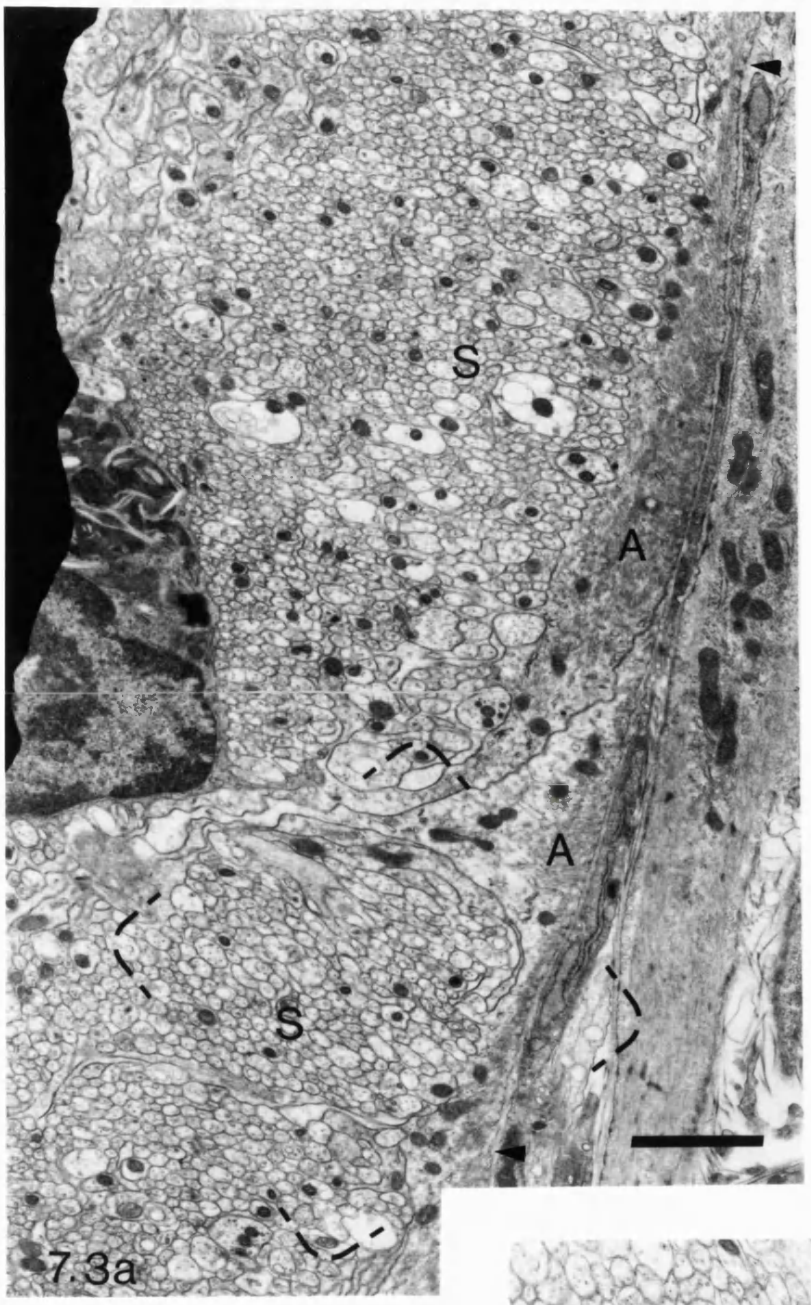
Many CNS sprouts were observed within the striatal neuropil near to and at the interface between brain and graft (**Fig. 7.3a** and **7.3b**). The number of sprouts observed around the grafts varied but did not seem to be related to graft survival time. These sprouts appeared as large bundles of small non-myelinated axons in a regular, almost crystalline array and were found in groups, surrounded by astrocyte processes (**Fig. 7.3a** and **7.3b**) or indented into astrocyte or oligodendrocyte cell bodies. These axonal sprouts had a similar appearance to regenerative CNS sprouts described in the thalamus around peripheral nerve grafts (G.Campbell *et al.* 1992) and around muscularis externa grafts in the corpus striatum (see chapter 3). It was noticeable that many sprouts were found close to blood vessels, as has previously been reported around peripheral nerve grafts in thalamus (G.Campbell *et al.* 1992).

Axons were found within all the grafts examined (**Fig. 7.1, 7.2, 7.4, 7.5, 7.6** and **7.7**). Some were present in bundles, often surrounded by glial processes and resembled the CNS axonal sprouts in the surrounding brain (**Fig. 7.4.**). Axons were also observed lacking glial contact (**Fig. 7.2**) and in contact with smooth cells (**Fig. 7.7**). In many bundles some of the axons were noticeably larger than others and had become myelinated by oligodendrocytes (**Fig. 7.4, 7.5** and **7.6.**). Although astrocyte processes were observed within the grafts, no astrocyte cell bodies were found to have migrated into the grafts.

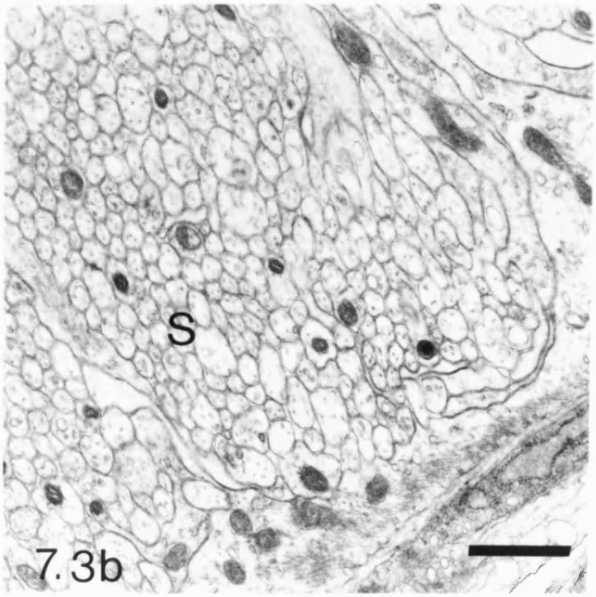
In two of the grafts examined there were examples of large diameter axons which were myelinated by Schwann cells (**Fig. 7.5** and **7.6.**). These Schwann cell-myelinated axons were not diffusely distributed, but occurred in discrete areas within the graft which also contained oligodendrocyte myelinated axons (**Fig. 7.5** and **7.6**).

Fig. 7.3a. Electron micrograph of interface between a colonic smooth muscle graft and the surrounding striatum, examined 6 weeks after implantation. The glia limitans is complete in this area of the graft, being composed of astrocyte processes (A) covered in a basal lamina (arrowheads). Two massive bundles of small non-myelinated axons (S) can be seen, on the CNS side of the glia limitans. Scale bar = 2μm.

Fig. 7.3b. High power electron micrograph of **Fig 7.3a.** The small diameter axonal sprouts (S) contain one or more microtubules and occasional mitochondria, neurofilaments and vesicles. Scale bar = 1μm.



