THE REGENERATIVE RESPONSE OF ADULT RAT CNS NEURONS TO THE IMPLANTATION OF PERIPHERAL NERVE GRAFTS INTO THE CORPUS STRIATUM

By

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A thesis submitted to the University of London for the degree of
Doctor of Philosophy

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"... [I was] sitting with my brain falling apart in my hands and suddenly there was a capsule around everything.

Oh no, I'm talking complete garbage!"

ABSTRACT

The study was focused on responses of CNS neurons in adult rats to autologous tibial nerve grafts implanted into the striatum, an area of particular interest due to its heterogeneity of cell content and clinical importance. Transmission electron microscopy identified many small non-myelinated axonal profiles, presumptive regenerating CNS axons, in the brain surrounding the graft. Similar axonal profiles also invaded living, but not freeze-killed, peripheral nerve grafts implanted into the striatum and retrograde labelling with HRP-conjugated ligands showed that the majority of regenerating axons originated from neurons in the substantia nigra pars compacta. Comparatively few striatal neurons, which showed some morphological similarities, were retrogradely labelled, perhaps indicating the preferential regeneration of particular populations. Many retrogradely labelled neurons were found within the ventral pallidum and the area close to the medial forebrain bundle but only if the graft tip terminated within these regions. Peripheral nerve grafts contained much higher levels of NGF than intact striatal tissue, but, although striatal NGF levels increased slightly after graft implantation, no concurrent up-regulation of the low affinity NGF receptor (p75NGFR) was detected in any striatal neurons, even those known to be NGF-sensitive. Two populations of striatal interneurons underwent perikaryal enlargement after the implantation of living, but not freeze-killed, grafts demonstrating that this response was mediated by factors produced by living grafts, and regenerating axons which appeared to originate from both these populations of striatal neurons were identified within the grafts as early as two weeks post operation. Additionally, a small set of large striatal perikarya was found to express mRNA for GAP-43 and the cell adhesion molecule L1, even in the control striatum; graft implantation had no apparent effect on their expression of either molecule. Similarly, neurons of the substantia nigra pars compacta, which most successfully regenerated long axons into the graft, contained high levels of these molecules irrespective of the presence of a nerve graft in the striatum.
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   (f) Postoperative care
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   (h) Inhibition of acetylcholine esterase (AChE)

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   (h) Inhibition of acetylcholine esterase (AChE)

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<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetyl cholinesterase</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4 chloro-3-indoly-phosphate</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BW</td>
<td>Browman-Wise</td>
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<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
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<td>ChAT</td>
<td>choline acetyltransferase</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
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<tr>
<td>DAB</td>
<td>3,3'- diaminobenzidine tetrahydrochloride</td>
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<td>DDT</td>
<td>dithiotreitol</td>
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<tr>
<td>DEPC</td>
<td>diethyl-pyrocarbonate</td>
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<tr>
<td>DFP</td>
<td>diiosopropyl fluorophosphate</td>
</tr>
<tr>
<td>DREZ</td>
<td>dorsal root entry zone</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix (molecule)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra acetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ELISA</td>
<td>enzyme linked immuno assay</td>
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<tr>
<td>EM</td>
<td>electron microscope</td>
</tr>
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<td>ER</td>
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<td>insulin-like growth factor</td>
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<td>JZ</td>
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<td>LIF</td>
<td>leukaemia inhibitory factor</td>
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<td>LM</td>
<td>light microscopy</td>
</tr>
<tr>
<td>LNGFR</td>
<td>low affinity NGF receptor</td>
</tr>
<tr>
<td>MB</td>
<td>Millonigs buffer</td>
</tr>
<tr>
<td>mfb</td>
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<td>NADPH</td>
<td>nicotine adenyl dinucleotide phosphate (reduced)</td>
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<td>NBT</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PN</td>
<td>peripheral nerve</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>po</td>
<td>post operative</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RGC</td>
<td>retinal ganglion cell</td>
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<td>PRSS</td>
<td>post reaction and storage solution</td>
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<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
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<tr>
<td>SP</td>
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<td>SSC</td>
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<td>TEM</td>
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ACKNOWLEDGMENTS

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* * * * * * *

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* * * * * * *

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Without doubt, my greatest thanks go to my generous parents, Peter and Gaibrielle, who have always supported me, Chris and Karen whenever we have needed it. They have done more towards the ultimate production of this thesis than I ever have, and it is with much love, and the greatest respect, that it is dedicated to them.
CHAPTER 1 - INTRODUCTION
Neurons of the mammalian central nervous system (CNS) are unable to regenerate axons after injury and damage to these neurons commonly results in complete functional loss in the affected pathway and often quite severe disability. Consequently, attempts to stimulate mammalian CNS axonal regeneration are of great neurobiological and clinical interest. One of the most significant discoveries in this area of research this century was that axotomized CNS neurons are able to regenerate long axons into segments of peripheral nerve tissue implanted into the CNS (Aguayo et al., 1985). However, although peripheral nerve grafts (PN grafts) have been shown to support axonal regeneration from neurons in a number of different CNS areas, this technique has, so far, not led to any clinical breakthrough in the treatment of CNS injury or disease. The neurobiology of the relationship between regenerating CNS axons and the environment of PN grafts is poorly understood, and this thesis attempts to clarify certain aspects of their interactions in a region of the brain of great clinical interest - the adult corpus striatum, which is affected by both Parkinson's disease and Huntington's disease and has been the host tissue site in a number of attempts to alleviate the symptoms of these diseases by grafting healthy CNS tissue into the diseased brain.

1 - Anatomy

This study concentrates on two distinct but related regions of the adult rat basal ganglia and midbrain: the corpus striatum and the substantia nigra.

Rat CNS anatomy is different from that of humans. In the human the corpus striatum consists of the caudate nucleus and the lentiform nucleus which, in turn, is subdivided into a lateral portion, the putamen, and a medial portion, the globus pallidus. The caudate nucleus and the lentiform nucleus are separated by a distinct white matter tract called the internal capsule, which consists of ascending and descending fibres connecting the cerebral cortex with lower centres including the thalamus, corpus striatum, brain stem and spinal cord. Although the separation is clear posteriorly, the anterior limb of the internal capsule is divided into fascicles passing through the fused rostrum portions of the caudate nucleus and putamen. It is this feature which gives the corpus striatum its name. In primates the caudate nucleus and putamen are grouped together under the collective name of the neostriatum, which is often shortened to striatum.

In the rat, however, no clear distinction exists between the caudate nucleus and the putamen; it appears as a homogenous structure traversed by many fine fibre bundles. The caudatoputamen, is commonly referred to as the striatum and lies between the internal capsule and the lateral ventricle, which form the medial boundary of the striatum, and the corpus callosum and lateral subcortical white matter which form the superolateral boundary. The globus pallidus of the rat is also different from that of
primates, lacking a clear division between internal and external sectors. The rat entopeduncular nucleus is recognised as the homologue of the primate internal pallidal segment (Morgan, 1927). The term striatum will be used throughout this study to refer to the caudatoputamen and not the globus pallidus. The identity of all rat brain regions referred to are taken from the Atlas of the Rat Brain by Paxinos and Watson (1986) which was also the source of all stereotaxic co-ordinates used for surgery.

(1a) The striatum

Although the striatum appears to be a homogeneous structure it is in fact divided up into discrete neurochemical and functional compartments which form a complex, but orderly, 3-D mosaic; the patch/matrix arrangement of striatal organization (for reviews see Graybiel, 1990 and Gerfen, 1992). This pattern of neurochemical arrangement was originally identified in the rat as a differential distribution of striatal μ opiate receptors (Pert et al., 1976) which was later correlated with the pattern of weak acetyl cholinesterase (AChE) staining areas in the cat already named 'striosomes' (Graybiel and Ragsdale, 1978). Since then discrete patterns of patch/matrix distribution have been determined within the adult striatum. In the rat, the majority of striatal choline acetyltransferase (ChAT) and neuropeptide Y (NPY) containing neurons are found within the matrix and the projection of their primary dendrites is restricted to within this division. However, a few of these neurons are located on the borders of the patch/matrix divide and are able to extend dendrites into both areas (Kubota and Kawaguchi, 1993). Conversely, levels of enkephalin, glutamic acid decarboxylase (GAD) and substance P (SP) are highest in the patches (Herkenham and Pert, 1981). Although medium spiny projection neurons containing γ-amino butyric acid (GABA) are found in both the patch and matrix compartments of the striatum their dendrites remain isolated within whichever division contains their perikarya. The axonal projection of these neurons also depends on their perikaryal placement; those perikarya found in the matrix extend axons to the GABAergic neurons of the substantia nigra pars reticulata (SNpr) and the globus pallidus while those in the patches innervate the dopaminergic neurons of the substantia nigra pars compacta (SNpc) (Gerfen et al., 1984; Kubota and Kawaguchi, 1993).

The distribution of afferents to the rat striatum is also related to patch/matrix organization; corticostriatal pyramidal projection neurons of deep layer 5 and layer 6 selectively innervate striatal patch neurons while those originating in superficial layer 5 and the supragranular layers project to the matrix only (Gerfen, 1989). It has also been suggested that the matrix is innervated by sensorimotor processing areas such as the agranular motor cortex and cingulate cortex while the patches receive inputs from structures involved in the limbic system such as the amygdala and SNpr (Gerfen, 1985; Donoghue and Herkenham, 1986; Ragsdale and Graybiel, 1988).
Cytology of the rat striatum

The rat striatum consists of one distinct population of projection neurons and many different types of interneurons heterogeneously mixed throughout the structure. The following descriptions all relate to rat cytology unless otherwise stated.

Medium spiny neurons

In addition to being the only class of projection neuron, the medium spiny neurons of the striatum are also the most numerous, making up approximately 95%-96% of the total number of striatal neurons (Grayeland et al., 1985; Smith and Bolam, 1990). These neurons have oval or fusiform perikarya and approximately four to seven primary dendrites which are spine free (Heimer et al., 1985). However, as the dendrites begin to branch, spine density increases, reaching maximal levels on the tertiary branches, giving Golgi impregnated neurons a characteristic 'bottle-brush' appearance (Dimova et al., 1980). Ultrastructurally, these neurons are unique in the striatum as they possess neither nuclear indentations nor nuclear inclusions (Dimova et al., 1980). Neurochemically they are characterized by high levels of GABA immunoreactivity (Kita and Kitai, 1988) and have been shown also to contain a number of other neuroactive molecules such as SP (Bolam et al., 1983), enkephalin (Pickel et al., 1980) and dynorphin (Vincent et al., 1982). The major projection of the medium spiny neurons is to the SNpr via the internal capsule (Heimer et al., 1985). While the inhibitory striatonigral fibres which contain GABA arise from medium spiny neurons distributed throughout the striatum (Staines et al., 1980; Jessell et al., 1978), those projection fibres which also contain SP, which has an excitatory effect, are predominantly from neurons found in the rostral striatum (Brownstein et al., 1976; Cuello and Kanazawa, 1978). As a result of the great number of these striatonigral projection fibres, the SNpr contains the highest levels of both GABA and SP in the adult brain (Fahn and Cote, 1969; Brownstein et al., 1976). The smaller striatal projections to the globus pallidus and entopeduncular nucleus use enkephalin and substance P as their neurotransmitter substances respectively (Cuello and Paxinos, 1978).

The medium spiny striatal projection neurons are the target for a number of different types of inputs from the cerebral cortex, SNpc, thalamus and intrinsic circuits of the striatum itself (Fallon and Loughlin, 1985). The position of synapses from these different incoming fibres are discretely placed according to their neuron of origin; hence, in the rat, inputs from extrastriatal neurons terminate on spines of the more distal portions of the dendritic tree and intrinsic connections from other medium spiny striatal neurons are formed more commonly on the more proximal dendritic shaft or even the perikarya, while synapses from large cholinergic interneurons are placed in an intermediate position (Smith and Bolam, 1990). Smith and Bolam (1990) hypothesize that this synaptic
arrangement allows for a regular and comprehensive integration of inputs onto the only efferent cells in the striatum.

**Medium aspiny neurons**

A population of medium-sized neurons with long, aspiny, locally ramifying processes has also been identified within the striatum (Dimova et al., 1980). These cells have spindle, triangular or fusiform perikarya, are evenly distributed throughout the striatum (Vincent et al., 1983) and comprise only 1-2% of the total number of neurons (Scherer-Singler, 1983). By electron microscopy (EM) these neurons have elongate, indented nuclei with intranuclear inclusions in both the rat and monkey (Graveland and Difiglia, 1985) and boutons containing mostly pleomorphic, and a small number of large granular, vesicles (Vincent et al., 1983; Takagi et al., 1983). Neurochemically, these neurons have been shown to be positive for somatostatin and avian pancreatic polypeptide (Takagi et al., 1983; Vincent et al., 1983), nitric oxide synthase (NOS) and NPY (Dawson et al., 1991), and nicotiamide adenyl dinucleotide phosphate (reduced) -diaphorase (NADPH-diaphorase; Kowall et al., 1985). Recently, mRNA for AChE has also been localized in these neurons (Bernard et al., 1995).

As interneurons, these cells only extend axons within the striatum, the majority of which synapse with the GABA-ergic medium spiny projection neurons (Vuillet et al., 1989a). In addition, medium aspiny striatal interneurons also receive afferent connections from nigrostriatal, pallidostriatal and corticostriatal fibres (Vuillet et al., 1989a; Aoki and Pickel, 1989; Bennet et al., 1993). It is not known whether this population of striatal neurons possess receptors for any specific neurotrophic factors or express significant levels of any known cell adhesion molecules (CAMs) or extra cellular matrix molecules (ECMs) in either an injured or uninjured state.

**Large aspiny neurons**

Golgi studies have identified a distinct population of large aspiny neurons in the striatum (Bolam et al., 1984). Up to three possible subdivisions of this phenotype may exist. However, all of those recorded so far share the ultrastructural features of large Golgi bodies and highly indented nuclei possessing large nucleoli and intranuclear inclusions (Dimova et al., 1980; Bolam et al., 1984). Using immunocytochemistry for ChAT and histochemistry for AChE, Eckenstein and Sofroniew (1983) reported that all ChAT positive striatal neurons contained AChE and vice versa. However, immunohistochemistry and cytochemistry have identified AChE in up to 6 different types of striatal neurons (Kaiya et al., 1980; Bolam et al., 1984), but only the large aspiny striatal interneurons have been shown, using in situ hybridization, to contain both AChE and ChAT (Bernard et al., 1995). This implies that the large aspiny neurons are the only cholinergic neurons in the striatum. Woolf and Butcher, (1981) demonstrated that the
large cholinergic striatal neurons were intrinsic interneurons and had no extrastriatal projections. The large aspiny cholinergic interneurons of the rat striatum make up approximately 2% of the total number of striatal neurons (Phelps et al., 1985). The majority of them have large, oval (51%) or fusiform (27%) perikarya (Butcher and Woolf, 1981) with many long dendrites which usually extend from the poles of their cell bodies (Butcher and Woolf, 1981; Bolam et al., 1984). There is some disagreement over the distribution of these cells within the striatum: Phelps et al. (1985) reported an even spread of ChAT positive cells through the anterior-posterior extent of the striatum, except for a few small medial and posterior areas which contained fewer cholinergic perikarya but Woolf (1991) found no difference in the density of these cells throughout the striatum. Kubota et al. (1993) stated that the topographical distribution of ChAT immunoreactive fibres and perikarya increased in a ventromedial to dorsolateral direction.

Large aspiny striatal interneurons in adult rats display immunoreactivity for neuronal cell adhesion molecule (N-CAM; Difiglia et al., 1989) and also contain high levels of mRNA for the growth associated protein (GAP)-43 (McKinney and Kent, 1994). Altar et al. (1991) found a medial to lateral gradient in the density of high affinity nerve growth factor (NGF) receptors in the striatum which, they stated, correlated with the distribution of ChAT positive neurons. In situ hybridization with probes against mRNAs for trkB and trkC, the tyrosine kinase high affinity receptors for brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) respectively, found relatively strong striatal labelling for trkC, much weaker labelling for trkB and no obvious gradient in their distribution (Altar et al., 1994). However, although the high affinity NGF receptor trkA has been identified on uninjured adult cholinergic aspiny interneurons (Steininger et al., 1993), there are only a small number of reports of the expression of p75 by these cells, and then only after striatal injury or exogenous infusion of neurotrophins into the ipsilateral lateral ventricle or into the striatum itself (Gage et al., 1989; Hagg et al., 1992).

The role of the large, aspiny cholinergic interneurons in the integration of the striatal network is reflected in the variety of different synaptic connections they form. In cats, Hassler et al. (1978) established that these neurons receive synapses from a small number of cortical afferents and also receive direct innervation from neurons in the intralaminar thalamic nuclei (Chung et al., 1977). Dopaminergic fibres from the SNpc have been found to form terminal connections with the dendrites and somata of striatal cholinergic interneurons in the rat (Chang et al., 1988) and it has also been established that the large aspiny interneurons also form synapses with other striatal cells including other neurons of their own phenotype (Pickel and Chan, 1990).

Other aspiny neurons

There is also evidence to suggest the existence of third, aspiny striatal
interneuron (Chang et al., 1982) which was first mistaken to be a population of glial cells by Dimova et al. (1980). These neurons are of medium size and have polygonal or fusiform perikarya which usually give rise to up to five primary dendrites and are considered to be interneurons (Theriault and Landis, 1987) and ultrastructurally, they possess deeply indented nuclei, often containing intranuclear rods (Cowan et al., 1990). It has been established that this sub-population of striatal neurons contain vasocactive intestinal polypeptide (VIP) and appear to be selectively distributed close to the fine white matter bundles which traverse the striatum (Theriault and Landis, 1987).

An additional population of GABAergic interneurons has also been identified within the striatum (Kita and Kitai, 1988). In 1990, two research groups established that these neurons were immunopositive for parvalbumin (Kita et al., 1990; Cowan et al., 1990). The somata of parvalbumin-positive neurons were found to be present in both the patch and matrix of the striatum and parvalbumin positive fibres were able to cross the boundaries between both compartments (Cowan et al., 1990).

(1b) Substantia nigra

The substantia nigra is found in the ventral midbrain tegmentum and comprises the pars compacta, the pars reticulata and the pars lateralis. This introduction will mainly concentrate on the dopaminergic neurons of the SNpc which project to the striatum forming the nigrostriatal pathway. The SNpc consists of a compact sheet of 4 to 10 layers of neurons lying over the more sparsely populated, ovoid SNpr (Fallon and Loughlin, 1985). The 'sheets' of dopaminergic SNpc neurons are morphologically different; the majority of cells in the most ventral layers have inverted pyramidal-shaped perikarya whose dendritic processes extend locally and into the SNpr. Dopamine (DA) has been identified in the perikarya, axons and dendrites of these cells (Björklund and Lindvall, 1975). The more dorsal layers have fusiform cell bodies which extend their processes to limbic and cortical structure, and Anden et al. (1966a) estimated that the axon of a typical human nigrostriatal dopaminergic neuron (taking account of its many branches) was approximately 55-77cm long (for review see Fallon and Loughlin, 1985).

Approximately 95% of the nigral neurons which comprise the nigrostriatal pathway are dopaminergic (Van der Kooy et al., 1981). Ascending axons collect in the rostral ventral tegmental area and medial SNpc before projecting to the striatum via the medial forebrain bundle and the internal capsule (Fallon and Loughlin, 1982). AChE has been identified in both the neuropil and cell bodies of nigrostriatal projection neurons (Lehman and Fibiger, 1978; Meibach and Weaver, 1979; Henderson, 1981) and, in human tissue, 13% and 24% of nigral tyrosine hydroxylase (TH) (a marker enzyme for dopamine) immunopositive neurons express mRNA for NT-3 and BDNF.
respectively (for review see Seroogy and Gall, 1993). The SNpc also expresses high levels of trk B, the high affinity receptor for the neurotrophin BDNF, and slightly lower levels for trk C, the high affinity receptor for NT-3 (Altar et al., 1994).

The projection pattern of nigrostriatal neurons is topographic; medially placed dopaminergic nigral neurons extend axons to the medial, ventral and anterior striatum, while the more laterally placed neurons project to the lateral and posterior regions of the striatum (Fallon and Moore, 1978b; Fallon et al., 1978b). Not all of the dopaminergic SNpc neurons project to the striatum, and nigroamygdaloid, nigroseptal and nigrocortical projections have all been identified as originating from this region (Fallon, 1981; Fallon and Loughlin, 1982).

2 - Neuronal response to injury

Much research has taken place into clarifying the different responses of mammalian peripheral and central neurons to axotomy in an attempt to understand their different outcomes; injured peripheral nervous system (PNS) neurons have the ability to regenerate and successfully reconnect with their original target tissues but injured neurons intrinsic to the CNS do not, resulting in the complete loss of function in the damaged pathway. Neurons which project from the CNS into peripheral nerves exhibit similar regenerative powers to neurons with cell bodies in the PNS. In contrast, the central processes of primary sensory neurons, which project from the PNS into the CNS are incapable of regeneration to their normal targets following axotomy (Carlstedt., 1983, Reier et al., 1983). This provides good evidence that the environment surrounding regenerating axons, rather than the environment surrounding their neuronal cell bodies, is a powerful influence governing the extent of axonal elongation. None the less, there is evidence that at least some intrinsic CNS neurons may differ from PNS neurons in their metabolic responses to axotomy, and that this in turn may also influence the extent of axonal regeneration. Thus research on CNS regeneration in mammals has been split between examining the somal response of the injured neurons (Barron et al., 1970; Lieberman, 1971) and investigating the physical and biochemical characteristics of their surrounding environment (Berry, 1982; Schwab and Caroni, 1988; Caroni and Schwab, 1988a and b). The response of adult mammalian CNS neurons to axotomy is in marked contrast to the situation in lower vertebrates where the ability to regenerate functional connections after injury is found in both the PNS and CNS (for review see Holder and Clark, 1988). Consequently there has also been some effort to identify the differences in the response to injury of lower vertebrate CNS neurons, and any differences in the environment found within the CNS of these animals when compared to mammals.

(2a) The effect of axotomy on PNS neurons

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The majority of damaged neurons of the PNS are able to successfully regenerate axons back to the regions of their original targets. It is in the somata of injured peripheral neurons that the first steps towards mounting a regenerative response take place; these changes within the cell body are known as the axon reaction and appear to be dependent on the retrograde transport of signals from the site of injury. The nature of the retrograde effects which stimulate the cell body response to axotomy is, as yet, unknown and the question remains as to whether it is a disruption in the normally continuous supply of some factor retrogradely transported to the cell body, either derived from the target tissue, as suggested by Cragg (1970), or perhaps a metabolite produced in the axon terminals, which signals that an injury has occurred, or if a specific injury signal is produced at the site of axotomy. Nja and Purves (1978) demonstrated that the retrograde transport of a target derived trophic factor such as NGF may play a role in suppressing the regenerative response of intact neurons; application of NGF to the cell bodies of axotomised sympathetic ganglion neurons prevented the onset of the axon reaction while the use of NGF antiserum induced this response in uninjured cells. Experiments by Singer et al. (1982) have, in contrast, shown that the administration of colchicine, which blocks axonal transport, at the time of axotomy was found to delay the onset of chromatolysis in injured rat peripheral neurons.

One of the most notable structural changes which occurs during the axon reaction takes place within 24-48 hours of injury and is characterized by the breakdown of Nissl substance within the cell body - an event known as chromatolysis. This process usually begins in the centre of damaged neurons and rapidly escalates, spreading towards the periphery of the perikaryon (Barron et al., 1970; Lieberman, 1971) although instances where chromatolysis begins in the margins of the perikaryon and progresses centrally have been reported (Cammermeyer, 1969). Axotomy has a number of other characteristic effects on the morphology of the affected neuron. Most noticeably, at the LM level, the perikarya of injured neurons enlarge and this is evident for up to 20 days after injury. Ribosomes are also lost from the rough endoplasmic reticulum (RER) resulting in an increase in the number of free ribosomes within the perikaryal cytoplasm. The nuclei of neurons undergoing chromatolysis characteristically adopt an eccentric position opposite the axon hillock (for reviews see Lieberman, 1971, 1974, Barron, 1989).

The increased expression of several molecules thought to be necessary for axonal regrowth is a prominent feature of the cell body response to axotomy in peripheral neurons. For example GAP-43 is up-regulated (see section 5b) and changes in the expression of cytoskeletal components also occur; PNS neurons show a marked down-regulation in the synthesis of neurofilament RNA and protein after injury and during regeneration (McQuarrie, 1983; Hoffman et al., 1987). However, the rate of transport
and synthesis of two tubulin genes which are involved with the axonal transport mechanism, T α I and class II β, is usually increased (Miller et al., 1989) although there is some variation in this between species and the nature of the injury experienced (Giulian et al., 1980; Oblinger and Lasek, 1988).

Changes also take place in the axon of the damaged neuron at the site of injury. The part of the axon distal to the damage undergoes Wallerian degeneration and the myelin sheath around the fibre degenerates and the debris is phagocytosed by attracted macrophages to leave only the basal lamina of the original fibre, which is soon filled with proliferating Schwann cells (Sunderland, 1978). The resultant Schwann cell filled basal lamina tubes are known as bands of Büngner. Macrophage recruitment takes place during the first 3 to 5 days after injury and, in keeping with their role of clearing debris, these cells are mainly found in areas which contain degenerating axons (Perry et al., 1987). Macrophages are essential for the process of debris removal and Schwann cell proliferation in damaged peripheral nerves (Beuche and Friede, 1984; Scheidt and Friede, 1987).

This was most clearly demonstrated in interesting experiments in which segments of transected mouse peripheral nerve were placed in diffusion chambers selectively impermeable to macrophage invasion with the result that, although myelin was discarded by the degenerating axons as normal, no phagocytosis occurred and the surviving Schwann cells did not proliferate. However, when chambers permissive to the invasion of macrophages were used phagocytosis did take place (Beuche and Friede, 1985). It is also possible that macrophages may not act alone when clearing debris from the degenerating nerve, as evidence exists which suggests that Schwann cells may play a minor role in this process either by independent phagocytosis (Allt et al., 1976) or acting synergistically with the macrophages (Nadim et al., 1991).

The role of macrophages in the initial onset of peripheral nerve regeneration was shown in a study of a strain of mice (C57BL/Ola) which show very slow, much reduced macrophage recruitment after peripheral nerve injury (Lunn et al., 1989). In these animals Wallerian degeneration does take place but extremely slowly. Surprisingly, peripheral nerve regeneration after crush injury takes place at the normal rate in these animals (Lunn et al., 1989; Brown et al., 1991, for review see Perry and Brown, 1992) but the axons mainly elongate along columns of formerly unmyelinating Schwann cells (Brown et al., 1992b) and columns of Schwann cells which previously contained motor axons which degenerate normally (Martini, personal communication).

Macrophages performing the phagocytosis of myelin debris have been shown to release factors into the area of damage which stimulate the proliferation of Schwann cells (Baichwal et al., 1988). The rate of Schwann cell proliferation in regenerating adult mouse sciatic nerve was found to be comparable to the rate of Schwann cell proliferation measured during development (Bradley and Ashbury, 1970). There is also evidence to
suggest that the cytokine interleukin-1 (which mediates the synthesis of NGF by non-neuronal cells, particularly Schwann cells) may be released by the incoming macrophages (Lindholm et al., 1987) although Matsuoka et al. (1991) reported that NGF mRNA levels in Schwann cells \textit{in vitro} were relatively resistant macrophage products. After injury, proliferating Schwann cells on both the proximal and distal side of a transected nerve form a bridge between the distal and proximal stumps (Scaravilli et al., 1986; LeBeau et al., 1988; Sunderland, 1978). Although the extracellular matrix of the original degenerated nerve, especially the basal lamina, is a permissive substrate for regenerating axons and is rich in growth supporting molecules such as laminin and fibronectin (Rogers et al., 1983; Bozyczko and Horwitz, 1986), it is the presence of living Schwann cells which offers the greatest support to regenerating peripheral axons. Most studies which have examined the regenerative response of axotomised peripheral neurons along segments of freeze-killed peripheral nerve, which contain no living Schwann cells, have shown that axons will only regenerate into the segments when either preceded or accompanied by Schwann cells migrating from the proximal stump (Anderson et al., 1983; Hall et al., 1986a). The rate of axonal regeneration after crush injury into peripheral nerves rendered acellular by freeze-thawing was found to be 40% slower than in control nerves containing living Schwann cells (Bresjanac and Sketelj, 1989) and this rate was decreased by a further 30% if the nerves were made acellular by scalding which, in addition to killing Schwann cells, also denatures basal lamina proteins (Sketelj et al., 1989). However, Tajima et al. (1991) found that destruction of the cellular components of PN grafts by freeze-thawing was advantageous in reducing the host immune-response to allografts from outbred donors of the same species, ultimately leading to better peripheral nerve regeneration than those receiving cellular grafts.

The denervated Schwann cells in the distal stumps of axotomised peripheral nerves provide a highly favourable substrate for regenerating peripheral nerve axons; they are known to upregulate the synthesis of neurotrophic factors such as NGF, BDNF, and NT-3 (Heumann et al., 1987; Meyer et al., 1992 and Maisonpierre et al., 1990 respectively) and express the cell surface adhesion molecules L1 and N-CAM which homophilically bind to the same molecules expressed on regenerating peripheral nerve growth cones and groups of growth supporting molecules are covered in greater detail in sections 5a and 5c.

Regenerating axonal sprouts have been noticed emerging from the proximal stump of damaged myelinated and unmyelinated peripheral fibres as early as a few hours after injury (Bray et al., 1972; Morris et al., 1972b; Friede and Bischauzen, 1980; McQuarrie, 1985). The tips of such regenerating sprouts are characteristically swollen and contain many different types of densely packed axoplasmic organelles including neurofilaments, smooth endoplasmic reticulum (ER), mitochondria and many microtubules (Bray et al., 1972; Bray and Aguayo., 1974; Morris et al., 1972, Duce and
Keen, 1976). Although many sprouts might arise from one damaged axon, survival and maturation of a regenerating fibre is dependent on making a successful target connection; those which do not achieve this fail to mature and may die back (Cragg and Thomas, 1964).

3 - Regeneration in the mammalian CNS

Neurons intrinsic to the mammalian CNS show a variety of responses to axotomy varying from cell death to changes in the size of perikarya and enzyme expression (reviewed by Leiberman, 1974). In general however, the effects of axotomy are less closely associated with regeneration and have been described as 'almost without exception a monotonously atrophic process' (Barron, 1989). This is very different from the response of neurons in the CNS of lower vertebrates such as fish and amphibians. Lesion of central neurons of animals in these phyla commonly results in the complete regeneration of nerve fibres back to their original area of target innervation, (Hibbard and Omberg; 1976). For example, the regenerating optic nerve fibres of fish appear to take a similar route to the tectum as they do during development (Attardi and Sperrry, 1963) and, unlike birds and mammals, are able to traverse areas of extensive glial scarring en route (Tuge and Hawanza, 1937; Koppanyi, 1955). However, as such ready regeneration does not take place in the mammalian CNS, its response to injury needs to be carefully examined in order gain any insight into the different physiological features of these two contrasting conditions.

(3a) The effects of axotomy on mammalian CNS neurons

Cell death

Cell death is a more common result of axotomy in the CNS than in the PNS. For example 90% of retinal ganglion cells (RGCs) in adult rats die within 1 month of intraorbital optic nerve section (Villegas-Perez et al., 1988; Sievers et al., 1989) compared with estimates of between 30 and 0% cell death in primary sensory neurons following peripheral axotomy (Rich et al., 1987). Observations on cell death in the CNS following injury date back to Marinesco (1909; for review see Lieberman, 1974) who described extensive cell death amongst Betz cells in the cerebral cortex of adult rabbits after axonal lesion in the internal capsule. Later experiments reported similar magnitudes of cell loss resulting from CNS damage; for example Morton (1969) reported the death of over 80% of human paraventricular and supraoptic nuclei neurons of 1 year after hypophysectomy. Extensive cell loss was recorded amongst magnocellular neurons of the red nucleus of adult and new born rats after hemisection of the mid-thoracic spinal cord (Prendergast and Stelzner, 1979), and dorsolateral lesion of the spinal cord of adult
cats at segment T12/L1 resulted in substantial neuronal loss from Clarke's nucleus at
segment L3 (Liu, 1955; Loewy and Schader, 1977). However, the number of cells no
longer detectable in any one region after central injury may not be the same as the number
which actually die. Gage et al. (1986) reported that lesion of the septohippocampal
pathway resulted in extensive apparent cell death within the septum but this was later
found to be severe neuronal atrophy giving the illusion of cell loss since subsequent
intraventricular infusions of NGF induced the reappearance of the axotomised neurons
(Hagg et al., 1987, 1988). A similar apparent neuronal loss has also been reported for
cholinergic brain-stem neurons; although over 95% of vagal neurons could no longer be
detected 7 days after unilateral axotomy compared to those in the contralateral control
side, there was no significant difference in the number found using Nissl stains or
retrograde labelling (Lams et al., 1988).

Cytological changes in injured CNS neurons

The cell body response of injured neurons in the CNS shares some
morphological characteristics with that of injured peripheral neurons, such as
chromatolysis and the displacement of the nucleus to an eccentric position (for review see
Lieberman, 1974; Barron, 1983 a and b). Again, chromatolysis begins rapidly after
axotomy and typically proceeds from a region part way between the nucleus and axon
hillock to include all the Nissl substance in the perikaryon (Lieberman, 1974; Barron,
1983 a and b) occasionally forming a perinuclear 'cap' of Nissl substance concentrated
around the nucleus (Barron, 1983a). This process is nicely illustrated by the response of
Betz cells in the cerebral cortex of adult cats after lateral funiculotomy as reported by
Egan et al., (1977). At the EM level chromatolysis begins with a shortening and
disruption of the linear organization of the cisternae of the RER and an increase in
cytoplasmic smooth ER (Lieberman, 1971; Barron, 1983 a and b). This process has
been observed in injured neurons of the rat red nucleus (Barron et al., 1975) and the
lateral geniculate nucleus of adult monkeys (Wong-Riley, 1972). Other organelles are
also disrupted during the processes which comprise the cell body response; the
vesiculation and vacuolation of the Golgi apparatus may occur (Barron et al., 1975), and
a variety of changes in mitochondria have been observed in different species, ranging
from mild disruption in rat red nucleus neurons after lesion of the rubrospinal tract (Egan
et al., 1977) to their extensive accumulation within injured cells of the monkey LGN after
ablation of the ipsilateral visual cortex (Wong-Riley, 1972). In central neurons however,
unlike those of the PNS, any hypertrophic response of injured cells is short lived and
typically consists of a transient perikaryal enlargement followed by severe cellular
atrophy and, in many cases, cell death (Barron et al., 1977, 1989; Egan et al., 1977).