7.3a



7.3b

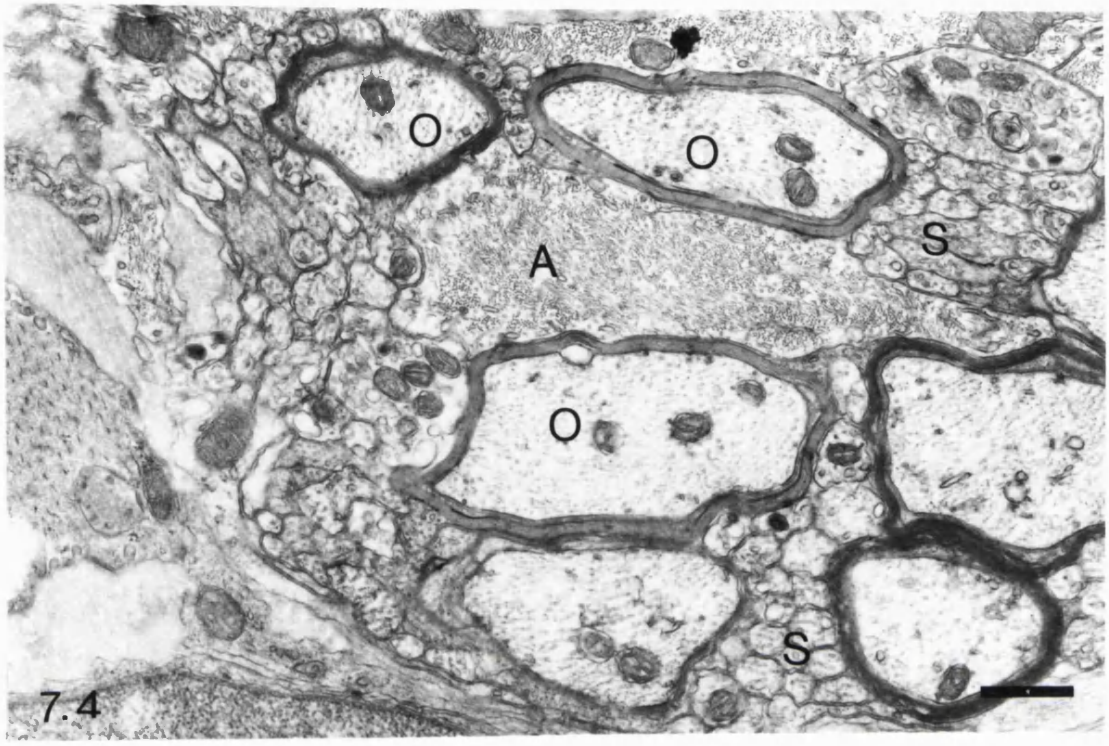
Large diameter non-myelinated axons were sometimes associated with glial cells whose identity it was difficult to discern (**Fig. 7.6**).

7.4.2. **Freeze-killed grafts.**

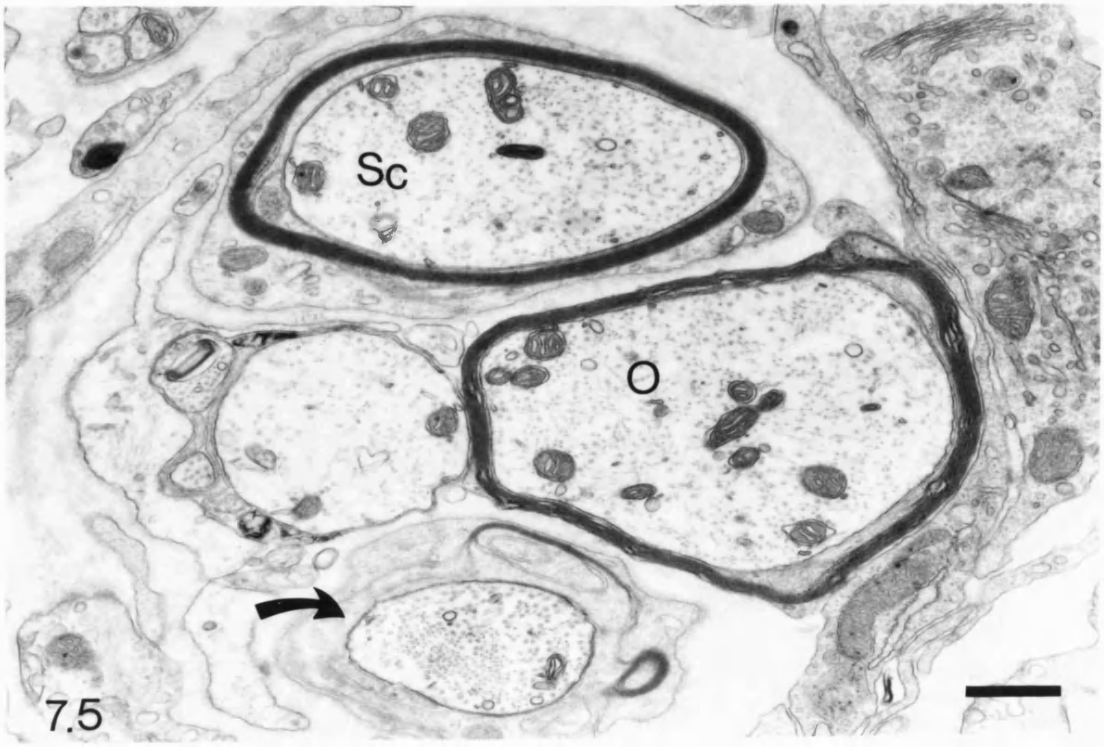
Grafts of freeze-killed smooth muscle cells were examined three and six weeks after implantation into the striatum and contained macrophages, fibroblasts, collagen and large areas of cellular debris and extracellular space. The striatum around the grafts contained many astrocyte processes and degenerating profiles, and although a number of small non-myelinated axonal profiles were observed, these were not observed in characteristic bundles and were much less numerous than around living grafts (compare **Fig. 7.3a** and **7.8**). A number of astrocyte processes were observed within the graft, but there were no examples of any axonal profiles within the freeze-killed grafts.

Fig.7.4. High power electron micrograph of a bundle of axons within a colonic smooth muscle graft, 3 weeks after implantation. The bundle contains both small non-myelinated axonal sprouts (S) and larger axons that have become myelinated by oligodendrocyte processes (O). Fibrous astrocyte processes (A) can be observed in the centre of the bundle and enclosing parts of the bundle. Scale bar = 500nm.

Fig. 7.5. High power electron micrograph of axons within a smooth muscle graft, 6 weeks after implantation. A Schwann cell myelinated axon (Sc) and an axon that appears to be myelinated by an oligodendrocyte process (O) can be seen. The third smaller axon is surrounded by disrupted myelin (curved arrow). Scale bar = 500nm.



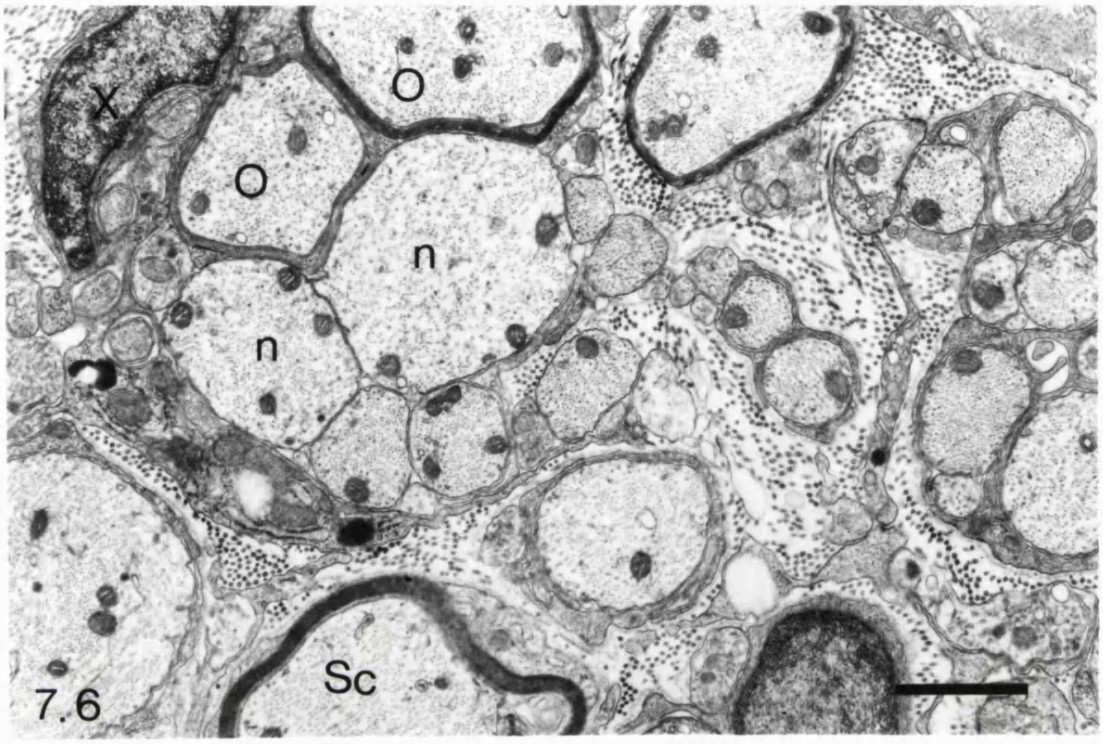
7.4



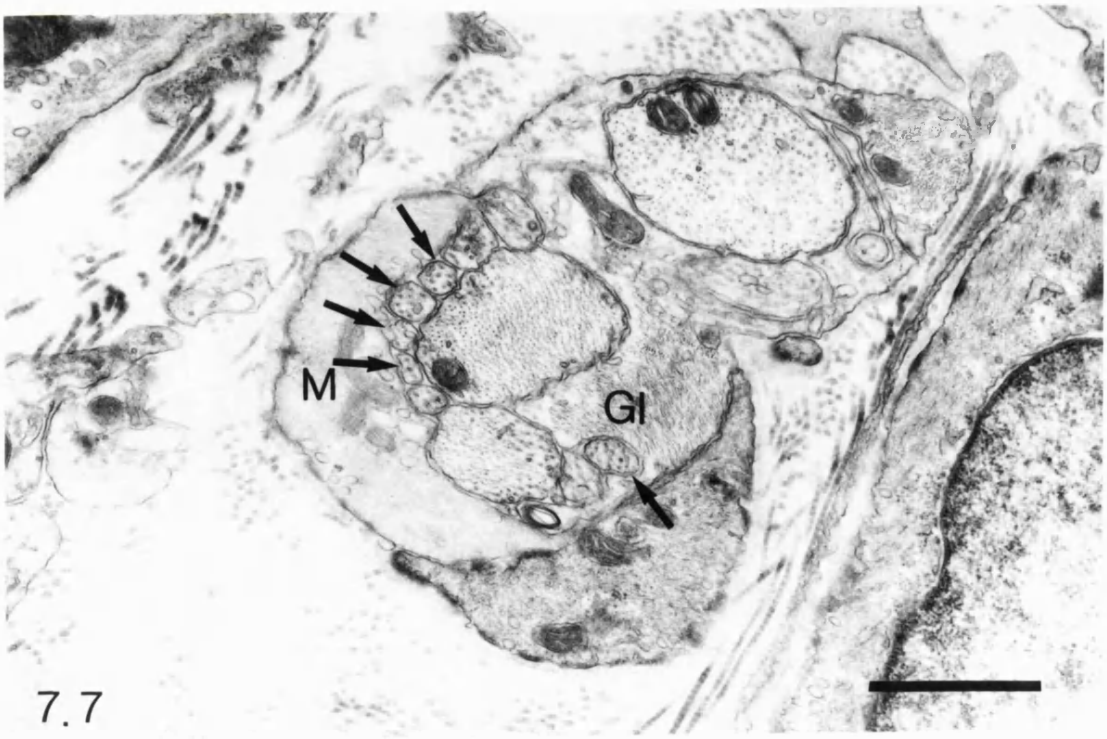
7.5

Fig. 7.6. Electron micrograph of bundle of axons, some of which are myelinated by oligodendrocyte (O) and Schwann cell (Sc) processes. A number of large diameter non-myelinated axons (n) can be seen, associated with a glial cell that can not be identified (X). Scale bar = 500nm.

Fig. 7.7. Electron micrograph of bundles of axons within a smooth muscle graft, examined 6 weeks after implantation. Axons (arrows) can be seen in association with a glial cell processes (Gl) and a pale smooth muscle cell (M). Scale bar = 1µm.

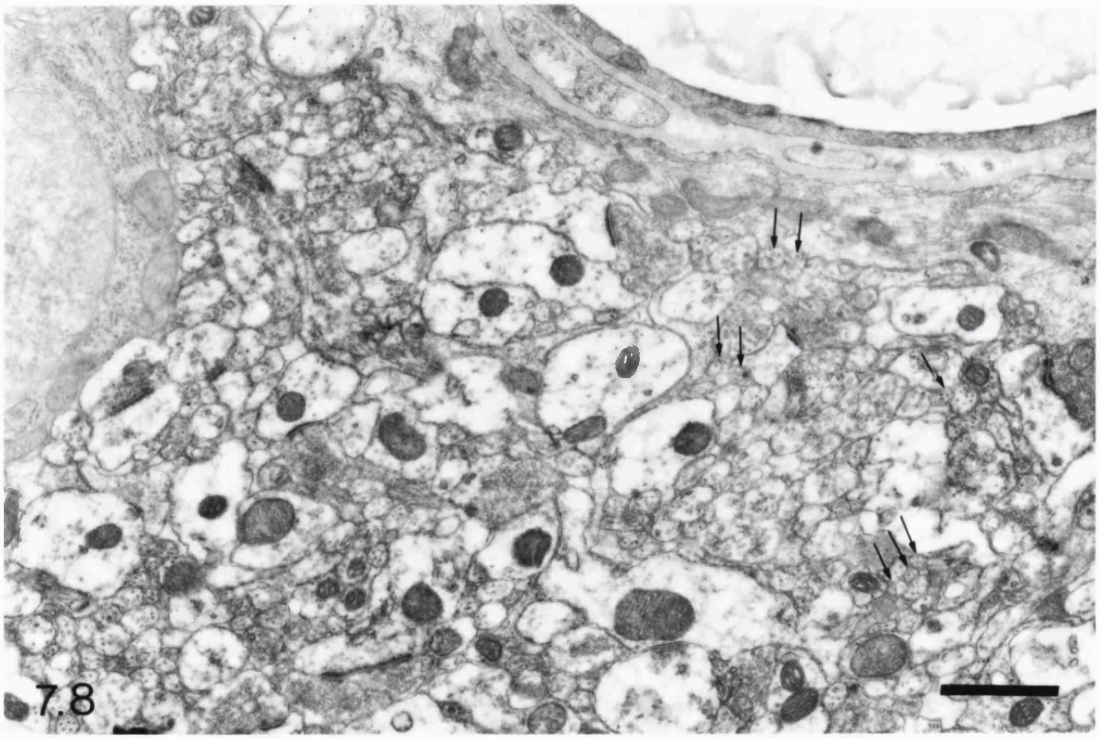


7.6



7.7

Fig. 7.8. Electron micrograph of the interface region between brain and graft of freeze-killed colonic smooth muscle. The striatal neuropil contains a number of small diameter non-myelinated axons (eg: small arrows), but they are not present in the characteristic bundles observed around grafts of living smooth muscle. Scale bar = 1μm.