Other aspects of normal neuronal metabolism of cytoskeletal proteins are
affected by injury in the CNS. As in the PNS, the neurofilament content of injured CNS
neurons is also affected by axotomy and levels vary widely between each case examined (see Barron et al., 1967, 1975; Egan et al., 1977). Rosenfeld et al. (1987) suggested that the increase in neurofilament protein levels witnessed in some injured CNS neurons (e.g. Oestreicher et al., 1988) may be a consequence of abnormal phosphorylation of neurofilaments and disruption of their transport from the perikaryon to their axons. Conversely, axotomised neurons in the red nucleus of adult rats showed a pronounced down-regulation of RNA and protein synthesis (Barron et al., 1977). In view of this, it is not surprising that neurotransmitter activity and the levels of related receptors and enzymes within injured CNS cells are also affected (reviewed by Lieberman, 1971). Lesion of the rubrospinal tract of adult rats at the cervical, thoracic or lumbar level of the spinal cord resulted in significant down-regulation of AChE and ChAT activity in the magnocellular neurons of the red nucleus (Gwyn, 1971) and a similar down-regulation in the activity of these enzymes was also reported in hypoglossal neurons after undergoing hypoglossal nerve crush (Wooten et al., 1978). Interestingly, the position of an injury has some effect on the subsequent modulation of enzyme levels; the effect of rubrospinal tractotomy at the cervical level is noticeable 4 days after injury but similar enzymic down-regulation is not apparent until 21 days after thoracic or lumbar tractotomy (Waldron and Gwyn, 1969; Gwyn, 1971).

(3b) Factors affecting the responses of intrinsic CNS neurons

Differences between species

Some unexplained variations have been identified between the neuronal response of different species to similar CNS injuries. Neurons in the lateral geniculate nucleus (LGN) of monkeys and rabbits display a swifter and more extensive response to cortical extirpation, with greater subsequent cell loss than those in the cat (Chow and Dewson, 1966; Mihailovic et al., 1971). Rats also showed a greater degree of neuron loss from the paraventricular and supraoptic nuclei after hypophysectomy and/or lesion of the hypothalamo-hypophyseal tract than did ferrets after the same injury (Adams et al., 1971; Raisman, 1973). The RGCs of rats (Villagas-Perez et al., 1988 a and b) and rabbits (Quigley et al., 1977) underwent much swifter atrophy, with more extensive subsequent cell death, after optic nerve lesion than did the RGCs of cats after the same injury (Holländer et al., 1985). The reasons for these differences are not obvious.

Age at time of injury

Differences were also found between the response of animals of the same species to analogous injuries dependent on their age. Lesion of the optic nerve leads to varying degrees of RGC loss in rodents of different ages. For example, after optic nerve transection, very few, if any (0-10%), RGCs of neonatal mouse or rat survive
(Muchnick-Miller and Oberdorfer, 1981; Allcutt et al., 1984) but up to 20% of adult mouse RGCs will survive for 10 days after optic nerve crush (Allcutt et al., 1984). Barron et al. (1986) reported that 32% of adult rat RGCs survive axotomy for up to 6 months after intraorbital optic nerve crush. However, the number of surviving neurons was lowered to only 10% at 1 month post lesion by full intraorbital nerve transection (Villagas-Perez et al., 1988 a; Sievers et al., 1989). Interestingly, Holländer et al., (1985) were still able to retrogradely label axotomised cat RGCs 15 months after initial axonal transection. Cerebellar lesion in neonatal mice and rabbits resulted in the loss of all neurons from the inferior olivary nucleus in all animals aged between 8 and 11 days old at the time of injury within 8 days of the operation, but only 30% - 50% of these neurons were lost in adult animals receiving the same injury (reviewed by Lieberman, 1974). Furthermore, the smaller degree of neuronal loss reported for adult animals in this study was not seen until 8-16 days after injury. Neonatal rats also lost more magnocellular neurons from the red nucleus after a contralateral hemisection of the mid-thoracic spinal cord than adult animals (Prendergast and Stelzner, 1976). Lesion of the brachium of the superior colliculus of syrian hamsters on or between postnatal days 1 and 3 was not found to disrupt visually elicited turning responses but this behavior was lost if the same lesion took place after postnatal day 3 (So et al., 1981). Significantly, if the lesion took place on or before the day of birth the animal displayed inappropriate turning behavior to the visual stimuli indicative that the developing fibres were unable to successively tranverse the region of injury to their correct targets if the pathway had not been already established prior to the ablation.

Proximity of the lesion to the cell body

One factor which has a demonstrable effect on the cell body response of injured central neurons is the distance between the site of axonal injury and the neuronal soma, the accepted hypothesis being that the more proximal the injury to the cell body, the quicker the consequent retrograde signal, the sooner the sequence of retrograde events in the perikaryon and more widespread the resultant neuronal degeneration (reviewed by Lieberman, 1974; Barron, 1983a and b). Lesion of the dorsolateral quadrant of the cat spinal cord at the T12 level lead to more extensive cell loss from Clarke's column (L3) than did a closer comparable injury at C4 (Liu, 1955). A later investigation of the same surgical paradigm revealed that a dorsolateral lesion of the spinal cord at the L1 level resulted in a loss of up to 30% of the large neurons of Clarke's column (L3) 3 months after injury but only 5% of these neurons degenerated after a medullary lesion of the dorsal spinocerebellar tract. Similarly, a greater number of neurons were lost from the red nucleus of adult rats after a cervical, compared to a thoracic, lesion of the rubrospinal tract (Egan et al., 1977). Sofroniew and Isaacson, (1988) suggested that a fixed spatial relationship existed between the degree of neuronal loss from the septum of adult rats
and their distance from the lesion in different portions of the fimbria-fornix. They estimated that approximately 70% of septal cholinergic neurons were lost (compared to the number seen in controls) if the fimbria-fornix lesion occurred within 2500μm of their perikarya compared to only 20% when the lesion was beyond 4500μm, with very small differences in the position of these lesions causing significant differences in the number of cells lost.

RGCs are also sensitive to the distance between axonal injury and their perikarya with estimates of up to 90% of adult RGCs degenerating within 1 month of intraorbital optic nerve transection (Berry et al., 1987; Villegas-Perez et al., 1988 a and b; Sievers et al., 1989). Intracranial optic nerve crush or transection however, resulted in much slower and less extensive loss of RGCs with 40% of RGCs surviving to be counted up to 7 months after injury (Misantone et al., 1984). The difference in the extent of neuronal survival appears to be finely graded between intraorbital and intracranial injury. Villegas-Perez et al., (1988b, 1989) found that while only 18% of RGCs survived a transection made 0.5mm from the eye (when compared to the number of RGCs counted in the contralateral control retina) the percentage of RGC survival increased steadily as the distance from the eye was increased, ie at 3mm from the eye 31% of RGCs survived, at 8mm 55% survived and at 10mm just over 70% of RGCs could still be counted 1 month after axotomy. Although this relationship exists, the nature of the multiple signals which induce the axon reaction in central and peripheral neurons is still unknown and why the response is greater following proximal rather than distal axotomy is still a matter for speculation.

4 - Why does axonal regeneration normally fail in the CNS?

The apparent inability of injured CNS neurons to regenerate is an important but widely researched and complicated phenomenon. As a result, many hypotheses have been proposed to account for the lack of axonal regrowth in the CNS (for example Ramon y Cajal, 1928; Clark, 1942 and 1943; Clemente, 1964; Kieman, 1979; Berry, 1982, 1983, 1985 and 1989; Aguayo, 1985) but no single hypothesis is supported by all the evidence revealed by almost a century of research. For many years it was believed that neurons of the mammalian CNS were simply intrinsically unable to regenerate due to some unidentified genetic restriction (Clark, 1942 and 1943; Barron, 1983 a and b). This view was largely abandoned following the success of experiments in which CNS axons were shown to regenerate into PN grafts (Aguayo, 1985; Berry et al., 1986a and b). In its place the role of the environment within the CNS was emphasized. The physical limitation imposed by extensive glial scarring after CNS injury was also identified as possible factor preventing CNS regeneration (Ramon y Cajal, 1928; Clemente, 1955; Windle, 1956; Reier, 1986; Reier et al., 1989). To compound this, molecules inhibitory
towards axonal regeneration have also been identified within the CNS (Berry, 1982; Schwab and Caroni, 1988; Caroni and Schwab, 1988 a and b). Coupled with the absence or low levels of those neurotrophic and growth permissive factors which abound in the PNS (as advocated by David and Aguayo, 1981; and Berry 1983) this makes the CNS a particularly unfavourable environment for axonal regeneration even from neurons possessing the potential for axonal regeneration.

(4a) The environment of the mammalian CNS is non-permissive for axonal regeneration

Astrocyte scars

The gliosis which occurs following injury to the CNS (comprising astroglial proliferation and hypertrophy) has long been regarded as an important factor inhibiting axonal regeneration (Ramon y Cajal, 1928; Reier et al., 1983, 1988). Glial scars are not entirely composed of astrocytes but often contain many other non-neuronal elements of the CNS, such as oligodendrocytes and microglia, and also macrophages and blood vessels (Clemente, 1955; Reier, 1986; Reier et al., 1989). None the less, they mainly consist of hypertrophied astrocytes (Reier, 1986).

Glial scars were originally thought to constitute a mechanical or physical barrier to axonal regeneration (Ramon y Cajal, 1928; Clement, 1953; Windle, 1956). Early histological investigation showed that regenerating CNS axons often entered the region of gliosis but their growth soon terminated within the scar (Ramon y Cajal, 1928; Sugar and Gerard, 1940). Perhaps the most compelling evidence that glial scars can inhibit regeneration comes from work involving elongating peripheral axons, either regenerating dorsal roots approaching the spinal cord or regenerating peripheral nerve fibres encountering transplanted CNS tissue.

Initially, either crush or transection of dorsal root fibres between the dorsal root ganglion and spinal cord stimulates a vigorous regenerative response from primary afferent neurons (Nathaniel and Nathaniel, 1973; Reier et al, 1983; Stensaas et al., 1987). Injured fibres readily extend along the peripheral portion of the dorsal root but cease elongation once they make contact with the CNS boundary, the dorsal root entry zone (DREZ). It is known that the DREZ contains large numbers of astrocyte processes (Carlstedt, 1985b). The effect of simulating astrocytic scar conditions on regenerating peripheral nerve fibres has also been examined by apposing a segment of living optic nerve tissue to the proximal stump of a transected peripheral nerve (Reier et al., 1983; Anderson and Turmaine, 1986; Hall and Kent, 1987; Anderson et al., 1989). The result in every case was that only a small number of regenerating peripheral fibres were identified in the CNS tissue and these were limited to the first 100μm of the graft (eg Anderson et al., 1989).

More recently it has been suggested that astrocytes provide a 'physiological stop
signal' to elongating axons (Rees et al., 1976; Lasek et al., 1987). Interestingly, a number of studies have reported the presence of occasional synapse-like structures at the terminal bulbs of the few axons which do manage to penetrate central astrocytic scars. Configurations which resembled vesicle-rich presynaptic endings were identified by Richardson et al. (1982) when using EM and autoradiography to examine the appearance of astrocytic scaring in the rat optic nerve 4 and 8 weeks after intracranial transection. Similarly, aggregations of neurotransmitter vesicles and synapse-like structures were reported at the terminals of regenerating cholinergic ventral root fibres which had penetrated short distances into the DREZ of adult rats 6 and 9 months after ventral root transection and anastomosis (Carlstedt, 1985). Further to this, Liuzzi and Lasek (1987) found that the cytoskeletal protein content of regenerating dorsal root axons differed between those which had suffered a complete transection or a crush injury. Those which came in contact with reactive astrocytes at the DREZ after L5 dorsal root transection showed no apparent build-up of neurofilament levels up to 3 months post transection at which time the neurofilament content of those neurons which had only been ligated had increased significantly. This lead to the hypothesis that the growth cones of regenerating axons and the reactive astrocytes of the injured CNS act upon each other to stimulate the activation of intra-axonal proteolytic enzymes which breakdown cytoskeletal proteins, such as neurofilaments and microtubules making axonal growth impossible. This corresponds with the normal course of events witnessed in the CNS during development when the appropriate synaptic contacts are established (Rees et al., 1976; Lasek et al., 1987). Application of a protease inhibitor to the DREZ after dorsal root injury abolished this effect and lead to a huge build-up of neurofilaments and other cytoskeletal proteins within regenerating neurons (Liuzzi, 1990).

However, astrocytic scaring does not have the same inhibitory effect on regenerating fibres in the CNS of lower vertebrates (for review see Berry, 1982) but this may be indicative of differences in the surface of astrocytes between lower vertebrates and mammals or in the regenerative abilities of their central neurons.

In light of the above reports it is surprising to find that in some instances reactive astrocytes have been found to form a favourable substrate for mammalian CNS axonal regeneration. In vitro, the rate of neurite extension and cell growth of cultured rat cerebella and spinal cord neurons plated on a monolayer of astrocytes was greater than on any other neuronal or non-glia substrates (Noble et al., 1984) and cultures of embryonic chick RGCs increased neurite outgrowth by 2.2 fold when plated on an astrocyte monolayer compared to laminin (Neugebauer et al., 1988). Examination of rat dorsal root ganglia (DRG) explants co-cultured with neonatal cortical neurons using EM revealed that the growing cells made preferential contact with astrocytic substrates compared to the collagen substrate also available to them (Ard and Bunge, 1988). However, Fawcett et al. (1989) reported that although monolayers of astrocytes supported axon growth in...
vitro, explants of adult retina and postnatal DRG neurons were unable to grow through purified three dimensional astrocyte cultures. Significantly, axons from both embryonic retinae and DRG neurons readily grew through these cultures replicating their behavior in vivo during development.

In vivo, a rise in the level of glial fibrillary acidic protein (GFAP) immunoreactivity in layer CA3 of the hippocampus, dentate gyrus and other connected nuclei between 1 and 30 days after lesion of the fimbria-fornix pathway was interpreted by Gage et al. (1988) as evidence that reactive astrocytes in these areas were acting as a permissive substrate for the injured intrinsic neurons. Kawaja and Gage (1991) also found that following excitotoxic lesion of the rat nucleus basalis with ibotenic acid and the subsequent implantation of modified fibroblast growth factor (FGF)-producing fibroblasts into the striatum, reactive astrocytes were seen to be closely associated with regenerating nucleus basalis axons, even as far entering the graft tissue with them. Embryonic astrocytes, coated onto specially designed Millipore implants, have also been used to promote the regeneration of DRG neurons through the DREZ into the adult spinal cord after crush injury (Kliot et al., 1990).

Axonal growth inhibiting molecules derived from oligodendrocytes

Berry (1982) proposed that CNS myelin, or some unidentified factor produced as a byproduct of its breakdown, may actively suppress axonal elongation, possibly by affecting growth cone structure or function, altering perikaryal protein synthesis (after being internalized within the damaged neuron) or disrupting their receptiveness to essential growth factors. Evidence supporting this hypothesis was drawn from experiments in which some non-myelinated, or even thinly myelinated, fibres showed a surprising degree of regeneration after chemical injury to the adult mammalian CNS (Bjorklund and Stenevi, 1979) and in which neurons were shown to regenerate axons but only in areas lacking CNS myelin, such as in the pituitary stalk (Adams et al., 1968; see Berry, 1982 for review). Additionally, evidence of regeneration in the CNS of neonatal animals at times before mature CNS myelin has been formed may also be taken to support the hypothesis (but see section 6 which addresses some of the problems involved in interpreting CNS regeneration).

Oligodendrocytes were later included with CNS myelin as having growth inhibiting properties. Differentiated, but not immature, oligodendrocytes were found to be highly non-permissive substrates for the outgrowth and adhesion of neurites extending from cultures of dissociated sympathetic or sensory ganglion neurons or foetal retinal cells (Schwab and Caroni, 1988). Poly-lysine coated culture dishes preabsorbed with isolated myelin from adult rat spinal cord had the same growth inhibiting effect on similar neuronal explants. Caroni and Schwab (1988b) then identified two highly growth-inhibitory membrane protein fractions with molecular weights of 35 and 250kD.
(determined by SDS-PAGE) from extracts of CNS white matter and named them NI-35 and NI-250 respectively. A monoclonal antibody, NI-1, was then raised against both these proteins and which bound with high affinity to the surface of differentiated oligodendrocytes and, in doing so, neutralized their inhibitory effect (Caroni and Schwab, 1988a).