7.8

7.5. Discussion.

In this report, we have demonstrated that freshly dissected, denervated smooth muscle taken from the colon of young rats is capable of surviving in the corpus striatum of adult rats for six weeks. A vigorous axonal sprouting response was observed in the brain around the graft, and many CNS axonal sprouts were seen to have extended into the graft, some maturing in contact with glial cells and becoming myelinated by oligodendrocyte processes or Schwann cells. The sprouting response in the striatum following the implantation of freeze-killed smooth muscle was greatly reduced and no axons invaded the grafts.

There are a number of reasons for believing the axonal profiles observed in the striatum around grafts of living smooth muscle are regenerating CNS axonal sprouts. Peripheral axons growing into CNS tissue do not appear as bundles of small non-myelinated axons (Anderson and Turmaine 1986, Anderson *et al.* 1989) and bundles of small non-myelinated axonal profiles arranged in a crystalline array (similar to the axons seen around smooth muscle grafts) are not observed in the intact striatum (see chapter 3 and 6) or in the striatum around grafts of freeze-killed smooth muscle cells (present results). The small non-myelinated axonal profiles observed in this study however, are similar to axons observed in the developing CNS (Henrikson and Vaughn 1974), and to regenerating CNS axonal sprouts observed in the transected optic nerve (Hall and Berry 1989, Zeng *et al.* 1991), the optic tract (Harvey *et al.* 1986) and the thalamus after implantation of peripheral nerve (G.Campbell *et al.* 1990, 1992).

The origin of axons within the grafts of living smooth muscle

is more difficult to determine. Many of the axons within the graft were morphologically similar to the CNS regenerative sprouts observed in the striatum around the grafts of living smooth muscle. Although it is possible that some of the other, larger diameter axons were derived from rogue enteric neurons, this is unlikely as grafts were carefully examined to exclude any that contained enteric ganglia (see sections 2.7 and 7.4), and the large diameter axons observed in this study contained few transmitter vesicles, unlike the enlarged axonal varicosities of enteric neurons (Cook and Burnstock 1976, Gabella 1972). The presence of Schwann cells and Schwann cell myelinated axons in the grafts may indicate that peripheral axons have invaded some smooth muscle grafts, as the Schwann cells probably originated from perivascular nerves which could also be a potential source of axons. It is also possible that the Schwann cells which were observed myelinating axons in some of the smooth muscle grafts could be residual cells, normally residing in the muscularis externa, associated with extrinsic sympathetic and parasympathetic nerves. However, Schwann cell myelinated axons were only observed in 2 of the 8 grafts studied, and so this may represent an anomalous situation. Thus it can be assumed that at least some of the axons observed within grafts of living smooth muscle are of CNS origin.

The phenomenon of CNS regenerative sprouting has been described after injury to the optic tract (Harvey *et al.* 1986) and optic nerve (Hall and Berry 1989, Zeng *et al.* 1991) and after the implantation of a number of different tissues (G.Campbell *et al.* 1992 and see chapters 3 and 6). It would appear that grafts of living colonic smooth muscle provoke a greater sprouting response than grafts of isolated enteric ganglia (see chapter 6) or grafts of muscularis

externa (myenteric plexus surrounded by smooth muscle: see chapter 3). However grafts of freeze-killed smooth muscle are unable to stimulate such a vigorous sprouting response, thus suggesting that a soluble substance derived from live smooth muscle cells affects axons within the brain. Although it is tempting to speculate on the production of a neurotrophic factor or cytokine by muscle cells, nothing is known of the neurotrophic factors produced by colonic smooth muscle, or of the identity and neurotrophic requirements of the CNS neurons whose axons grow into the grafts.

Recent investigations into the short-term effect of colonic myenteric plexus and smooth muscle on striatal neurons has shown that dedifferentiated smooth muscle (taken from 2-4 day old guinea pig, and cultured for 10 days) has no neurite-promoting effect on cultured neonatal striatal neurons (taken from 7 day old rat: Höpker *et al.* 1994). This apparently conflicting result may be due to a number of factors. For example, the culture experiments were short-term (24-48 hour) and utilized tissues of different ages from those investigated in the present report. It is also possible that afferents to the striatum or axons *en passage* formed regenerative sprouts in the striatum after implantation of smooth muscle grafts, populations of neurons not investigated in the culture experiments (Höpker *et al.* 1994). Another major difference between the two experimental paradigms is that smooth muscle cells used in the co-culture experiments were de-differentiated (Höpker *et al.* 1994); it is likely that the different phenotypes of smooth muscle cell produce different chemicals.

Previous experiments utilizing grafts of smooth muscle (including pioneering work in the early 1970's) demonstrated that when grafts were implanted so as to lesion the medial forebrain

bundle (the tract that carries dopaminergic axons from the substantia nigra) catecholaminergic axons invaded the grafts (Björklund and Stenevi 1971). The extent of innervation depended on the type of graft; fibres only grew into the periphery of tissue which was normally "lightly" innervated (eg: uterus), while tissue that normally received a heavier innervation (eg: iris) were invaded by fibres to a greater extent (Björklund and Stenevi 1971). It is not clear whether this reflects increased trophic support from heavily innervated smooth muscle or the presence of a greater number of Schwann cells in the tissue. Central axons have also been reported to grow into grafts of other peripheral tissue such as skeletal muscle (Heinicke 1980, Anderson *et al.* 1988) and skin (Heinicke and Kiernan 1978, Heinicke 1980), where the extent of innervation has been correlated with the presence of extravascular protein (Heinicke 1980).

Axons which invaded smooth muscle cell grafts were associated with a variety of cells. Although invading axons were also observed in contact with astrocyte processes and Schwann cells, a number were observed lacking cellular contact, while other axons were fasciculated, growing on other axons, or associated with fibroblasts. In previous investigations of grafts of muscularis externa (comprising myenteric plexus with surrounding smooth muscle: see chapter 3), axons were observed to invade the graft in contact with astrocytes or enteric glia. A detailed study of the invasion of central fibres into peripheral nerve grafts in the thalamus has demonstrated that CNS axons initially invade the peripheral nerve graft unaccompanied by astrocytes/glia, but once in the graft become quickly associated with Schwann cells and continue to elongate and

mature in association with Schwann cells columns (G.Campbell *et al.* 1992). The ability of axons, at least in some instances, to grow with no glial covering within smooth muscle grafts is of interest as it suggests that CNS axonal sprouts which invade grafts of smooth muscle, muscularis externa or peripheral nerve will grow on glial cells if present, but are also capable of elongating on other cells, or without any cellular contact. This is in marked contrast to the situation in skeletal muscle grafts, where the invading CNS axons are almost exclusively found on the surface of Schwann cells (Anderson *et al.* 1988).

As in previous investigations of intrastriatal grafts of muscularis externa and myenteric plexus (see chapters 3 and 6), many ingrown sprouts had matured and become myelinated by oligodendrocytes. However, in two of the grafts examined, Schwann cell myelinated axons were observed within the graft, a situation not encountered in previous investigations of intrastriatal grafts of myenteric plexus and muscularis externa (see chapters 3 and 6). Schwann cells performing myelinating roles have only been observed within the CNS after lesions which have a deleterious affect on CNS glial cells (Blakemore *et al.* 1990, Dusart *et al.* 1992). It is thought that perivascular Schwann cells enter the CNS when the glial limitans is disrupted, possibly stimulated by a reduction in the number of oligodendrocytes (Dusart *et al.* 1992). Thus it is possible that the implantation of colonic smooth muscle in those two grafts produced a greater striatal lesion, sufficient to allow the invasion of Schwann cells.

Large diameter axons have previously only been observed surrounded by CNS myelin, within clusters of ingrown CNS sprouts (see chapter 3 and 6). The presence of clusters of large-diameter

unmyelinated axons in this study may reflect the fact that there is a lack of oligodendrocyte processes or Schwann cells capable of myelinating these axons.