Many other experiments measuring the extent of neurite outgrowth from single neurons seeded onto different substrates have identified CNS white matter, which is rich in myelin and oligodendrocytes, as being non permissive for axonal growth from a whole range of developing and adult neurons including embryonic chick DRG neurons (Carbonetto et al. 1987) sympathetic ganglion explants (Crutcher, 1989) and neocortical cells (Watanabe and Murakami, 1989) and embryonic rat DRG explants (Bedi et al., 1992), neonatal RGCs and prenatal and adult DRG neurons (Schewan et al., 1993).

Savio and Schwab (1989) showed that preincubation of white matter sections (adult rat spinal cord) with the NI-1 antibody made them permissive substrates for neuroblastoma cells. Using video time-lapse microscopy, neurites from dissociated neonatal DRG neurons have been shown to cease elongation as soon as their growth cones made firm filopodial contact with oligodendrocytes from the CNS of young rats. Although this was often followed by complete growth cone collapse, one third of the neurites observed were able to retract from the unfavourable substrate after a brief period of paralysis (Bandtlow et al., 1990). Significantly, in vivo studies have shown that intraventricular grafts of hybridoma cells secreting NI-1 encourage the formation and growth of axonal sprouts up to 7-11mm caudal to a mid-thoracic transection of the rat corticospinal tract within 2-3 weeks of injury (Cadelli and Schwab, 1991) and application of the NI-1 antibody to ipsilateral retinofugal fibres after postnatal ablation of one superior colliculus prevented the usual abnormal sprouting of these fibres across the tectal midline to innervate the remaining superior colliculus but allowed them to cross the transiently oligodendrocyte-rich stratum opticum and to terminate deeper within the superficial gray than was seen using control vehicle only (Kapfhammer et al., 1992). Application of these antibodies in vivo has also been shown to enhance axonal regeneration of lesioned corticospinal tract fibres across grafts of embryonic spinal cord tissue implanted into the lower thoracic spinal cord of adult rats (Schnell and Schwab, 1993).

However, experiments involving Browman-Wyse (BW) mutant rats have demonstrated that absence of oligodendrocytes and the inhibitory factors associated with them from the CNS is not enough to promote neuronal regeneration within the adult CNS after injury. The BW rats lack both oligodendrocytes and CNS myelin from a region of their proximal optic nerve near the retina. This region, which varies in length (2-4 mm) between animals, consists mainly of unmyelinated RGC fibres, astrocytes and occasionally, Schwann cells and the few myelinated fibres found within this region are
myelinated by Schwann cells (Berry et al., 1989). The optic nerve distal to this oligodendrocytes-free region appears to be normal (Berry et al., 1989). After optic nerve lesion, injured RGCs were only able to extend fibres into those B-W optic nerves which also contained endogenous Schwann cells; the oligodendrocyte-free regions alone, which contained astrocytes only, were unable to support regeneration (Berry et al., 1992). The RGC of control B-W rats which contained normal CNS myelin along with Schwann cells in their optic nerves, were also unable to regenerate after injury (Berry et al., 1992). In the periphery Hall et al., (1992) found that transected sciatic nerve fibres were unable to regenerate across bridges of oligodendrocyte-free optic nerve tissue unless Schwann cells were present too. This demonstrates that the absence of CNS myelin and oligodendrocytes from a region of the CNS is not enough to promote axonal regeneration after injury. However, the presence of Schwann cells, which can provide a growth promoting environment is not enough to overcome the strong inhibitory effects of CNS myelin.

Adult CNS oligodendrocytes and myelin are not growth-inhibitory to all neurons. Two research groups have recently found that different types of embryonic neural tissue are able to extend axons for long distances along the CNS white matter tracts of adult rats. Wictorin et al. (1990) reported that human telencephalic neuroblasts (cells taken from 8 - 10 week old aborted foetuses) transplanted into the excitotoxically lesioned striatum of immuno-suppressed adult rats were able to grow axons for up to 20mm along the internal capsule and myelinated fibre tracts. Similarly, by 13-20 weeks after implantation into the excitotoxically lesioned nigrostriatal pathway of adult rats, fibres from human neuroblasts from the dissociated ventral mesencephalon of 6-8 week old embryos were able to extend neurites rostrally into the denervated striatum. Significantly, cortical neuroblasts implanted into the same region developed very few projections into the striatum but their axons densely invaded the frontal cortex instead (Wictorin et al., 1992a). Davies and colleagues found that embryonic mouse hippocampal neurons microtransplanted into the fimbria (Davies et al., 1993) and into the corpus callosum and cingulum (Davies et al., 1994) of adult rats extended along host myelinated fibre tracts as fast as 1mm per day. These fibres, found singly or grouped into fascicles, were intimately integrated and aligned with the host glia and left the white matter tracts at the correct place in order to invade their appropriate terminal fields.

Intraspinal grafts of human neuroblasts in lesioned thoracic spinal cord displayed the same extensive fibre projection as seen in the brain; single fibres were identified, extending both caudally and rostrally along the white matter tracts, up to 10mm from the site of implantation (Wictorin et al., 1990). The rate of neurite extension by embryonic mouse hippocampal neurons transplanted into the corticospinal tracts was slower (0.5mm per day) than those transplanted into the brain (Li and Raisman, 1993). The surprising speed and accuracy of growth by embryonic neurons within what is
usually considered to be a most unfavourable environment has led to speculation that they are unable to 'perceive' the nature of their surroundings as they may not possess the same receptors as adult neurons for the inhibitory factors which are so prevalent in the CNS.

5 - The lack of growth promoting factors in the mammalian CNS

(5a) Neurotrophic factors

Certain molecules have been identified which have pronounced effects on neurite outgrowth from, and the regeneration and even survival of, distinct neuronal populations. These molecules have been named growth or 'neurotrophic' factors. They have effects on in the PNS and CNS of both embryonic and adult animals and are strongly conserved across many different species. There are many different recognised factors having distinct neurotrophic properties for different classes of neurons. Consequently elucidating the role of each factor in every neuronal population throughout its development, maturation and response to injury is an enormous task and, to date, no account of neurotrophic factors, their number or their functions, can be taken as complete.

Current reviews include the following molecules as having neurotrophic properties:

- Nerve growth factor (NGF),
- Brain derived neurotrophic factor (BDNF),
- Neurotrophin-3 (NT-3),
- Neurotrophin-4/5 (NT-4/5),
- Neurotrophin-6 (NT-6),
- Ciliary neuronotrophic factor (CNTF),
- Glial derived neurotrophic factor (GDNF),
- Basic and acidic fibroblast growth factors (bFGF and aFGF),
- Epidermal growth factor (EGF),
- Insulin-like growth factor 1 (IGF-1),
- Platelet derived growth factor (PDGF),
- Transforming growth factor-b (TGF-b),
- Leukaemia inhibitory factor (LIF).

Those trophic factors which conform to certain criteria (production in neural target tissue, availability in limited amounts, retrograde axonal transport; see Thoenen, 1991) and have a close genetic homology are known as neurotrophins (NGF, BDNF, NT-3, NT-4/5 and NT-6). This review will focus attention on those factors which are particularly interesting in relation to the types of adult CNS neurons injured in this thesis;
the neurotrophins, the cytokine CNTF, and GDNF - a member of the transforming growth factor-β superfamily of growth factors.

Nerve growth factor

The neurotrophic effect of NGF was first identified by Hamburger and Levi-Montalcini in 1949 and since then it has become the most well researched and best known of the neurotrophin family of growth factors. Its neurotrophic properties were originally identified when Bueker (1948) transplanted different types of mouse tumors into chick embryo spinal cords. One tumor, the mouse sarcoma '180', was found to secrete a substance which induced hypertrophy of neighbouring DRG and stimulated the growth of many sympathetic and sensory nerve fibres (Hamburger and Levi-Montalcini, 1949, 1951, 1953). In order to further define the nature of this substance, samples were treated with snake venom, which is an excellent source of phosphodiesterases, to reveal whether the growth promoting fraction was a nucleic acid. Surprisingly, the control samples in this experiment stimulated an even greater neurotrophic response from cultured chick sensory ganglia than the sarcoma extract. The snake venom was found to contain a very similar protein to that produced by the mouse sarcoma '180' (Cohen and Levi-Montalcini, 1956; Levi-Montalcini and Cohen, 1956). In the snake, the highest concentration of this protein was found in the venom gland, and the corresponding organ in the male mouse, the submaxillary gland, was also found to contain very high levels of the neurotrophic factor which, by then, had been named nerve growth factor (Cohen, 1960).

Since then, methods of NGF purification from mouse submaxillary gland have been established (Bocchini and Angeletti, 1969) and the amino acid sequence of the protein has been determined (Angeletti and Bradshaw, 1971). The molecular characteristics of NGF have also been investigated; it is a complex consisting of three subunits - α, β and γ in the ratio of 2:1:2. The β subunit is the neuroactive part of the complex (Greene et al., 1971) and is also referred to as 2.5S NGF (Gage et al., 1988; Williams and Rylett, 1990; Fischer et al., 1991). The combined molecular weight of the complex is around 130,000.

NGF has been shown to be essential for the normal development of the PNS; in vivo injections of antibodies raised against NGF into neonatal mice were found to selectively destroy the peripheral sympathetic nervous system (Cohen, 1960), and exposure of foetuses to the same antibodies resulted in an 80% reduction in the number of dorsal root and trigeminal ganglion neurons in rats and guinea pigs (Johnson et al., 1980, 1986; Pearson et al., 1983 respectively). Primary sensory neurons derived from the neural crest, such as those in the DRG, appear to be sensitive to NGF and its depletion, whereas those neurons derived from the ectodermal placodes, eg nodose ganglion neurons, seem to be unaffected by NGF modulation. This has been
demonstrated in vivo (Pearson et al., 1983; Roher et al., 1988b) and in vitro (Rosenthal et al., 1990). DRG neurons retain their sensitivity to NGF through to adulthood (Johnson et al., 1986), although NGF is no longer essential for their survival (Lindsay, 1988). Sympathetic trunk ganglia are also sensitive to NGF in culture (Rohrer and Barde, 1982) as has been claimed for adult RGCs (Carmignoto et al., 1989, although see also Thanos, 1989, and Cohen et al., 1994, who reported that NGF had little or no influence on the survival of, or outgrowth of neurites from, adult RGCs in vitro).

Although the role of NGF in the developing CNS is not yet clear, there is plenty of evidence to suggest that NGF is important in the normal functioning of the adult brain even though the majority of CNS neurons are insensitive to the molecule. Most of this comes from studies where the level of NGF available to dependent neurons has been altered and the subsequent cellular response monitored. The majority of adult rat NGF-sensitive brain neurons are found in the basal ganglia and basal forebrain, the best known being the cholinergic neurons of the septum. The latter cells do not synthesize NGF (Korsching et al., 1985) but retrogradely transport it from their target neurons in the hippocampus (Whittemore et al., 1988) back to their perikarya in the medial septum (Schwab et al., 1979). Reduced levels of NGF found in the medial septum and nucleus basalis in aged rats have been linked to age-related memory impairments (Fischer et al., 1991; although Hellwig et al. (1990) published results which contradict this). The down-regulation of phenotypic marker proteins such as AChE or ChAT (Fischer et al., 1987), neuronal perikaryal atrophy (Fischer and Björklund, 1991, Hagg et al., 1989) and apparent neuronal cell death (Kromer, 1987; Hagg et al., 1988) have also been associated with a reduction in normal NGF levels. All of these symptoms can be alleviated, or even reversed, by intracerebral infusions of NGF (Fischer et al., 1987, 1991; Fischer and Björklund 1991, Hagg et al., 1989). Intraventricular infusions of NGF increase the synthesis of phenotypic markers, such as ChAT and AChE, beyond the levels seen in normal healthy cells, and cause perikaryal hypertrophy of normal cholinergic neurons in the adult medial septum (Fischer et al., 1987; Hagg et al., 1989a). The cholinergic neurons of the adult striatum are also sensitive to such infusions, although they do not respond as strongly as those in the medial septum (Fischer et al., 1987).

In the adult PNS, non-neuronal cells of the normal sciatic nerve synthesize NGF at a low level (Heumann et al., 1987). However, after transection the rate of NGF synthesis by Schwann cells of the distal stump changes significantly; Heumann et al. (1987) recorded a 15-fold increase in the amount of detectable mRNA for NGF as early as 6 hours after transection of the nerve. The accumulation of NGF by injured nerves has been shown to be biphasic; the first increase in NGF levels is rapid and the result of NGF retrogradely transported from the target tissue, accumulating at the site of axotomy. Heumann et al., (1987) confirmed this by demonstrating that although the levels of accumulated protein in the distal stump increase by 6 hours post transection there is no
significant up-regulation in the local synthesis of NGF mRNA before 3 days post transection. The second increase is concurrent with the invasion of macrophages into the injured nerve, and lasts for several weeks (Heumann et al., 1987, Lindholm et al., 1987). If this invasion is prevented the synthesis of NGF by local non-neuronal cells is inhibited (Lindholm et al., 1987). Date (1994) reported that the level of NGF up-regulation in the distal stump of mouse sciatic nerve, transected 24 hours earlier, was significantly higher in aged (24 month) mice than in aging (12 month old) mice or in young (1 month old) mice when compared to the level in their contralateral non-transected sciatic nerves.

Brain derived neurotrophic factor

BDNF, isolated from samples of pig brain in 1982 (Barde et al., 1982), has a molecular weight of 12,300 and has an approximate 50% amino acid homology with NGF (Maisonpierre, 1990). The levels of BDNF mRNA in the CNS are higher than those of NGF mRNA, particularly in the spinal cord and superior colliculus which are projection sites for many BDNF sensitive neurons. This conforms with the hypothesis that BDNF is a target derived neurotrophic factor (Leibrock et al., 1989). BDNF and NGF show some overlap in specificity; in the PNS both developing (Davies et al., 1986) and adult (Lindsay et al., 1985) dorsal root ganglion neurons are sensitive to NGF and BDNF in vitro, and in the brain both molecules have a trophic effect on cholinergic basal forebrain neurons (Alderson et al., 1991). In addition, BDNF is able to offer in vitro neurotrophic support to particular neuronal populations which are insensitive to NGF, such as placod crest-derived sensory neurons (Lindsay et al., 1985), and can sustain the survival and differentiation of some non-cholinergic neurons of the CNS in vitro, particularly dopaminergic neurons of the ventral mesencephalon (Hyman et al., 1994). However, in vivo, BDNF is unable to provide axotomised nigro-striatal dopaminergic fibres the same degree of protection against degeneration that NGF can give to axotomised medial septal neurons after fimbria-fornix transection (Knüsel et al., 1991; Morse et al., 1993). In intact adult peripheral nerve, the level of immuno-detectable BDNF is negligible. However, upon transection there is a great up-regulation in the rate of BDNF mRNA synthesis (Meyer et al., 1992). This elevated BDNF synthesis takes place in the non-neuronal cells of the nerve, mostly in the Schwann cells, although Meyer et al. (1992) estimated that around 10% of the fibroblasts identified in the nerve were also BDNF immuno-positive. However, the upregulation of BDNF by denervated Schwann cells shows a different pattern of temporal modulation to that of NGF. The level of BDNF mRNA expression is not up-regulated until 3 days post axotomy and then rises steadily, peaking 3 - 4 weeks later at a maximal level ten times greater than that seen for NGF (Meyer et al., 1992).
Neurotrophin-3

A third protein member of the neurotrophin family of growth factors was identified in 1990 (Maisonpierre, 1990). NT-3 has a 57% homology with the amino-acid sequence of NGF and a 58% homology with that of BDNF in the mature rat. These three proteins have a 48% amino acid homology (Maisonpierre, 1990). Not surprisingly there is also an overlap in the functions of NGF, BDNF and NT-3. Although NGF, but not BDNF, can support sympathetic trunk ganglia in culture and BDNF, but not NGF, can support sensory nodose ganglia in culture (Barde et al., 1982). NT-3 shows a much broader specificity and can support both neuronal populations in vitro (Maisonpierre, 1990). However, NT-3 has no effect on cultures of chick ciliary ganglia in vitro, which are supported by the cytokine CNTF (Maisonpierre, 1990) but it is potent in promoting neurite outgrowth from E10 embryonic chick sympathetic neurons (Davies, 1989) and has a neurotrophic effect on E10 nodose ganglia neurons and on DRG neurons, although less than that seen with NGF (Rosenthal et al., 1990). Morfin et al. (1994) found that NT-3 enhanced the neurite outgrowth and branching of embryonic rat hippocampal neurons in vitro and speeded the development of neuronal polarity. In the adult rat, NT-3 has been located in the viscera - the heart, kidney, liver, spleen, and lung (Rosenthal et al., 1990). Given that it is a retrogradely transported neurotrophin it could act as a target derived growth factor for many sympathetic and sensory neurons from these sites. It is also found in the cerebellum, medulla oblongata and hippocampus (Rosenthal et al., 1990). The number of neurons containing retrogradely transported NT-3 was found to differ within the DRG of adult rats; more were found in cervical (36% of total) and lumbar (38%) than in thoracic ganglia (18%). NT-3 immunoreactivity within these ganglia was also found to be confined to a sub-population of larger DRG neurons (Zhou et al., 1994). In the adult rat NT-3, was able to rescue both ChAT and GAD positive striatal neurons from degeneration after excitotoxic lesion of the striatum when infused into the site of injury immediately after an injection of quinolinic acid, reducing the loss of ChAT immunopositive neurons by 23% and GAD immunopositive neurons by 33% when compared to control infusions of vehicle alone (Engber et al., 1994). NT-3 mRNA is easily detectable in the normal adult sciatic nerve but, unlike NGF or BDNF, levels of mRNA for this neurotrophin in the distal portion of the nerve are decreased by 90% at 6 hours after and had returned to the levels seen before injury by 2 weeks post transection (Funakoshi et al., 1993).

Neurotrophin-4/5

In 1991 the genomic DNA sequence of NT-4/5 was isolated independently by Hallböök et al., (1991) from the ovaries of Xenopus toads (and named it NT-4), and Berkemeir et al., (1991) from rat and human tissue (who named it NT-5). As a compromise the term NT-4/5 is now used to refer to this neurotrophin. The amino-acid
sequence of mammalian NT-4/5 is closely related to those of the other members of the neurotrophin family displaying a 65%, 62% and 51% homology with BDNF, NT-3 and NGF respectively (Hallböök et al., 1991). NT-4/5 does share some of the neurotrophic properties of its relations; in vitro NT-4/5 can support cultures of E14 dopaminergic neurons of the rat mesencephalon, as can BDNF and NT-3 (Hyman et al., 1994). NT-4/5 had the greatest effect on these cultures and increased the number of surviving TH-positive neurons by 700% over 7 days while BDNF, in a similar study lasting 8 days, induced only a 500% increase in cell number (Hyman et al., 1991). NT-4/5 can induce similar amounts of neurite outgrowth from chicken DRG cultures as BDNF, but less than NGF, and can induce weak but consistent outgrowth from chicken nodose ganglia (Hallböök et al., 1991). Unlike NGF, NT-4/5 was not seen to promote any such activity in cultures of sympathetic ganglia (Hallböök et al., 1991). Intravitreal injections of NT-4/5 directly after optic nerve transection have been shown to prevent the loss of adult rat RGC at 7 days post operation (po; Clarke et al., 1994); 99% of these cells survived compared to 52% of those which received the transection alone. However by 14 days po the survival promoting effect was almost non-existent. NT-4/5 was also found to selectively promote the survival of SP and somatostatin immunoreactive neurons, the medium spiny projection neurons and medium aspiny interneurons respectively, in organotypic slices of neonatal mouse striatum. NGF, BDNF and NT-3 failed to show any effect on the numbers of these neurons surviving (Ardelt et al., 1994). Funakoshi et al., (1993) found that the levels of NT-4/5 measured in the distal stump of transected sciatic nerve at 2 weeks post transection were 800% greater than those measured in intact control nerves.

**Neurotrophin-6**

NT-6 is the most recently isolated members of the neurotrophin gene-family and was isolated from teleost fish by Götz et al. (1994) who report that its structure complies with the known molecular features of other members of the neurotrophin gene family. This brief report also presents evidence that recombinant rabbit NT-6 is able to promote the survival of E8 chick sympathetic and sensory DRG in vivo, which is similar in extent to that of NGF, but has no effect on nodose or ciliary embryonic chick ganglia, unlike BDNF or CNTF respectively (Lindsay et al., 1985; Helfand et al., 1976).

**Glial cell line derived neurotrophic factor**

GDNF is one of the most recent neurotrophic factors to be isolated. It was originally identified as a neurotrophic factor for dopaminergic neurons of the substantia nigra (Lin et al., 1993). It is not a neurotrophin but is a member of the transforming growth factor-β gene superfamily (Oppenheim et al., 1995). GDNF mRNA has been isolated in Schwann cells in the PNS (Henderson et al., 1994) and in a cell line of type 1
astrocytes derived from the neonatal CNS (Schaar et al., 1993). In the CNS, high levels of GDNF mRNA were found in the embryonic substantia nigra and postnatal striatum of the rat, suggesting a local and target derived trophic role of GDNF for dopaminergic neurons during development. However, no expression of GDNF mRNA was seen in either the striatum or SN of adult animals, and even 6-hydroxy dopamine (6-OHDA) lesion of the nigro-striatal pathway in the medial forebrain bundle failed to induce its re-expression in either region (Schaar et al., 1993). Many of the effects of GDNF in vivo and in vitro are still being investigated but already there is plenty of evidence to suggest that it is a potent neurotrophic factor both in the embryonic and adult nervous system. In vivo, GDNF can totally prevent motor neuron cell death following facial nerve transection in neonatal rats if injected daily from the time of transection (pO). This compares with a 90% cell loss in lesioned control animals (Yan et al., 1995). In the same study Yan et al., (1995) found that adult sympathetic neurons were unable to retrogradely transport GDNF in vivo, and that in vitro cultures of sympathetic neurons failed to show any trophic response to this factor. Cultures of neonatal sensory DRG neurons did respond to GDNF, however, again suggesting that this trophic factor selectively acts on certain neuronal populations. In vivo, daily nigral injections of GDNF, starting immediately after axonal transection, had a protective effect on adult dopaminergic neurons of the SNpc; 86% of these neurons survived with the GDNF treatment compared to only 52% in the control experiments (Beck et al., 1995). So far, in the literature available at the time of writing, no reference has been made to the role of GDNF in injured peripheral nerves.

Ciliary neuronotrophic factor

CNTF is not a member of the neurotrophin family of growth factors but a cytokine, and was originally described as a growth factor for cholinergic ciliary neurons (Helfand et al., 1976; or Alder et al., 1979). CNTF does not fulfill the classical definition of a neurotrophin as it is not available to sensitive neurons from their target fields (Stockli et al., 1989) and is apparently not retrogradely transported by uninjured chick peripheral nerves (Smet et al., 1991). However, following its injection into the sciatic nerve, 125I-labelled CNTF undergoes retrograde transport by adult rat DRG sensory neurons. The rate of retrograde transport increased by 500% when the nerve underwent a crush injury 7 days before injection (Curtis et al., 1993). Although CNTF has been shown to encourage neurite outgrowth from some NGF sensitive neurons (Barbin et al., 1984) it also has some trophic properties quite distinct from those of NGF. In vitro, CNTF can support the survival of ciliary ganglion neurons (Helfand et al., 1976) and embryonic spinal motor neurons (Arakawa et al., 1990); 63% of these embryonic spinal motor neurons survived for 3 days with CNTF while NGF and BDNF had no effect on their survival. Interestingly, b-FGF was able to support 51% of the
spinal motor neurons and a combination of CNTF and b-FGF had an additive effect and induced 100% neuronal survival for up to 7 days post-plating. In vivo, infusions of CNTF can rescue axotomised facial nerve motor neurons in the neonatal rat (Sendtner et al., 1990) and prevent the degeneration of median septal neurons following fimbria-fornix lesion (Hagg et al., 1992). Receptors for CNTF have been identified on both adult and immature DRG and motor neurons indicating that a role may exist for CNTF throughout development (Ip et al., 1991; Dobrea et al., 1992).

High levels of CNTF are found in unlesioned adult peripheral nerves (Manthorpe et al., 1986) but, unlike NGF, not in their target tissues (Stockli et al., 1989). CNTF has been immuno-localized to Schwann cells, indicating that they are the probable source of the molecule in peripheral nerves (Rende et al., 1992). Schwann cells drastically change their rate of CNTF synthesis upon denervation (Sendtner et al., 1992); the level of CNTF bioactivity in the distal portion of transected nerves was reduced to 30% of the pre-lesion level. Interestingly, the location of CNTF within the nerve also changes upon transection; after injury most of the immunoreactive CNTF is found within the extracellular space rather than being in the cytoplasm of intact Schwann cells (Sendtner et al., 1992), presumably, making CNTF more readily available to regenerating axons.

(5b) GAP-43

The growth associated molecule which is now known as GAP-43 was originally isolated by a number of different groups researching a variety of different aspects of neuronal physiology and was consequently assigned many different names. It was first identified by Skene and Willard (1981a and b) who isolated two growth-associated proteins common in the developing nervous system of the rabbit and, in reference to their different molecular weights, named them GAP-23 and GAP-43. Once synaptogenesis was complete, the synthesis of these molecules differed in the adult nervous system; GAP-23 continued to be synthesized in uninjured neurons while GAP-43 was significantly down-regulated. However, after peripheral axotomy GAP-43 synthesis was up-regulated in successfully regenerating neurons whereas GAP-23 expression appeared to be unaffected by injury. Neurons which were not able to regenerate, such as adult RGCs, did not re-synthesize GAP-43, drawing Skene and Willard (1981) to the conclusion that the inability of mammalian CNS neurons to regenerate after injury could possibly be a consequence of their inability to re-synthesize GAP-43 once damaged.

A protein identical to GAP-43 was also isolated from synaptic plasma membranes, named B-50, and shown to be involved in polyphosphoinositide metabolism (Oestreicher et al., 1983; van Hoof et al., 1988). It was isolated from the hippocampus, named F-1; and, in this site, its phosphorylation by protein kinase-C was correlated with
long term potentiation (Nelson and Routtenberg, 1985; Routtenberg, 1986). It was also identified as pp46, a major phosphoprotein of growth cone membranes (Katz et al., 1985; Meiri et al., 1986), and, under the name of p57 (or neuromodulin), was isolated from bovine cerebral cortex and shown to preferentially bind calmodulin in the absence of calcium (Andreason et al., 1983). Direct comparisons of the molecular sequences of these functionally different proteins have proved that these diverse functions can all be attributed to the same molecule (for review see Skene, 1989).

GAP-43 is a membrane bound protein which, in neurons, is transported by the rapid phase of axonal transport from its site of synthesis in the cell body to its active sites in the growth cone or nerve terminals (Benowitz et al., 1981). The exact calculation of the molecular weight of this protein is hampered by its variable migration on SDS gels of different concentration, but estimates range from 43 - 57 kDa (Jacobson et al., 1986; Benowitz et al., 1987). Originally, GAP-43 was thought to be an entirely neuron specific protein (Benowitz et al., 1988) but there is now evidence to suggest that GAP-43 is also synthesized by non-neuronal cells of the developing and adult nervous system (Vitkovic 1988, 1992; Vitkovic and Mersel, 1989; Curtis et al., 1992).

Immunohistochemical localization of the protein in explants of developing neurons in vitro has shown that growth cones have very high levels of GAP-43 (Meiri et al., 1988) and Skene et al. (1986) estimated that GAP-43 comprises approximately 1% of the total of growth cone protein content. During development GAP-43 is highly concentrated in the growth cones of developing axonal, but not dendritic, processes (Goslin et al., 1989).

Many studies have provided evidence which correlates the expression of GAP-43 with axonal growth. Transfection of GAP-43 cDNA into GAP-43 deficient non-neuronal COS cell lines which, under normal culture conditions extend very few processes, results in the formation of many fine, long filopodial processes (compared to cells transfected with control plasmids, Zuber et al., 1989). The introduction of antisense oligonucleotides against GAP-43 into cultures of primary hippocampal neurons resulted in the inhibition of process outgrowth (Fidel et al., 1990). The extent of process outgrowth was directly related to the dose of antisense oligonucleotide administered. In a similar investigation Jap Tjoen San et al., (1992) showed that antisense oligomers directed against GAP-43 mRNA block both the NGF induced outgrowth of processes from PC12 cells in vitro and the enhancement of GAP-43 immunoreactivity with which neurite outgrowth is normally correlated. Antibodies directed against GAP-43 have also been shown to block neurite extension by PC12 cells in vitro (Shea et al., 1991). However the continued extension of differentiated, GAP-43 immuno-negative dendritic processes (Goslin et al., 1989), and the observation of NGF, bFGF and cAMP mediated neurite extension by a GAP-43 deficient cell line (Baetge and Hammang, 1991) demonstrate that this protein is not essential for the elongation of all types of neuronal
processes.

In the developing brain, levels of GAP-43 peak during the first postnatal week, when a great deal of synaptogenesis occurs, but declines by over 90% in most neurons by 2-3 weeks after birth (Jacobson et al., 1986). Samples of foetal ventral mesencephalic tissue and foetal striatal tissue implanted separately into the adult striatum continue to synthesize GAP-43 for some time after grafting. High levels of immunoreactivity have been identified in the neuropil of foetal ventral mesencephalon grafts at 15 days post implantation which were significantly down-regulated 15 days later and by 13 weeks post implantation the levels of GAP-43 protein in the graft were approximately the same as those of the surrounding host tissue (Clayton et al., 1991). Grafts of foetal striatal neurons showed the same temporal pattern of expression; GAP-43 mRNA expression was highest at 15 days post implantation and was greatly reduced by 30 days post implantation, by 3 months post implantation the level of GAP-43 mRNA synthesis was not significantly different from that of the surrounding host tissue (Sirinathsinghji et al., 1993). In both cases the modulation of GAP-43 synthesis by the grafts followed a similar temporal pattern to that of developing neurons which down regulate GAP-43 synthesis once most of their synaptic connections are made. It is possible that the down-regulation of GAP-43 synthesis observed in these grafts was also an indicator that they had formed a synaptic connection with the host tissue.

Expression of GAP-43 mRNA does persist in some discrete populations of adult CNS neurons. Levels are particularly high in neurons of the neocortex and CA3 neurons of the hippocampus, which retain some synaptic plasticity into adulthood (Benowitz et al., 1990), and other regions of the adult brain such as the locus coeruleus, SNpc and the granule layer of the cerebellar cortex (Kruger et al., 1993, Bendotti et al., 1991). The distribution of GAP-43 protein in the adult brain is slightly different from that of its mRNA and its immuno-localization in the projection areas of those regions high in GAP-43 mRNA, such as the striatum and layer 1 of the cortex, reflects the characteristically rapid transport of GAP-43 protein from its site of synthesis to nerve terminals (Benowitz et al., 1988). GAP-43 protein has been shown to be abundant throughout the neuropil of the normal adult rat striatum, particularly in small unmyelinated axons, but was not identified in any neuronal somata (DiFiglia et al., 1990) and in situ hybridization for GAP-43 mRNA combined with immunohistochemistry for ChAT demonstrated that the expression of GAP-43 by adult striatal neurons is confined to the aspiny cholinergic interneurons (McKinney and Kent, 1994).

Although normally down-regulated to very low levels in the adult nervous system, some adult neurons and non-neuronal cells have been shown to up-regulate their synthesis of GAP-43 in response to some forms of injury. Following damage to the adult rat sciatic and facial nerves both the synthesis and axonal transport of GAP-43 protein are increased (Tetzlaff et al., 1989). Bisby et al., (1988) showed that within 24 hours of a
sciatic nerve crush significantly higher levels of GAP-43 protein were retrogradely transported along the nerve towards the DRG, rising to a 60 fold increase by 14 days post injury. From this evidence he suggested that GAP-43 synthesis in adult peripheral nerve cells is regulated by a suppressing factor derived from their peripheral target; if their supply of this factor is interrupted, for example by axotomy, GAP-43 synthesis is up-regulated until levels of the suppressing agent are restored by regeneration as by 114 days post injury the levels of GAP-43 protein synthesis had returned to control levels. However, Chong et al., (1992) found that the expression of GAP-43 mRNA in DRG and spinal cord motor neurons after sciatic nerve transection was for a finite period only and was eventually down-regulated even when no regeneration took place. Conversely high levels of GAP-43 protein were still found in peripheral axons which had regenerated into freeze-killed PN grafts although they had ceased to regenerate any further (Chong et al., 1992). This evidence implies that expression of GAP-43 by itself is not sufficient to ensure regeneration in the injured PNS.

However, despite all the evidence to the contrary, the presence of high levels of GAP-43 within injured neurons does not automatically confer on them the ability to regenerate. Developing neurons of the mammalian CNS, which express high levels of GAP-43, were thought to be able to regenerate successfully and form appropriate target synapses after axotomy. In 1979 Kalil and Reh reported the apparent topographically correct regrowth of severed pyramidal tract axons in neonatal (P5) hamsters. However 11 years later, after the publication of a number of reports which contradicted this work (eg Tolbert and Der, 1987), Kalil refuted their previous interpretation of the results stating that the axonal sprouts observed in the cut corticospinal tract could not be the result of regrowth of severed axons (Merline and Kalil, 1990). It was established that these fibres were actually late developing axons which were not present in the corticospinal tract when the lesion occurred (but which would be expected to contain high levels of GAP-43).