Although much larger bundles of CNS axons entered smooth muscle grafts than grew into peripheral nerve grafts in the thalamus (G.Campbell *et al.* 1992) or striatum (Woolhead and Anderson, personal communication), the size and shape of the grafts in the present study precluded any estimate of the distance over which such axons can regenerate through smooth muscle grafts. Peripheral nerve grafts can support the regrowth of CNS axons for several centimetres (Aguayo 1985), but we have no evidence to suggest whether smooth muscle can achieve the same result. However, this could be determined by implanting smooth muscle cells within the CNS/striatum in a biologically inert linear structure, such as a synthetic tube (eg: Williams *et al.* 1983) or piece of acellular peripheral nerve (eg: Hall and Berry 1989). Irrespective of the ability of smooth muscle grafts to support prolonged axonal elongation, the stimulation of axonal sprouting from CNS neurons by smooth muscle grafts opens the possibility that small grafts of smooth muscle could enhance local repair processes in the injured brain or spinal cord. Both Schwann cells and adrenal medullary cells implanted in the corpus striatum can ameliorate the behavioural effects of nigrostriatal pathway lesions (see chapter 1.3.2.2). It is likely that both types of graft act via the release of trophic factors (see chapter 1.3.2.2 and 1.4.6). Smooth muscle cells may prove to be a more readily obtainable source of potent trophic influences on axons within the corpus striatum.

CHAPTER 8.

GENERAL DISCUSSION

The objective of this study was to determine whether the myenteric plexus could survive transplantation into the CNS, as an initial step in examining the potential of this model as an approach to treating neurodegenerative diseases. In the experimental chapters, this has been shown to be possible. However, many problems and questions have been raised by these experiments and some are discussed in detail in this chapter, especially those which might influence the direction of future work in this field.

8.1 General summary of the results of ENS transplantation; comparison with other studies.

The experimental work presented in this thesis has demonstrated that grafts of enteric ganglia with and without surrounding smooth muscle coats are capable of surviving within the corpus striatum regardless of whether it received a prior quinolinic acid (QA) lesion. When examined electron microscopically, grafted enteric ganglia and smooth muscle remained morphologically similar to ganglia and smooth muscle observed within the gut wall. Grafted enteric neurons containing the enzyme NADPH-diaphorase extended fibres into both the QA lesioned and unlesioned striatum, and tyrosine-hydroxylase (TH)-containing fibres of central origin invaded grafts of enteric ganglia with surrounding smooth muscle. When examined electron microscopically, grafts of enteric ganglia and smooth muscle produced a sprouting response in the surrounding striatum, with smooth muscle grafts stimulating the most vigorous response.

Investigations undertaken in other laboratories have also examined grafts of enteric ganglia taken from young rats implanted in the hippocampus (Lawrence *et al.* 1991) and enteric ganglia with surrounding smooth muscle layers, taken from adult rats and implanted

into the spinal cord (Jaeger 1993, Jaeger *et al.* 1993). Although both groups have reported that enteric ganglia taken from young (Lawrence *et al.* 1991) and adult (Jaeger 1993, Jaeger *et al.* 1993) rats remain morphologically similar to ganglia seen in the gut wall, there are a number of differences between their reported observations and those presented in this thesis. For example, it has been reported that a population of enteric glial cells implanted together with enteric neurons in the cholinergically denervated hippocampus form end-feet arrangements with blood vessels in the grafts, and display caveolae at the cell surface (Lawrence *et al.* 1991). However presumptive enteric glial cells with caveoli were observed only rarely within grafts of adult rat enteric ganglia and their surrounding smooth muscle implanted into the corpus striatum (chapter 3) and were not observed in grafts of young rat enteric ganglia without surrounding smooth muscle tissue (chapter 6). Enteric glia in the gut wall do not display these characteristics (Gabella 1981a). It is not clear whether enteric glia exhibiting many caveoli and forming close relationships with blood vessels are produced by influences from the hippocampus, or by the absence of influences from enteric neurons.

Another difference between the results presented in this thesis and those reported by groups examining enteric ganglia grafted into the hippocampus and spinal cord relates to the outgrowth of grafted neurons. NADPH-diaphorase-containing enteric neurons extend processes into the surrounding striatum, regardless of whether it has received a prior excitotoxin lesion (see chapter 5). This is in contrast to the observations of grafted enteric neurons in the hippocampus and spinal cord, where AChE-containing grafted neurons did not extend processes beyond the boundaries of the graft (Lawrence *et al.* 1991,

Jaeger *et al.* 1993). This difference may reflect that NADPH-diaphorase-containing enteric neurons have a greater ability to extend processes into foreign environments or that the striatum represents a more conducive environment for the elongation of axons from enteric neurons (for more detailed discussion see chapter 5).

A third difference between the results presented in this thesis and those reported in the literature relates to the vigorous sprouting response observed around grafts in the striatum. Grafts of myenteric plexus with or without smooth muscle stimulate a sprouting response and are invaded by putative CNS axonal sprouts, some of which mature and become myelinated by oligodendrocyte processes (see chapters 3 and 6). Whether enteric ganglia grafted into the hippocampus stimulate a sprouting response or are invaded by oligodendrocyte myelinated or unmyelinated CNS axons was not mentioned in the report by Lawrence *et al.* (1991). Similarly, whether enteric ganglia with surrounding smooth muscle cells implanted in the spinal cord stimulate a sprouting response in the CNS is unclear, although grafts are reported to be invaded by axons which become myelinated by Schwann cells (Jaeger *et al.* 1993), including the central processes of dorsal root ganglion neurons (Jaeger 1993).

It is also apparent in the observations presented in these studies, that enteric ganglia are capable of becoming closely associated with the surrounding striatum (see chapter 6), a situation not observed when enteric ganglia are implanted into the hippocampus (Lawrence *et al.* 1991) or into the spinal cord (Jaeger *et al.* 1993). Many of the differences outlined above may reflect the implantation site, as the striatum has been demonstrated to be capable of great alterations in morphology, for example in response to excitotoxin lesions (Roberts and DiFiglia 1990b), however further investigations

will need to take place to determine whether similar responses can be observed in other CNS areas (see section 8.7).

8.2. Problems of identification of CNS axonal sprouts.

There are a number of reasons why the bundles of small non-myelinated axons observed around grafts of myenteric plexus and/or smooth muscle have been identified as regenerating CNS axonal sprouts. Firstly, they show a striking similarity to developing CNS axonal sprouts (Henrikson and Vaughn 1974). Secondly, they are morphologically distinct from regenerating PNS axonal sprouts innervating the CNS (eg: Reier *et al.* 1983, Carlstedt 1985, Anderson *et al.* 1989), which grow for short distances in the CNS, becoming varicose and full of organelles. Thirdly, they resemble the CNS axonal sprouts observed in the transected optic nerve (Hall and Berry 1989, Zeng *et al.* 1991) and sprouts produced in response to peripheral nerve (PN) grafts in the thalamus (G.Campbell *et al.* 1992) and in the striatum (Woolhead, personal communication). Immunocytochemical studies have shown that similar small non-myelinated axons (identified as regenerating CNS axonal sprouts), observed in and around PN grafts in the thalamus are coated with tenascin and embryonic NCAM, two molecules not present on regenerating PNS axons (Martini 1994). Thus, there are a number of reasons for believing that the majority of small non-myelinated axons observed around grafts of myenteric plexus alone and/or smooth muscle, are regenerating CNS axonal sprouts. However, it should be pointed out that it is impossible to determine the origin of any particular axonal profile observed in the electron microscopic studies described in this thesis on morphological grounds alone.