In contrast, the failure of adult CNS neurons to regenerate after axonal injury has been correlated with their inability to up-regulate GAP-43 synthesis. Transected adult optic nerves have often been used as an easily accessible model of axonal injury within the CNS environment (Skene and Willard., 1981a; Berry, 1986a and b). Regeneration of injured RGC axons is complete in fishes and some amphibians, but in adult mammals regeneration is limited to abortive axonal sprouting in the period 2-14 days after optic nerve injury (Zeng et al., 1994). Skene and Willard reported GAP-43 was transported into injured toad RGC axons (Skene and Willard., 1981a) but not into injured mammalian optic nerves (Skene and Willard., 1981b; other studies also provided evidence that not all injured mammalian CNS neurons up-regulate GAP-43, for example Benowitz and Routtenberg, 1987). In recent years it has become apparent that GAP-43 expression is increased in at least some mammalian CNS neurons following various forms of injury (Ng et al, 1988; Oestreicher et al., 1988).
Doster et al., (1991) demonstrated that the amount of GAP-43 protein found in mammalian RGCs after injury was dependent on the lesion distance from their neuronal perikarya; injury within 3mm of the eye caused the accumulation of GAP-43 immunoreactivity in RGC perikarya but injury any further from the orbit did not. However, this apparent increase in GAP-43 synthesis was only transient and could have been the result of an accumulation of protein within the axonal stump which, before the pathway was mechanically interrupted, was normally anterogradely transported away from the cells perikarya to the axon terminal. However, Jones and Aguayo (1991, in abstract form, using radio-labelled in situ hybridization) found that both proximal and distal optic nerve transection lead to similar increases in the level of GAP-43 mRNA detectable in RGCs from 3 days after operation. From this they concluded that that axotomy alone, without subsequent fibre elongation, was sufficient to stimulate an increase in GAP-43 mRNA synthesis in injured RGCs and its modulation was independent from the changes observed in GAP-43 protein levels after the same form of injury.

In the brain there is evidence that certain populations of adult neurons are able to up-regulate their level of GAP-43 synthesis during the regeneration of axons into PN grafts. Campbell et al., (1991) identified many GAP-43 immuno-positive CNS axons around and within segments of common peroneal or tibial nerve 3-14 days after the nerves were grafted into the thalamus of adult rats. GAP-43 also accumulates within some thalamic perikarya 11-14 days after grafting. Further investigation showed that although mechanical injury was a sufficient stimulus to induce the transient expression of GAP-43 mRNA in thalamic neurons, particularly in the thalamic reticular nucleus, prolonged synthesis of GAP-43 mRNA was only seen in the presence of a graft (Vaudano et al., 1995). A direct correlation was also made between the capacity of neurons to up-regulate and maintain synthesis of GAP-43 mRNA expression and the ability to extend axons long distances within the graft. This relationship was also seen amongst specific populations of cerebellar cells - neurons of the deep cerebellar nuclei exhibited prolonged upregulation of GAP-43 mRNA synthesis after implantation of a PN graft and were able to regenerate many axons into the grafts. However, Purkinje cells of the cerebellar cortex, which do not up-regulate GAP-43 expression in response to graft implantation, did not show any regenerative ability (Vaudano et al., 1993). Doster et al., (1991) made the observation that the conditions which seem to encourage an increase in GAP-43 expression, such as proximal axotomy, are similar to those which favour the regrowth of injured CNS axons into PN grafts. Curiously, proximal axotomy of CNS neurons also leads to much more neuronal cell death than distal axotomy (see section 3b).

It is also possible that GAP-43 alone may not be essential for the development of the nervous system. Contrary to all previous evidence on the importance of GAP-43 for neurite outgrowth, a strain of mutant mice, deficient for the GAP-43 gene, is able to
develop an apparently normal nervous system (Strittmatter et al., 1995). Although the mice die in the early post-natal period (only 10% of the knockout animals survive to 3 weeks after birth), the growth rate and formation of the nervous system up to that time is grossly normal. Significantly, cultured DRG explants from mutant mice were able to extend neurites or growth cones in a manner which was indistinguishable from control cultures demonstrating that GAP-43 is not essential for growth-cone formation. However, slight aberrations were found in the pathfinding ability of some GAP-43 deficient neurons in vivo; DiI tracing demonstrated that, unlike those in wild type animals, developing RGCs in the GAP-43 knock-out mice were unable to extend axons past the optic chiasm to the contralateral superior colliculus. Instead of coursing through the chiasm towards the contralateral optic tracts, axons in the mutant mice continue to extend but within the chiasm itself, becoming tangled and increasing its overall size. Strittmatter and colleagues concluded that within the normal developing nervous system GAP-43 is involved with the amplification of pathfinding signals from the growth cone, particularly the G-protein signal transduction system which is involved with the modulation of growth-cone function (Strittmatter et al., 1994b). The insensitivity of growth-cones to subtle pathfinding signals within the optic chiasm may adequately explains their apparent confusion within that area and inability to find their correct path.

Neurofilament negative cells, possibly Schwann cells, distal to a peripheral nerve lesion also expressed GAP-43 protein (Tetzlaff et al., 1989) and are closely associated with regenerating axons. There is strong evidence that non-myelinating Schwann cells of degenerating adult peripheral nerves synthesize GAP-43; Bisby et al., (1991) found a significant increase in the level of GAP-43 cDNA in the non-myelinating Schwann cells of axotomised facial nerves in vivo, and Curtis et al., (1992) demonstrated that although GAP-43 expression is only found in the non-myelinating Schwann cells of uninjured adult sciatic nerve, nearly all Schwann cells of the distal stump up-regulated GAP-43 expression by 4 weeks after sciatic nerve transection.

(5c) Cell adhesion molecules and extracellular matrix molecules

Selective populations of adult CNS neurons are able to regenerate after injury. This ability is partly dependent on the 'permissiveness' of the environment with which the regenerating axons interact; segments of peripheral nerve implanted into the brain or anastomosed to severed optic nerve have been shown to be particularly favourable substrates for such regeneration (Aguayo, 1985; Berry et al., 1988a). However, this capacity is apparently dependent on active interactions between the regenerating axons and their substrate as freeze-killed PN graft preparations do not promote such axonal regeneration (Smith and Steveson, 1987; Berry et al., 1988a; Hall and Berry, 1989). It is possible that Schwann cells, the growth supporting cells in these grafts, are providing
molecular cues to regenerating axons in the form of components of the cell surface or factors secreted into the extracellular matrix (Fawcett and Keynes, 1990). During development, CNS axons, and probably axons forming peripheral nerves, elongate on cells other than Schwann cells; if developing axons require the same growth promoting cues, these other non-neuronal cells must also be capable of supplying the molecules ie it is the nature of these cues and signals which is important for regeneration rather than the substrate supplying them. This chapter introduces two classes of such molecules which mediate axon-glia interactions during axonal regeneration and/or modulate axonal elongation: cell adhesion molecules (CAMs) and extra cellular matrix molecules (ECMs).

Cell adhesion molecules

Three major groups of CAMs have been identified; the cadherins, the integrins and immunoglobulin-like molecules (Takeichi, 1990; Reichardt and Tomasseli, 1991). This study will examine the expression of two specific immunoglobulin-like molecules - L-1 and N-CAM during the regeneration of CNS axons.

L-1 is a transmembrane glycoprotein and has an extracellular section consisting of six immunoglobulin-like domains and five fibronectin type III repeats (Rathjen and Schachner, 1984). Its molecular structure shows close homology with that of the rat large NGF induced external glycoprotein NILE (Stallcup and Beasley, 1985) and three very similar glycoproteins isolated from chickens; neuron-glia-CAM or Ng-CAM (Grumet and Edelman, 1984), 8D9 (Lemmon and McLoon, 1986), and G4 (Rathjen et al., 1987). L-1 has an apparent molecular weight of 200-230 kDa. N-CAM is also a transmembrane glycoprotein and is the product of a single gene although, as a result of alternative splicing, it can be expressed in the nervous system as at least three different isoforms with molecular weights of 120, 140 and 180 kDa (see Rutishauser and Goridis, 1986, for review). In all isoforms the extracellular section comprises five immunoglobulin domains and two fibronectin type III repeats (Barthels et al., 1987; Cunningham et al., 1987).

In the PNS L-1 and N-CAM have similar patterns of expression during development. In embryonic mice N-CAM is found on the surface of fasiculating axons, their growth cones and filopodia, and on Schwann cells (Martini & Schachner, 1991). L-1 has also been identified on the peripheral nerves of embryonic mice and is restricted to a sub-population of developing Schwann cells, most likely the non-myelinating kind (Faissner et al., 1984). If myelination takes place L-1 and N-CAM synthesis is down-regulated by the Schwann cells, but they remain expressed on non-myelinating Schwann cells, and their associated axons, through to adulthood (Martini & Schachner, 1986). Immunoelectron microscopy has demonstrated that once myelinating Schwann cells have made approximately one and a half turns around an axon the expression of L-1 becomes almost completely undetectable in both Schwann cells and their axons and although they
continue to express N-CAM at a very low level (Martini & Schachner, 1986). Bartsch et al. (1989) demonstrated that a similar down-regulation in the level of axonal L-1 occurs in the CNS with the onset of myelination by oligodendrocytes (which do not express L-1). In vitro, antibodies generated from immature mice have been used to demonstrate the adhesive properties of these molecules; antibodies against L-1 disrupt neuron-neuron adhesion and the fasciculation of axons, while antibodies against N-CAM cause the disruption of neuron-neuron and astrocyte-astrocyte adhesion, in addition to neuron-astrocyte adhesion (Keilhauer et al., 1985).

Interestingly, transection of the adult sciatic nerve results in the re-expression of both L-1 and N-CAM in Schwann cells of the distal stump (for review see Martini, 1994). At 4 days post transection 10-20% of previously myelinating Schwann cells, which had down regulated their synthesis of L-1 and N-CAM in the embryo, were positive for both CAMs, and by 2 weeks all denervated Schwann cells were L-1 and N-CAM immunoreactive (Martini and Schachner, 1988). Northern blot analysis determined that Schwann cells up-regulated mRNA for L-1 by 4 days post transection and mRNA for N-CAM by 6 days post transection for N-CAM (Tacke and Martini, 1990). In vitro, dissociated Schwann cells displayed a similar pattern of N-CAM re-expression although the induction of N-CAM mRNA synthesis was more rapid than in vivo (Jessen et al., 1987). It is possible that the re-expression of these molecules by the injured PNS may be an attempt to recreate a similar growth permissive environment to the one which existed during development to encourage axonal regeneration. Zhang et al., (1995) found that Schwann cells in segments of sciatic nerve implanted into the thalamus of adult rats expressed mRNA for L-1 and N-CAM for up to 13 weeks po. L-1 and N-CAM were found on regenerating CNS axons which grew into the grafts and was specifically upregulated by the major neuronal population which regenerated axons into the grafts (the thalamic reticular nucleus; TRN).

Extra cellular matrix molecules

As with the CAMs, many ECMs have been associated with the development and regeneration of the nervous system such as heparin sulphate or chondroitin 6-sulphate proteoglycan and tenascin-C, (see Reichardt and Tomaselli, 1991; for review). This introduction will only cover one of these molecules - tenascin-C. Tenascin-C, and some very similar related molecules, have been isolated from numerous species and tissue types and been given many different names (for review see Erikson and Bourbon, 1989). Unlike L-1 and N-CAM, tenascin-C is an oligomeric glycoprotein composed of six disulphide-linked subunits (Erickson and Bourdon, 1989.). Each arm of this molecule has a globular domain at the amino terminal which is followed by several EGF-like repeats and fibronectin type III motifs.

During development of the PNS both tenascin-C and laminin are involved in
neuron-glia interactions and tenascin-C is strongly associated with the perineurium and Schwann cells of peripheral nerves (Martini and Schachner, 1991), particularly near the nodes of Ranvier (ffrench-Constant et al., 1986). In embryonic peripheral nerves molecules of tenascin-C are found around developing axons (Martini, 1994). In the developing mouse optic nerve tenascin-C levels are low but increase in neonatal and young (P14) animals. By 4 weeks, expression of tenascin-C is completely downregulated and remains so in the adult optic nerve (U. Bartsch et al., 1992). A similar pattern of tenascin-C expression is also found within the developing brain - tenascin-C mRNA is expressed in all layers of the developing cerebella cortex but is significantly down-regulated in the adult where its expression is restricted to the granular layer only (S. Bartsch et al., 1992).

*In vitro* studies have illustrated the multifunctional nature of tenascin-C. In culture, various types of disassociated embryonic neurons can be successfully cultured on a polylysine/tenascin-C substrate. Antibodies against the tenascin-C completely inhibited outgrowth (Weherle and Chiquet, 1990). In the same study spinal cord explants were cultured on a tenascin only substrate. The neurites found were poorly attached to the substrate, grew more rapidly and were rarely branched. Lochter et al., (1991) found that single cell cultures of E18-19 rat mesencephalon or hippocampal neurons grew 40% more neurites when plated on a tenascin-C/polyornithine substrate than those plated on polyornithine alone. However, when these cells were cultured on normally permissive substrates such as laminin, fibronectin or polyornithine with a medium containing a soluble form of tenascin-C this outgrowth was completely inhibited. *In vitro*, substrates of purified tenascin-C were not able to support either the attachment or growth of cerebella astrocytes while embryonic mesencephalon, hippocampal or cerebella neurons always chose a tenascin-C free substrate if one was available (Fai ssner et al., 1990). The perikarya of cells which are able to adhere to tenascin-C coated substrates were not as flattened or as spread out as those plated on fibronectin or laminin (Erikson and Bourbon, 1989).

In the adult CNS, levels of tenascin-C are usually low (Laywell et al., 1992). However certain types of injury can induce the re-expression of this molecule in some non-neuronal cells. Stab wounds to the cerebella and cerebral cortices of adult mice resulted in enhanced expression of tenascin-C around the lesion site, associated with a discrete population of GFAP positive astrocytes (Laywell et al., 1992). Nitrocellulose implants into the cerebral cortex of adult mice stimulated the expression of tenascin-C but only around the implanted tissue. Again this expression was co-localized to a population of GFAP positive astrocytes. These astrocytes were only found immediately around the lesion site within the cortex; no tenascin-C expression was seen in astrocytes elsewhere within the ipsilateral cortex or corpus callosum (McKeon et al., 1991). In the same study samples of glial tissue from around the implant were taken from adult and neonatal
animals and plated out as a substrate for neuronal explants. The neonatal samples were able to support neurite growth and extension while the adult ones were not, which McKeon et al., (1991) suggested may be a result of the higher tenascin levels in reactive cells around the adult implant and may demonstrate the limiting effect of tenascin-C on regenerating neurons of the CNS.

Lesion of adult peripheral nerve stimulates the re-expression of tenascin-C along the entire length of the severed distal stump (Martini et al., 1990). At 2 days po tenascin-C expression in the distal stump, at the ultrastructural level, is limited to the basal laminae of Schwann cells. Five days later, expression is also associated with fibroblasts, macrophages and endothelial cells; by 14 days po its expression is confined to the extracellular matrix around Schwann cells and nerve fascicles (Martini et al., 1990). At 14 days po, tenascin-C levels peak in the distal stump but the molecule has almost vanished once again by 35 days po. The time course of tenascin-C downregulation in the distal stump correlates with axonal regeneration from the proximal stump; if the distal stump is chronically denervated, eg by removal of the proximal stump, this downregulation of tenascin-C is not seen (Martini et al., 1990) suggesting that the restoration of Schwann cell-axonal contact, or axonal target reinnervation, may provide the stimulus for downregulation. As lesioned peripheral nerves successfully regenerate it is probable that tenascin-C has a growth permissive, rather than inhibitory, role in the injured adult PNS. However, upregulation of tenascin-C is not seen in either the proximal or distal segments of transected adult optic nerve which does not normally regenerate (U. Bartsch et al., 1992). Taken together, this evidence implies a role for this molecule in regeneration in the PNS (Martini et al., 1990) and that the absence of tenascin-C may be directly involved in the failure of regeneration of neurons of the mammalian CNS (Laywell et al., 1992).

6 - CNS axonal regeneration into PN grafts

(6a) Early research into CNS axonal regeneration into PN grafts

For most of this century it was assumed that the majority of CNS neurons were intrinsically incapable of regenerating axons. This view was largely overcome by the experiments of Aguayo, Berry and others who took advantage of recently developed retrograde tracing techniques to investigate axonal regeneration into PN grafts in the CNS. Early evidence that CNS axons could grow into PN grafts did already exist, for example Tello (1911, cited by Ramon y Cajal, 1928) reported that small sections of predegenerated sciatic nerve placed in the cortex of adult rabbits for between 12-40 days did become innervated with host fibres, the number of which decreased with time so that by 40 days post implantation significantly fewer fibres could be seen within the grafts.
He also noticed that fresh grafts, those implanted immediately after transection rather than left to degenerate for a number of days, did not have such a strong neurotropic effect on host CNS axons and determined that the best results were obtained using grafts in which the Bands of Büngner were well developed. In the same review of CNS injury, Ramón y Cajal (1928) observed that segments of peripheral nerve inserted into the brain became temporarily invaded by axons and that 'sprouts wandering through the scar can be nourished and oriented by the cells of Schwann that [form] Bands of Büngner in the grafts' but neither Tello nor Ramón y Cajal were able to demonstrate definitively the CNS origin of the axons in the grafts. Significantly he also hypothesized that the failure of central fibres to regenerate after injury 'derives from external conditions, the presence or absence of auxiliary factors that are indispensable to the regenerative process'. It has long been thought that such auxiliary factors could be derived from peripheral nervous tissue, stimulating the use of PN grafts, but what are the features of PN grafts which make them so successful in promoting CNS regeneration?

(6b) Subsequent research into CNS regeneration through PN grafts

Le Gros Clark, in the early 1940's, clarified Tello's work and showed that while regenerating nerve fibres could be found in the graft tissue, they were few in number and not central in origin, most likely perivascular fibres from the periphery (Le Gros Clark, 1942, 1943). Unfortunately, the majority of early work was compromised by two difficulties: it was impossible to prove that the axons which grew into PN grafts in the CNS did indeed originate from CNS neurons and many experiments were confusing because of the lack of understanding of transplantation immunology and grafting of material between outbred hosts and donors or even different species. Consequently research into the regeneration of CNS neurons into PN grafts could not significantly progress until the importance of using autografts or inbred animals was understood and the origin of fibres invading the grafts could be demonstrated conclusively.

Fortunately, the development of immunology and the introduction of retrograde tracing techniques using HRP allowed the critical experiments to be done and Richardson et al. (1980) followed by David and Aguayo (1981) were able to demonstrate unequivocally the central origin of fibres regenerating into PN grafts implanted into the CNS. The latter study showed that when peripheral nerve tissue was implanted proximally into the medulla oblongata and distally into the spinal cord of adult rats and mice, CNS fibres from the brainstem and ascending spinal tracts regenerated into these grafts for distances up to 3.5cm in the rat and 2cm in mice, far longer than they would normally extend in the intact animal. Unfortunately axons ceased to elongate beyond a few millimetres of their re-entering CNS tissue. It was also noted that the perikarya of the
majority of the regenerating brain stem and spinal cord neurons were found very close to the implanted end of the graft, within 4mm and 6.5mm respectively. Association with the target regions did not seem to be essential to promote the regeneration of CNS axons and grafts whose distal end was not reimplanted into neural tissue but left, for example, under the scalp or sutured to adjacent muscle also supported axonal regeneration.

It remained to be demonstrated, however, that the CNS axons growing into PN grafts were indeed from axotomised neurons. So and Aguayo (1985) also used HRP and two different fluorescent tracers to show that all of the axons they labelled regenerating into a PN graft inserted into the retina of adult rats originated from axotomized RGCs and were not the result of collateral sprouting from undamaged fibres. Similarly Berry et al. (1986) showed unequivocal regeneration of axons from optic nerves severed in the orbit into PN grafts and, in doing so, developed what is now the most popular model for studying regeneration into grafts. If the axons of brain stem neurons which had already regenerated fibres into PN grafts were lesioned once more, this time within the graft, they were able to regenerate once again, the graft continuing to support them (David and Aguayo, 1985). Munz et al., (1985) showed that CNS axons which had regenerated into PN grafts from brain stem nuclei could conduct action potentials but exhibited signs of significantly reduced or altered synaptic inputs and activation and discharge patterns. However, Keirstead et al., (1989) reported the reformation of functional connections by adult hamster RGCs regenerating through PN grafts sutured to the proximal stumps of intraorbitally transected optic nerves, distally implanted into the ipsilateral superior colliculus. The ultrastructure of the presynaptic profiles of similar regenerating axons contacting neurons in the superior colliculus of the adult rat appeared normal (Vidal-Sanz et al., 1991). Interestingly, newly formed synapses were found to be confined to the layers of the superior colliculus which normally receive retinal inputs (Carter et al., 1994, using adult hamsters) and the extension of regenerating axonal sprouts into the CNS parenchyma was still restricted to within 350μm of the distal end of the graft even when functional regeneration was observed (Vidal-Sanz et al., 1991, using adult rats). Most significantly, Thanos et al. (1992) established that the pupillary light reflex of adult rats could be regained after similar PN graft implantation into the pretectal region.

(6c) Interactions between CNS tissue and PN grafts

As with any injury to the CNS, implantation of a PN graft stimulates a strong wound response from non-neuronal cells, particularly reactive astrocytes. After mechanical lesion to the CNS reactive astrocytes at the site of injury establish a complex and permanent glial scar (Berry et al., 1988). In grafted animals the glial scar forms at the graft/brain interface, resembling the dorsal surface of the CNS and is thus referred to as the glia limitans. This layer forms quickly and can be identified within as little as 5
days after graft implantation. Initially, at the EM level, the glial limitans consists of many astrocytic processes and an incomplete basal lamina surrounding the graft, this layer becomes continuous by 14 days after grafting and eventually forms a complex and multilayered stratum which is considered to be impenetrable to regenerating axons by 2 months after grafting (Campbell et al., 1992). Curiously, when PN grafts are anastomosed to severed optic nerves there is an initial period when the GFAP-positive astrocyte processes appear to be withdrawn from the immediate site of interaction between the CNS and peripheral tissue (Berry et al., 1988). Sprouting axons appear first in this GFAP-negative zone, followed by astrocyte processes. It is not known whether a similar sequence of events occurs around grafts in the brain but the withdrawal of tenascin-C immunopositive astrocyte processes has been reported to take place from injuries to the fimbria/fornix (Lips et al., 1995) and astrocyte processes do not appear to lead regenerating axons into grafts in the thalamus (Campbell et al., 1992).

It has also been demonstrated that although relatively few RGCs are able to extend axons into PN grafts sutured to the proximal stump of intraorbitally transected optic nerves, compared to the total number found in the retina, a much greater number benefit from their presence. Berry et al. (1987) recorded a 25% reduction in the number of RGCs lost from whole mounts of retinae 30 days after intraorbital PN grafting compared to the number lost from retinae whose lesioned optic nerves did not receive grafts. Similarly, Villegas-Perez et al. (1988a) estimated that although over 90% of RGCs retrogradely labelled from their cut ocular stumps were lost from adult rat retinae 30 days after optic nerve transection, 20-40% more surviving RGCs were found in retinae which had received grafts at the time of axotomy 1-3 months earlier. When acellular, freeze-killed PN grafts were sutured to the transected optic nerve 28,000 RGCs out of an estimated 111,000 (25%) were found to survive (Berry et al., 1987).

Excised segments of peripheral nerve implanted into the CNS appear to undergo the same structural changes after injury as the distal portion of transected peripheral nerves left in situ. The original peripheral axons quickly degenerate; Campbell et al (1992) were unable to detect any recognisable axonal profiles within PN grafts by 3 days after implantation into the thalamus. As in degenerating peripheral nerves left in situ, axonal and myelin debris is phagocytosed by invading macrophages and by 8 days after implantation columns of Schwann cells surrounded by basal lamina (Bands of Büngner) can be identified within the graft (Hall and Berry, 1989; Campbell et al., 1990, 1992). In view of the importance of denervated Schwann cells during peripheral nerve regeneration, as described earlier (see section 2a), it seems likely, and is also assumed, that these cells continue to display similar properties once implanted into the CNS. There is some evidence for this: for example Schwann cells in PN autografts placed in the rat thalamus rapidly re-express the low affinity nerve growth factor receptor p75NGFR (Vaudano et al., 1995) and also re-express genes for the cell adhesion molecules N-CAM.
and L-1 and the extracellular matrix molecule, tenascin, in a similar pattern to lesioned peripheral nerves (Zhang et al., 1995, 1995a and b, in press).

A variety of non-neuronal elements, such as fibroblasts, macrophages, collagen and microfibrils have been seen to be associated with Schwann cell columns in grafts in the thalamus (Campbell et al., 1992). Campbell et al. (1992) also reported the appearance of astrocyte processes within the Schwann cell columns in proximal parts of the grafts from 14 days post graft implantation onwards, most noticeably in the graft periphery and often in association with Schwann cell process in bands of Bungner. However, astrocyte cell bodies were never seen to invade the grafts.

It is generally assumed that the most important aspect of the environment within PN grafts for promoting CNS axonal regeneration is the presence of living Schwann cells. This is based on knowledge of the axonal growth-promoting properties of Schwann cells and the results of experiments using freeze-killed grafts. The latter have an intact extracellular matrix (initially) but no living Schwann cells and are not invaded by CNS axons (Berry et al., 1987, 1988; Smith and Stevenson, 1987; Zeng, 1994). However, acellular PN grafts treated with purified b-NGF before implantation into the lesioned septal-hippocampal pathway were found to support almost as many regenerating central axons as fresh cellular grafts (Hagg et al., 1991), indicating that one important role of the living cells within PN grafts is to provide neurotrophic factors to the regenerating tissue. It is consequently not surprising that attempts have been made to promote CNS regeneration not just with PN grafts but also with purified Schwann cells.

(6d) The use of Schwann cells to promote CNS regeneration

Recently, much interest has been taken in the use of cultured Schwann cells and their ability to promote CNS regeneration either with or without an external matrix structure. Although the step to transplant Schwann cells into the injured adult CNS has a sound neurobiological basis, the amount of whole nerve autograft material available per subject is limited. However, when using laboratory animals, Schwann cells can be cultured from in-bred litter-mates to provide a reservoir of cells ready for transplantation. Schwann cells can be enwrapped by some form of artificial matrix, such as tubes or rolls of polymerised collagen before being implanted into the CNS (Paino et al., 1994; Bunge, M.B. et al, 1994) restricting them to one particular region of damage, or dissociated Schwann cells can be injected directly into brain parenchyma, allowing them to migrate away from the site of implantation. Results from these experiments have, so far, been favourable. Xu et al. (1992, 1993 and 1994) have consistently found that guidance channels seeded with cultured Schwann cells promote and support extensive regeneration of spinal cord axons when grafted between the proximal and distal stumps of transected adult rat spinal cord. In addition, the concurrent application of neurotrophins (Xu et al.,
1994) or neuroprotective agents, such as methylprednisolone (Chen et al., 1994), significantly increases the number of spinal cord axons regenerating through the channels. Using a similar technique, cultured Schwann cells, derived from embryonic rat DRGs, were seeded onto polymerized collagen and rolled to form tubular 'Swiss roll'-like implants to bridge dorsal spinal cord lesions in adult rats. By 28 days post implantation large numbers of both unmyelinated and myelinated axons (in a ratio of 20:1) were identified within the rolls compared to control rolls, implanted without the Schwann cells, which did not contain any (Paino et al., 1994).

Observations of dissociated rat Schwann cells, derived from neonatal peripheral nerve, carefully seeded into the CNS of syngenic adults have shown that these cells can readily survive within the CNS (Brook et al., 1993). Once in contact with host tissue, Schwann cells adopted an elongate morphology, extending long processes either side of the ovoid nucleus, and appeared to migrate freely along host blood vessels and fibre tracts where they assumed an orientation in parallel with the existing interfasciular rows of CNS glia and axons. However, no peripheral myelin was formed by these cells within the time period examined (up to 3 months post transplantation). With regard to promoting CNS regeneration, Schwann cells, carefully transplanted into the brain in the form of a slender vertical tract running between the thalamus and the hippocampus via the choroid fissure, were able to support the elongation of regenerating host axons from the thalamus to the hippocampus (Brook et al., 1994). In the spinal cord, suspensions of cultured Schwann cells, derived from neonatal sciatic nerve, were able to promote the regeneration of both ascending and descending fibres across upper cervical lesions of the corticospinal or ascending dorsal column tracts in adult rats where PN grafts had failed (Li and Raisman, 1994).

(6e) Differential axonal regeneration into PN grafts in the CNS

As described above, some of the earliest work examining the different regenerative responses of mammalian adult peripheral and central neurons established two basic principles on which the majority of CNS regeneration research has since been based; 1) that the majority of adult CNS neurons are able to regenerate after injury but components of their surrounding environment inhibit this response, and 2) that this is based on the provision of a number of factors to which CNS axons can respond in the growth permissive environment of the PNS.

Interestingly, examination of the literature provides several examples of selective or differential axonal regeneration into PN grafts in the CNS and even provides evidence that the success of regeneration can be linked to the physiological state of the injured neurons. For example, in the cat more α-class RGCs regenerate axons into PN grafts anastomosed to the proximal stump of transected optic nerve than either the β or γ classes
(Watanabe et al., 1993). Benfey et al., (1985) showed that the great majority of CNS axons which grow into sciatic nerve grafts implanted into the thalamus of adult rats originated from the TRN rather than from thalamic projection neurons which form the major population around the graft tip. This was confirmed by Morrow et al. (1992) who also showed that the number of TRN neurons which did regenerate was correlated to the size of the graft implanted. Similarly Benfey and Aguayo (1982) demonstrated that regeneration by different neocortical neurons was not equal and Dooley and Aguayo (1982) reported, in abstract form only, that cerebella cortical neurons never grow into PN graft but several other neurons from the central cerebella nuclei and brain stem nuclei projecting to the cerebellum were able to regenerate axons into the same grafts. This difference in regenerative response was later correlated to different molecular changes within the cell bodies of the two neuronal populations after injury (Vaudano et al, 1993, abstract form only) indicating perhaps that there may be intrinsic determinants particular to each CNS neuronal population which establish the extent of their predisposition towards a state of possible regeneration after injury.

Another population of central neurons which show a strong regenerative response to PN graft can be found in the basal ganglia - the corpus striatum (Benfey and Aguayo, 1982). They reported experiments involving the implantation of a segment of autologous sciatic nerve through a craniotomy in the lateral frontal bone to lie close to the basal ganglia. Of the 20 adult female Sprague-Dawley rats which received these grafts only 8 had grafts which terminated within the striatum itself. However, almost half of the total 444 neurons found to regenerate were located within the caudal-lateral striatum. The regenerated neurons were retrogradely labelled using Gelfoam pads soaked in a 20% solution of HRP (Type VI, Sigma) applied to the cut distal end of the graft for 50 minutes 48 hours before the animals were transcardially perfused with a 3% glutaraldehyde/0.1M phosphate buffer fixative. To prevent seepage of the tracer onto the surrounding tissue the exposed portion of the graft was placed on a plastic sheet and coated with Vaseline. The HRP was visualized using the tetramethylbenzidine and hydrogen peroxide method described in Mesulam (1978) and, in keeping with similar studies, 79% of the labelled neuronal perikarya were found within 1.5mm of the implanted graft tip.

7 - Aims of this study

Despite the many examples of successful CNS regeneration into PN grafts described above, very few detailed studies exist of the morphological and neurochemical events which occur during the interactions between the PN graft and CNS host tissue. Of these, the most extensive studies have been limited to PN grafts in the optic nerve and thalamus (Berry et al., 1988; Hall and Berry, 1989; Campbell et al., 1990). In view of the great neuronal diversity which exists within the CNS and the
possibly distinct regenerative responses of each neuronal population, more detailed studies of the interactions between graft and host tissue are required to establish whether similar events occur in different areas of the CNS after PN graft placement. This thesis will examine PN grafts implanted into the striatum of adult rats and their interactions with the host brain tissue.

The striatum represents an interesting region for these experiments, it is a large, heterogeneous structure which is separated from neighbouring nuclei by distinct boundaries (see Chapter 1, section 1a). It contains a variety of different neuronal classes, including one small population of cells with an established neurotrophic sensitivity (the large cholinergic aspiny interneurons), and its afferent and efferent connections are well established. It has already been established that both striatal neurons and neurons which project into the striatum can extend long axons into PN grafts (Benfey and Aguayo, 1985, Chapter 3) but little information is available about the morphological and neurochemical phenotype of these neurons. In addition to this, this thesis will examine the ultrastructural relationship between the host and grafted material (Chapter 4). Peripheral nerves are known to up-regulate the production of many growth promoting factors after injury (see Chapter 1, section 5a) but the neurotrophic properties of PN grafts after implantation into the CNS are unclear. This thesis will try to establish the level of NGF found within PN grafts and the surrounding striatal tissue (Chapter 5) and will investigate whether striatal neurons undergo similar changes following PN graft implantation to those which occur following exposure to neurotrophins. These include neurotrophin receptor expression (Chapter 5) and perikaryal hypertrophy (Chapters 6 and 7) after grafting. In the PNS, injured neurons are able to re-express growth associated factors (such as GAP-43, Chapter 8) or growth promoting factors (such as L1, Chapter 9) which are normally seen only in the developing nervous systems but are down-regulated in most adult neurons. The influence of PN grafts on the expression of these factors by the different populations of striatal neurons will also be examined in an attempt to understand how PN grafts can stimulate the extensive regeneration of CNS axons.
2 - METHODS
1 - Animals

All experiments were performed on outbred adult female Sprague Dawley rats (200-300g) supplied by the UCL Biological Services. Animals were housed 5 to a cage in a 12 hour light, 12 hour dark illumination cycle and received food pellets and water ad libitum.

2 - General surgery

(2a) Sterilization of equipment

All surgical instruments, drapes and swabs were autoclaved for 20 minutes at 121°C prior to use.

(2b) Anaesthesia

In most instances initial anaesthesia was induced using a mixture of 1.5% oxygen, 3% nitrous oxide and 4% Halothane (Fluothane, ICI) delivered through a scavenging nose cone from an IME portable anaesthesia station. Once deep anaesthesia was established, i.e. no corneal blink reflex, the Halothane level was reduced to 2%. However where this apparatus was not available, anaesthetic ether (Rhône-Poulenc) was used to achieve a light anaesthesia to allow intramuscular injection of 0.003ml/10g Hypnorm neuroleptalgic (Jassen Animal Health) and intraperitoneal injection of 0.005ml/10g Diazapam muscle relaxant (Roche Pharmaceuticals). Animals were then left in a warm environment until deep anaesthesia was established.

(2c) Preparation

The right hind leg and scalp were shaved using veterinary hair clippers and the exposed skin cleaned with 70% ethanol and left to dry. Animals were then placed on a heated pad covered with a sterile drape with the anaesthesia nose cone secured in place. Apart from cutaneous incisions all surgery was performed using a Zeiss operating microscope.