8.3 Sprouting response of the CNS to the implantation of different tissues.

The experiments described in this thesis have demonstrated that the implantation of myenteric plexus with or without the surrounding smooth muscle layers is capable of producing a vigorous sprouting response from axons in the corpus striatum. We believe these sprouts are of CNS origin (see above), although it has not been determined whether the axons arise from striatal neurons, afferents to the striatum, or axons travelling *en passant* through the striatum. However, the observation that there were many fewer CNS regenerating axonal sprouts around myenteric plexus grafts in the quinolinic acid lesioned striatum than around grafts in the unlesioned striatum would suggest that many of the sprouts observed around those grafts arose from intrinsic striatal neurons killed by the excitotoxin (eg: medium spiny striatal projection neurons and medium aspiny interneurons; see chapter 6 for fuller discussion).

The axonal sprouts seen around grafts of enteric plexus and/or smooth muscle were not short-lived structures; they persisted for at least six weeks. In this regard, they resembled sprouts seen in the thalamus and cerebral cortex around PN grafts (G.Campbell *et al.* 1992), but were quite different from axonal sprouts found in optic nerves transected in the orbit (which are numerous three to seven days after injury, but rare 14 days after injury: Zeng *et al.* 1991). These observations may imply that not only are sprouts produced in response to the implantation of myenteric plexus and or smooth muscle, but that they also receive sufficient support to remain in existence for long periods. The absence of such large numbers of axonal sprouts around freeze-killed tissue grafts (see chapter 3 and

7) illustrates that the sprouting response requires influences, presumably soluble factors, from living cells in the grafts.

When comparing the extent of sprouting observed in the striatum around different types of graft, it is apparent that fewer CNS regenerative sprouts were observed around grafts of myenteric ganglia than around grafts of muscularis externa containing myenteric plexus and grafts of smooth muscle alone. In order to determine why there was a difference in the sprouting response to the implantation of different types of tissue, it is necessary to examine what factors or combination of factors are important for the induction of a sprouting response or the successful maintenance of regenerative sprouts.

It is possible that grafted tissue may release trophic factors, stimulating injured neurons to produce regenerating axonal sprouts, or allowing axonal spouts produced in a non-specific response to injury to survive. There are a wide variety of defined trophic factors and cytokines that have such an effect. For example, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), are capable of stimulating septal neurons to regenerate axons to the hippocampus following lesions of the fimbria-fornix (Hagg *et al.* 1991, Kawaja *et al.* 1992, Gage *et al.* 1993, Morse *et al.* 1993). Similarly both NGF and neurotrophin 3 (NT-3) are reported to stimulate the regeneration of corticospinal axons after spinal cord lesion (Schwab *et al.* 1992, Tuszynski *et al.* 1993, Schnell *et al.* 1994). Both BDNF and basic fibroblast factor (bFGF) have been shown to enhance the survival of cultured dopaminergic substantia nigra neurons (Knüsel *et al.* 1990, Hyman *et al.* 1991, Spina *et al.* 1992) although it has been demonstrated that BDNF cannot prevent degeneration of nigral neurons following axotomy (Knüsel *et al.*

1992). More recently a number of groups have reported the presence of a molecule derived from astrocytes, glial derived growth factor (GDNF), that is present in the postnatal rat brain (Schaar *et al.* 1993, Strömberg *et al.* 1993) and promotes the survival of cultured embryonic dopaminergic neurons *in vitro* (Engele *et al.* 1993, Lile *et al.* 1993, Lin *et al.* 1993, Park and Mytilineou 1993) and *in vivo* (Strömberg *et al.* 1993) and stimulates the transient production of sprout-like axons and growth cones within the striatum when injected into the ipsilateral substantia nigra (Henry *et al.* 1993). Basal forebrain neurons, corticospinal neurons and dopaminergic neurons innervating the striatum are all possible candidates for producing axonal sprouts in the striatum around enteric grafts. Cholinergic basal forebrain axons have been shown to invade grafts of NGF-secreting fibroblasts implanted in the striatum (Kawaja and Gage 1991), corticospinal axons pass through the striatum and nigral neurons innervate the striatum. The responses to neurotrophins by intrinsic striatal neurons are not, in most cases, well defined. Cholinergic interneurons respond to NGF by undergoing hypertrophy (Gage *et al.* 1989) and express trkA (Merlio *et al.* 1993). Most of the small and medium sized cells in the striatum express trkB and trkC (Merlio *et al.* 1993), and would be expected to respond BDNF, NT-3 and neurotrophin-4/5 (Ip *et al.* 1993). Whether these factors are capable of causing axonal sprouting of intrinsic striatal neurons is not known.

There have been no extensive studies investigating which of the defined neurotrophins are produced within the ENS, although NT-3 has been demonstrated to play a role in promoting the differentiation of enteric glia and neurons (Chalazonitis *et al.* 1993). Studies in this laboratory have shown that striatal neurons co-cultured with enteric

ganglia extend processes further than similar neurons co-cultured with de-differentiated smooth muscle or fibroblasts, suggesting that a stimulatory factor is produced by enteric neurons and/or enteric glial cells (Höpker *et al.* 1994: see chapter 7 for a more detailed comparison of the results presented in this study and those of Höpker *et al.* 1994).

Another possible explanation of the difference in the sprouting response observed around grafts is that the different tissues examined may have differing effects on the non-neuronal cells in the surrounding striatum. Glial cells (and/or neurons) may respond to various stimuli by expressing neurotrophins, cytokines and other molecules capable of enhancing axonal outgrowth. For example, astrocytes in the transected optic nerve contain increased ammounts of NGF mRNA compared to those observed in the intact optic nerve (B.Lu *et al.* 1991), and glial fibrillary acidic protein-containing reactive astrocytes around electrolytic and mechanical lesions contain NGF-like immunoreactivity (Altar *et al.* 1992, Olderfeld-Nowak *et al.* 1992). Other neurotrophic molecules have been observed within astrocytes including ciliary neurotrophic factor (Stockli *et al.* 1991), bFGF (Vijayan *et al.* 1993), and GDNF (see above). Although the living enteric grafts could well be acting via influences over the expression of trophic molecules by CNS neurons and glia, there is no direct evidence to suggest how this may occur.

In addition, macrophages may also play a role in the observed sprouting response around grafts of enteric grafts. Macrophages have been shown to invade sites of injury in both the PNS and CNS, although the invasion in the CNS is much less extensive (Perry *et al.* 1987). Macrophages have also been shown to release factors that

stimulate the proliferation of Schwann cells (Beuche and Friede 1984) and astrocytes (Giulian *et al.* 1989), and causes an increase in NGF synthesis and low-affinity NGF-receptor expression in Schwann cells (Heumann *et al.* 1987). *In vitro* studies have demonstrated that macrophages may be able to alter the non-permissive environment in the CNS following lesions, into an environment permissive for CNS axonal regeneration (David *et al.* 1990). Thus the less extensive macrophage-invasion observed in the CNS (compared to the PNS) may be related to the less extensive regeneration. Consequently, it may be envisaged that grafts of enteric ganglia or smooth muscle have a greater stimulatory effect on macrophage invasion, and thus stimulate a greater regenerative response, although there is no evidence to support this idea.

Whilst all grafts of living tissue were invaded by axons that became myelinated by oligodendrocyte processes, the smooth muscle grafts were also invaded by axons that became myelinated by Schwann cells (chapter 7). The Schwann cells may have originated in perivascular nerves, or have been present in the smooth muscle on dissection (see discussion in chapter 7). The fact that Schwann cells and Schwann cell myelinated axons are not observed in grafts of enteric ganglia (with and without smooth muscle) may indicate that the Schwann cells are inhibited from myelinating by the presence of enteric ganglia or that smooth muscle grafted without enteric ganglia stimulates the invasion of Schwann cells from perivascular sites, or perhaps that smooth muscle grafts had a more disruptive effect on the glial limitans, allowing perivascular Schwann cells to invade the smooth muscle grafts.

8.4 Role of enteric glia.

One of the interesting observations arising from these studies is that ingrowing CNS regenerative sprouts were observed in large bundles that originated in the brain, but became associated with glial cells in the graft (see **Fig. 3.7a and b** in chapter 3). This suggested that enteric glial cells were producing a substance that stimulated the growth of CNS axonal sprouts, or attracted them into the grafts. In order to examine this possibility, an experiment was designed, where a purified population of enteric glia were implanted in the striatum. Because it is difficult to obtain large quantities of colonic myenteric plexus from rats, enteric ganglia were taken from young guinea pig taenia coli and grown in culture and the centre of each culture (where the majority of enteric neurons are found) was scrapped away, thus leaving a partially purified population of enteric glial cells (Bannerman *et al.* 1988b). After 7-10 days in culture, enteric glial cells were harvested and a suspension of cells were injected into a peripheral nerve that had been repeatedly frozen and thawed (four times) thus rendering it acellular. The injected nerve was then implanted in the striatum from adult rat and the animal received daily injection of the immunosuppressant, cyclosporin A (10mg/kg;ip.). Ten days and three weeks after implantation, the animals received an overdose of pentabarbitone, before being transcardially perfused for electron microscopy (see chapter 2 for fixative and EM processing procedure). Detailed examination of the ultrathin sections revealed that there was no evidence for the presence of regenerating axons or living enteric glial cells within the freeze-killed peripheral nerve. This suggests either that the immunosuppression regimen was insufficient to prevent rejection of the grafted guinea-pig cells, or that enteric glial cells were unable

to survive within the acellular nerve, or perhaps that insufficient glial cells were implanted in the first place. The major difficulty with pursuing this line of research remains the problem of obtaining sufficient numbers of glial cells, preferably of rat origin.

Thus, it remains to be determined if enteric glial cells are capable of stimulating the regenerative growth of CNS axons such as those from striatal neurons or afferents to the striatum. There is a large amount of data suggesting that glial cells can produce trophic factors, for example Schwann cells synthesize NGF and BDNF (Meyer *et al.* 1992). GDNF, a molecule derived from astrocytes has been shown to have a number of trophic effects on developing and mature CNS neurons (see above). Recently tissue culture experiments have shown that enteric ganglia taken from 2-4 day old guinea-pig colon (taenia coli) have a neurite-promoting effect on co-cultured striatal neurons taken from young rats 7 day old, although it appears that enteric neurons are necessary for this response (Höpker *et al.* 1994).

8.5. How do myenteric plexus grafts compare with other types of tissue- are they a good source of material for intracerebral implantation in neurodegenerative diseases?

In order to assess the possible significance of grafts of enteric ganglia, and smooth muscle, in the treatment of neurodegenerative diseases, it is necessary to compare the results outlined in this thesis with those of other grafts implanted in the striatum.

8.5.1. Myenteric plexus grafts compared with striatal grafts.

To date the most extensive integration of grafted material with the surrounding brain has been observed with intrastriatal grafts of foetal striata (see Introduction, section 1.3.2.4.). As previously

outlined, these grafts have been demonstrated to extend fibres to many of the areas innervated by normal striatal neurons, and receive axons from areas normally projecting to the striatum. Functionally also, these grafts have demonstrated the greatest ability to ameliorate lesion-induced behavioural deficits, although not all such deficits are improved (for more detailed discussion see 1.3.2.4.). It can be suggested that this exceptional response comes about because the grafted tissue is placed in an environment similar (although more mature) to the one in which it would normally develop, and so many of the cues for growth may be present, or are re-expressed after striatal lesioning. It would be expected that grafted enteric ganglia, like other types of tissue implanted in a "strange"/non-target areas, would be unable to replicate this "best result".

For enteric plexus grafts to exhibit the ability to functionally reinnervate the lesioned striatum, it would be necessary that the grafted neurons reconstruct the circuitry of the striatum (eg: receive afferent connections similar to those received by striatal neurons, and exhibit receptors for the various transmitters that act on striatal neurons), in order that the activity of grafted neurons could be modulated. Grafted neurons would also need to extend axons to the correct target areas and release appropriate transmitters, in order that they could produce a relevant response in striatal projection areas. It is difficult to imagine these criteria being met by grafts of enteric neurons.

8.5.2. **Myenteric plexus grafts compared with grafts of nigral neurons.**

As has been discussed above (see section 1.3.2.1) grafts of foetal nigral tissue extend fibres into the dopamine-denervated

striatum, acting to modulate the activity of striatal neurons, and ameliorate many of the behavioural deficits observed in animal models of Parkinson's disease. Clinical trials, utilizing grafts of foetal ventral mesencephalon implanted in the caudate and putamen of Parkinsonian patients or patients with MPTP-induced parkinsonism, have also produced clinical improvements in motor function (see section 1.3.2.1.7) although not to the extent observed in studies using experimental animals. It has been reported that there are only a small population of enteric neurons within the human intestine that contain TH, and so are capable of producing DA (Wakabayshi *et al.* 1989). Thus, it is feasible that grafted enteric neurons genetically modified to produce DA may be able to act, to some extent, in a similar way to grafted foetal nigral cells, since we have demonstrated that the grafted enteric neurons extend fibres into the surrounding striatum. However we have no evidence to suggest that grafted enteric neurons form functional synapses on striatal neurons, or receive a meaningful afferent input capable of modulating their activity.

8.5.3. **Myenteric plexus grafts compared with grafts of adrenal chromaffin cells, genetically modified cells or cell lines.**

The third type of graft that has been extensively examined within the striatum involves cells that produce trophic factors or neurotransmitter molecules (eg: DA). These include inactive donor cells that have been genetically engineered to produce specific neurotrophins or transmitters (see section 1.3.2.3) or cells which normally produce trophic factors or transmitters (eg: cell lines, see section 1.3.2.3, or possibly adrenal medullary cells, see section 1.3.2.2).

Since some CNS neurons have been demonstrated to regenerate

their axons when supplied with appropriate trophic factors (eg: NGF acting on septal cholinergic neurons; see section 1.5.5), it would seem reasonable to suggest that the addition of such a trophic factor may aid or stimulate a regenerative response, in particular CNS axonal sprouting.

It may be speculated that the CNS axonal sprouts observed around grafts of myenteric plexus, with and without surrounding smooth muscle and around grafts of intestinal smooth muscle may have been produced in response to the release of one or more trophic factors; however as there is no information regarding the neurons giving rise to CNS axonal sprouts, it is difficult to suggest the identity of the possible factor(s).

Besides the possibility of utilizing enteric cells as a source of potential unknown trophic factor(s), it may be possible to genetically engineer enteric neurons to produce, for example BDNF, a neurotrophic factor that has been shown to stimulate the outgrowth of nigrostriatal dopaminergic neurons in culture (see above). Enteric neurons represent a particularly interesting source of cells, as we have shown that they are capable of extending processes into the surrounding striatum (see chapter 5) and thus are potentially able to release the product of the transfected gene throughout a large volume of brain tissue. However, there are a number of problems associated with genetically manipulating cells. For example, retroviruses are ineffective at introducing genes in postmitotic cells (eg: neurons), and, while herpes simplex viruses appear to offer more promise, there are some theoretical problems. In order to safely transfect neurons with modified herpes simplex virus, the virus must be made defective, so it is unable to replicate, but it is possible that the defective

virus may recombine with wild-type virus to form an infective/lethal form (Breakefield *et al.* 1992). A second problem is whether it is possible to see long-term expression of the gene-product (a problem which has affected other investigators; see section 1.3.2.2). Another problem that has affected other trials of genetically engineered cells has been the production of tumours by the uncontrolled growth of intracerebrally injected immortal cell lines (see section 1.3.2.3), although this has not been reported to occur when primary cell cultures are used (see section 1.3.2.3).

8.6. Disease states where grafts of enteric ganglia or intestinal smooth muscle may have a beneficial effect.

8.6.1. Parkinson's disease.

As outlined above and in the Introduction (section 1.6.2.1) grafts of foetal nigral tissue have proven the most capable of ameliorating behavioural abnormalities seen in animal models of Parkinson's disease and symptoms in Parkinsonian patients. For grafts of enteric ganglia to have any therapeutic effect in this disease, grafted cells would need to produce DA. Although there is evidence of catecholamine-containing enteric neurons within different intestinal regions of many species (eg: TH-containing neurons in the human oesophagus, Wakabayshi *et al.* 1989; dopamine-β-hydroxylase-containing neurons in the rat colon, Schultzberg *et al.* 1980), catecholamine-containing enteric neurons are generally not present in large quantities, and thus it would be necessary to genetically engineer enteric neurons to produce DA, via the introduction of the gene responsible for the production of TH. Even then, there is no evidence that grafted cells genetically engineered to produce DA would be able to normalize lesion-induced alterations in mRNA levels of

transmitters and synthetic enzymes in striatal projection neurons, as has been reported after implantation of foetal nigral tissue (see section 1.3.2.1).

8.6.2. **Huntington's disease.**

Although grafts of enteric ganglia or smooth muscle are unlikely to be effective in Huntington's disease (HD: see above), grafts may be an ideal vehicle for any protective factor which may halt or delay the onset of HD. This is especially relevant, since the gene responsible for HD has recently been isolated (The Huntington's Disease Collaborative Research Group 1993) opening the way for definitive genetic tests for the disease; and since HD is a late-onset degenerative disease and it appears that the degeneration proceeds at the same rate as the symptoms, there is time to act before neurodegeneration begins.

8.6.3. **Alzheimer's disease.**

It can be postulated that grafts of enteric neurons may have a beneficial effect in Alzheimer's disease (AD), since they contain cholinergic neurons (Furness and Costa 1987, Lawrence *et al.* 1991, Jaeger *et al.* 1993), one of the neuronal types which degenerate early on in AD. However, it has been demonstrated that cholinergic enteric neurons are not capable of extending axons into the hippocampus or spinal cord (Lawrence *et al.* 1991, Jaeger *et al.* 1993). The reasons for this are obscure, but have been discussed more fully in chapter 5.

Another therapy that has been suggested for AD is NGF treatment. This treatment has been demonstrated to ameliorate spatial memory impairments and cholinergic neuron atrophy in aged rats (Fischer *et al.* 1987) and clinical trials in a single patient with AD

have reported some modest improvements (Olson *et al.* 1992, Olson 1993). Although there is no evidence to suggest that grafted enteric neurons would produce NGF, there still remains the possibility that enteric neurons could be genetically engineered to produce NGF, which could potentially be released over a large area. However, it remains to be seen whether genetically engineered enteric neurons would be capable of producing and releasing the gene product in the long-term (see above).

8.7 **Future Work.**

As with any project of this nature, there are far more questions left unanswered than have been answered within this thesis. Thus the list of potential future directions is long, but I would like to take this opportunity to discuss a number that interest me.

1. Retrograde tracing from globus pallidus and substantia nigra, to determine whether fibres extend from the graft to normal target sites for striatal neurons. It would be possible to combine this study with an immunohistochemical study to determine the neurochemical identity of the grafted neurons extending fibres. Since the grafts are small in size, it would be quite difficult to trace axons innervating the grafts by retrograde tracing.
2. Anterograde tracing from thalamus, frontal and cingulate cortex and substantia nigra, to determine if grafts of enteric ganglia or smooth muscle are innervated by normal afferent to the striatum. If tracers such as WGA-HRP and PHA-L (localized immunocytochemically with avidin-biotin and visualized with diaminobenzidine) were used, it would be possible to examine the grafts at the EM level, to determine if these afferents formed synapses on the grafted enteric neurons. The nature of any such synapses would be of great interest.

3. Investigations into the fate of grafted enteric neurons containing different neurotransmitter types. Colocalization studies have demonstrated that substance P (SP), vasoactive intestinal polypeptide (VIP) and enkephalin (ENK) are found in different enteric neurons in the rat proximal colon (Schultzberg *et al.* 1980) Since we have determined that NADPH-diaphorase-containing enteric neurons (which probably also contain VIP: Aimi *et al.* 1993) are capable of extending axons into the surrounding brain when implanted into the striatum, it would be interesting to examine whether other types of enteric neurons (eg: those containing SP or ENK) were able to extend fibres when implanted in the striatum.

4. The survival and axonal outgrowth of grafted myenteric neurons within other areas of the CNS. Since cholinergic neurons in enteric ganglia grafted into the cholinergically-denervated hippocampus or spinal cord do not extend fibres into the surrounding CNS (Lawrence *et al.* 1991, Jaeger *et al.* 1993), it would be of great interest to determine whether this occurs because these environments are inhospitable to ingrowth of ENS axons, or because cholinergic neurons are poor at regenerating their axons. A relatively simple experiment to investigate this problem would be to examine the behaviour of NADPH-diaphorase-containing enteric neurons grafted into the cholinergically denervated hippocampus or the spinal cord.

5. Behavioural effects of grafts. It would also be of interest to determine whether enteric neurons were able to replace striatal neurons lost in the QA-lesioned rat striatum (or enhance the plasticity of any surviving striatal neurons), especially if there was evidence from tracing studies that grafts extended fibres to some striatal target areas and were innervated by normal afferents to the

striatum. Suitable behavioural tests include simple tests (eg: amphetamine-induced rotational behaviour) and more complex tests (eg: skilled forelimb use), that have been demonstrated to show improvements after the intrastriatal implantation of foetal striatal tissue (eg: Dunnett *et al.* 1988, Montoya *et al.* 1990 and see section 1.3.2.4.5).

Similarly, it would be of interest to determine if grafted enteric neurons or smooth muscle were capable of stimulating the sprouting of lesioned nigrostriatal neurons and of ameliorating the behavioural deficits produced by partial nigrostriatal lesions (as produced by 6-OHDA). If amelioration were observed to simple behavioural tests (such as amphetamine-induced rotation), it may be of interest to determine whether such grafts were also capable of ameliorating more complex deficits/tests (for example of such tests see sections 1.2.3.2., 1.3.2.3. and 1.3.2.2.

6. One of the main disadvantages of this work is that there is no information regarding the possible origin of the CNS regenerative sprouts observed around grafts. In order to determine the origin of such sprouts, it may be possible to perform EM-immunolabelling with antibodies raised against molecules present in afferents to the striatum (eg: TH for dopaminergic nigral neurons). Such information may give a hint as to which neurotrophic factor may be involved in stimulating the sprouting response.

7. As discussed earlier, it is possible that enteric neurons could be genetically engineered to produce enzymes responsible for neurotransmitter synthesis (eg: TH), or to produce trophic factors (eg: NGF/BDNF), which may be able to stimulate a regenerative response or act as neuroprotectors. However, this type of study is very much in its infancy although enteric neurons (unlike fibroblasts

or skeletal muscle) represent an advantageous source of tissue, since we have demonstrated that they are capable of extending fibres for comparatively long distances into the neighbouring areas of the brain.

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