(2d) Tibial nerve graft preparation

An incision was made in the skin of the right thigh following the direction of the femur and the underlying quadriceps muscles were exposed and separated. The sciatic nerve bundle was then located and the tibial branch was carefully separated from the common peroneal and sural nerves using fine watchmakers forceps. A section of tibial nerve approximately 15 mm in length was excised, carefully trimmed of all adhering blood vessels and fat and laid on the exposed quadriceps and moistened with Hanks' Balanced Salt Solution (HBSS) (Gibco, Life Technologies Ltd). A point was marked
6mm from the proximal end of the graft segment with a 10/0 monofilament suture (Ethicon Ltd). The suture thread was left uncut and the graft was returned to lie between the quadriceps muscles bathed in HBSS until implantation, or underwent at least 5 cycles of freeze-thawing in liquid nitrogen to act as a freeze-killed control before being replaced in the leg.

(2e) Graft implantation

The scalp was opened along the midline and the periosteum was cleared from the right hand side using a scalpel blade to expose the sagittal and midline cranial sutures. The position of the underlying corpus striatum was defined using stereotaxic co-ordinates from Paxinos and Watson, (1986) and a deep depression was made in the skull at this point using a dentists' drill (burr size RdP.C. 7, round) leaving only a very thin layer of bone over the brain. The remaining bone was carefully removed using micro blood vessel forceps to form an oval aperture in the skull approximately 3mm long by 2mm wide. The dura mater was opened with a fine syringe needle to allow access to the brain.

The graft was transferred to lie under the scalp on the right temporalis muscle in a drop of HBSS. The proximal end of the graft was positioned over the dural incision and, using a glass micropipette, was implanted vertically into the brain to a depth of 6mm. The graft was secured to the dura at the 6mm mark using the existing 10/0 suture. The exposed part of the graft was then covered with a pad of sterile tissue soaked in HBSS to prevent dessication while the distal end of the graft was sutured to the fascia of the temporalis muscle using an 8/0 suture (Ethicon Ltd.). This was done to prevent graft displacement once the scalp was closed. The tissue pad was removed and the scalp wound sutured using Mersilk 4/0 braided suture thread (Ethicon Ltd.). The leg wound was closed using the same method or with 10mm Michell clips (International Market Supply) placed every 5mm along the wound.

(2f) Postoperative care

Postoperatively all animals received 15mg/kg Clamoxyla (Beecham) a long term antibiotic, and 2.5mg/kg Finedyne (Flunixin) a long term analgesic (Schering-Plough Animal Health) subcutaneously. Animals were left to recover full consciousness in a heated environment before being returned to the animal house until the time of sacrifice.

(2g) Application of retrograde tracers to the graft

Animals were anaesthetised and prepared as above. The scalp was reopened, taking care not to disturb the graft lying underneath the skin, and pulled away from the skull surface laterally to the right to expose all of the graft. Any connective tissue and blood vessels formed around the graft were carefully cleared away using a scalpel blade and fine forceps until the entire distal segment of the graft could be seen. Any bleeding
from the disrupted blood vessels was swabbed using sterile tissue pads. An 8/0 suture was then passed through the fascia of the temporals muscle immediately beneath the graft approximately 2mm from its distal end and loosely knotted over the graft.

A small nick was made in the perineurium of the distal end of the graft with a pair of fine spring bow scissors. A fine canular attached to the barrel of a clean 5μl Hamilton syringe containing the retrograde tracer solutions (1μl of sterile water for injection, Antigen Pharmaceuticals, mixed with 0.5μl of cholera toxin subunit-B conjugated HRP solution, Quadratex, and 0.5μl of wheat germ agglutinin conjugated HRP solution, Sigma) was inserted and pushed along the inside of the graft so that the tip of the needle was at least 2 mm past the 8/0 suture mark but not over or within 2mm of the craniotomy. The suture knot was gently tightened around the barrel of the needle and the plunger of the syringe was slowly depressed over approximately 60 seconds to avoid rupturing the graft and flooding the surrounding tissue with the solutions. As the needle was withdrawn the suture was tightened and knotted to form a ligature around the graft to prevent the tracer solutions leaking out of the graft via the nick in the perineurium. The graft was pinched twice carefully with fine micro blood vessel forceps to improve axonal uptake of the tracers. During this procedure care was taken to avoid desiccation of the graft by regularly applying drops of HBSS onto the exposed graft surface. The scalp wound was closed and the animal received the same post operative care as following graft implantation.

(2h) Inhibition of acetylcholine esterase (AChE)

All animals used for AChE histochemistry received an intramuscular injection of 1.8mg/kg of diisopropyl fluorophosphate (DFP, an irreversible AChE inhibitor suspended 10mg/ml in peanut oil, Sigma) and 10mg/kg of atropine sulphate (Sigma) in sterile water for injection (Antigen Pharmaceuticals) intraperitoneally 4½ hours prior to perfusion.

3 - Perfusion

(3a) For general light microscopy

Light anaesthesia was induced using anaesthetic ether vapor and the animal was injected intraperitoneally with an overdose of Sagatal (sodium pentobarbitone, Rhône Mérieux). Breathing was monitored carefully until the corneal blink response was lost and the animal was transferred to lie ventral surface upper most in a dissecting dish in a fume cupboard. The abdominal cavity was rapidly opened longitudinally to the xiphisternum using a large pair of scissors, two cuts were then made at right angles to this exposing the diaphragm. The diaphragm was cut and the rib cage opened with two longitudinal cuts at the side and pulled back and secured with clamps to reveal the heart.
The pericardium was carefully removed and the right atrium opened. A blunt cannula leading from a Watson-Marlow peristaltic pump was then forced through the wall of the left ventricle and up into the aorta. The animal was first exsanguinated with 100ml of 0.1 M phosphate buffer at a flow rate of 22-33 ml/minute and then perfused with differing types and amounts of fixative dependent on the histochemical reaction to follow as listed below.

(3b) For histochemistry and immunohistochemistry

Animals received 400ml of filtered 4% paraformaldehyde (BDH) in 0.1M phosphate buffer adjusted to a pH of 7.4 using drops of 1M sodium hydroxide (BDH) at a flow rate of between 22-33 ml/minute.

(3c) For general electron microscopy

Animals were treated as above but were not prewashed with phosphate buffer before rapid perfusion with 400ml 4% paraformaldehyde, 0.5% glutaraldehyde and 0.5% glucose (BDH) in 0.1M phosphate buffer, pH 7.4, at between 40-50 ml/minute. The brains were removed immediately and full brain blocks were stored in the same fixative at 4°C until processed.

(3d) For electron microscopic immunocytochemistry

Animals were rapidly perfused with 400ml 4% paraformaldehyde and 0.25% glutaraldehyde made up in 0.1M standard phosphate buffer without any prior phosphate buffer prewash. One litre of this buffer was made by adding 10.6g of sodium dihydrogen orthophosphate 1-hydrate (BDH) and 56g of di-potassium hydrogen orthophosphate (BDH) to 1 litre of distilled water. 4ml of 0.5% calcium chloride (BDH) was added to the fixative once the above ingredients were dissolved. The final solution was filtered and taken to a pH of 7.2 - 7.4 using drops of sodium hydroxide.

(3e) For retrograde tracing with cholera toxin subunit B/HRP and wheat germ agglutinin/HRP conjugates

Animals were perfused with 500ml of filtered 1% paraformaldehyde (BDH) and 1.25% glutaraldehyde (Agar Scientific Ltd.) in 0.1 M phosphate buffer adjusted to a pH of 7.4 as above at a flow rate of 11-22 ml/minute.

(3f) Preparation for in situ hybridization

Animals were overdosed with Sagatal as described above and decapitated once the corneal blink reflex was lost.

(3g) Enzyme-linked immunoassay
Animals were overdosed with Sagatal as described above and decapitated once the corneal blink reflex was lost. Tissue samples were microdissected and the individual wet weight for each tissue sample from each animal was recorded. The samples were rapidly frozen on solid carbon dioxide and stored at -70°C until the time of assay.

4 - Dissection

When any of the following techniques were used in tissue preparations for *in situ* hybridization all instruments and equipment were sterilized prior to use either by autoclave (20 minutes at 121 °C) or by treatment with 100% ethanol.

(4a) Brain tissue

Animals were decapitated using large scissors to sever the neck muscles and pliers to cut through the spinal cord. The scalp was then opened and the connective tissue covering the skull was cut away leaving the external portion of the graft covering the craniotomy intact. The temporalis and neck muscles were trimmed away from the bone using a scalpel. Starting at the foramen magnum, bone forceps were used to chip away the skull covering the cerebellum and the left temporal bone. The right temporal bone was carefully removed without disturbing the graft so that from a lateral view the graft could be seen extending from the craniotomy through the subcranial space into the brain. Fine scissors were carefully inserted into the space and the graft cut from the overlying bone. The frontal bone was removed without disturbing the graft in the brain and the olfactory bulbs and cranial nerves were trimmed off as the brain was lifted out of the skull. The whole brain was then trimmed into two blocks ready for cryoprotection.

(4b) Tibial nerve, dorsal root ganglia (DRG) and spinal cord

Where tibial nerve graft tissue had already been taken from an animal the contralateral thigh was opened and the quadriceps muscles parted to reveal the sciatic nerve bundle. The sciatic nerve was traced back to the L4, L5 and L6 ventral rami and the vertebral canal was opened via a laminectomy with bone forceps. The L4, L5 and L6 dorsal roots were traced to the spinal cord and the L4, L5 and L6 DRGs and the L4, L5 and L6 segments of the spinal cord were removed.

(4c) Cryoprotection of tissue

After dissection all blocks of tissue perfused for light microscopy were left in 30% sucrose (BDH) in 0.1 M phosphate buffer at 4°C until they had sunk before being sectioned.

5 - Sectioning tissue
For LM immunohistochemistry and histochemistry

Tissue blocks were rapidly frozen with solid carbon dioxide and positioned on a Reichert freezing microtome using OCT mounting medium (Miles Inc.). Coronal sections were cut in a dorsal to ventral direction, to reduce the possibility of the blade pushing the graft from the brain tissue, and from the rostral to caudal portion of the block. Sections were collected into the appropriate buffer dependent on the histochemical reaction to follow. Brain sections containing grafts were cut at a thickness of 40μm while smaller tissue blocks such as spinal cord and DRGs were cut at 60μm.

For in situ hybridization

Non perfused tissue blocks were mounted, caudal side downwards, onto a metal cutting chuck using OCT mounting medium and slowly frozen by standing the chuck in a bath of liquid nitrogen. This allowed the cold to conduct slowly up the chuck stem preventing too rapid freezing of the block and later fracturing. The block and chuck were then placed in a Jung-Reichert Cryocut 1000 cryostat set at -18 °C and left to equilibrate. Sections were cut coronally at 20μm thick for in situ hybridization using oligonucleotide-labelled probes and at 12-14mm thick for in situ hybridization using digoxigenin-labelled probes. Sections were cut moving caudally through the block, and collected directly onto sterilised gelatinised slides for in situ hybridization using oligonucleotide-labelled probes and saline treated slides for in situ hybridization using digoxigenin-labelled probes. Sections were rapidly air dried using a cold fan for in situ hybridization using oligonucleotide-labelled probes, but not for the digoxigenin-labelled probes, and processed immediately.

6 - Preparation of slides

Clean slides were washed in distilled water before being dipped in a bath of 1g Gelatine (Sigma) and 0.1g Chrome alum (BDH) in 100 ml distilled water. During preparation the solution was heated to 50°C to ensure the gelatine was fully dissolved before being filtered. Slides were then dried in an oven at 60°C.

For in situ hybridization using alkaline phosphotase-labelled probes

Autoclaved slides were washed in DEPC water (diethyl -pyrocarbonate, Sigma, 100μl/500ml distilled water, autoclaved 4 hours after mixing) and dipped in 1.87g gelatine and 187mg chrome alum in 750ml DEPC water dissolved at 70°C before filtration. Slides were held in autoclaved slide trays and wrapped in autoclaved foil before being oven dried. Slide trays were allowed to cool to room temperature before
use.

(6c) For in situ hybridization using digoxigenin-labelled probes

Autoclaved slides were washed in Decon-90 detergent (Rhone-Poulenc) for two hours, rinsed in running water for 20 minutes followed by a 5 minute rinse in distilled water. The slides were then immersed in 15ml 37% HCl (BDH) and 985ml 95% ethanol (BDH) for 15 minutes followed by a 2 minute rinse in distilled water and dried at 160°C over night. The next morning the slides were removed from the oven and allowed to cool before being dipped in 6% saline in 100% acetone (BDH) for 5 minutes. The slides were then washed twice in 100% acetone (BDH) for 3 minutes and then twice in distilled water for 3 minutes and left to air dry at room temperature.

7 - Histochemical procedures

(7a) Tetramethylbenzidine (TMB) method of HRP visualization

Free floating sections were collected into 0.1 M phosphate buffer and stored at 4°C for not more than 24 hours before reacting. Sections were first rinsed in distilled water for 10 - 15 seconds six times before being immersed in incubation solution for 20 minutes at room temperature (RT) on a rocker table. The incubation solution consisted of 2 solutions prepared separately but combined seconds before the introduction of the tissue sections. Solution A: 100g sodium nitroferricyanide (Sigma), 5ml pH 3.3 acetate buffer in distilled water (acetate buffer: 200ml 1M sodium acetate (Sigma) and 190ml 1M hydrochloric acid in distilled water adjusted to a pH of 3.3 with concentrated acetic acid / sodium hydroxide, BDH). Solution B: 5mg 3,3', 5,5' TMB (Sigma) dissolved in absolute ethanol (Analar, BDH). One ml of 0.03% hydrogen peroxide in distilled water was then added to the incubation solution and the sections were left for a further 20 minutes or until reaction product was seen in the surrounding solution. Sections were then rinsed for 5 minutes six times in post reaction and storage solution (PRSS, 200ml of the acetate buffer in 800ml distilled water) at 4°C and mounted from PRSS onto clean gelatinised slides and left to air dry for not more than 24 hours at room temperature.

(7b) Acetylcholine esterase

Free floating sections were collected into 0.1M phosphate buffer, pH 7.4, and stored at 4°C for not more then 24 hours before further processing. Sections were briefly rinsed in distilled water and immersed in a solution of 7.2mg ethopropazine (Sigma), 72mg glycine (Sigma), 50mg copper sulphate (BDH), 120mg acetyltiocholine (Sigma) and 680mg sodium acetate (Sigma) in 100ml distilled water, adjusted to pH 5 using drops of glacial acetic acid (BDH), for 60 minutes on a rocker table. Sections were then rinsed six times for one minute in distilled water before development for 2 1/2
minutes in a solution of potassium ferricyanide (Sigma) dissolved to a final concentration of 10% by weight in PRSS. Sections were then rinsed six times for thirty seconds before being mounted on clean gelatinised slides and air dried for twenty four hours at room temperature.

(7c) NADPH-diaphorase

Free floating sections were collected in 0.1M Tris (hydroxymethyl) methylamine (Tris, BDH) buffer and left at 4°C for not more than 24 hours before reacting. A solution of 27mg malic acid (Sigma) in 9ml 0.1M Tris buffer was then added to 2mg of nitro blue tetrazolium (Sigma) in 900μl 0.1M Tris buffer with 100μl of 10% Triton-X (Sigma). A separate solution of 10mg NADPH (Sigma) was prepared in 100μl of distilled water. If the solution appeared yellow rather than colourless, indicating that the enzyme may have been degraded, it was discarded and replaced using new NADPH. All solutions were combined and the sections were incubated free-floating in the final mixture for either 30 minutes at 37°C or 90 minutes at room temperature. The reaction was stopped by transferring the sections into fresh 0.1M Tris buffer and sections were mounted straight from this onto clean gelatinised slides and air dried at room temperature for 24 hours.

(7d) Thionin staining

Free-floating sections were collected into 0.1M phosphate buffer, mounted directly onto clean gelatinised slides and left to air dry at room temperature for 24 hours. Slides were then placed in a glass staining tray and immersed into the following series of solutions for the times stated below in Table 2.1. Sections were coverslipped using DPX mountant (Sigma) directly from the last bath of Histoclear and left to air dry at least 3 hours before inspection.
SOLUTION | TIME
---|---
70% ethanol | 10 seconds
95% ethanol | 10 seconds
Absolute ethanol | 10 seconds
Histoclear | 15 minutes
Absolute ethanol | 2 minutes
95% ethanol | 2 minutes
70% ethanol | 2 minutes
50% ethanol | 2 minutes
Distilled water | 2 minutes
Absolute ethanol | 2 minutes
Absolute ethanol | 2 minutes
Histoclear | 2 minutes

Table 2.1 Thionin counterstaining and dehydration of prepared and mounted sections

(7e) Immunohistochemistry

The basic protocol for choline acetyl transferase (ChAT, anti-choline acetyltransferase antibody, clone 1E6, Biogenesis), low affinity nerve growth factor receptor (LNGFR, anti-p75 antibody, clone 192, Boehringer Mannheim), GAP-43 (anti-GAP-43, 9-1E12 antibody, a gift from Dr. D. Schreyer, Dept of Physiology, Queens University, Kingston, Canada) and tyrosine hydroxylase (TH, anti-tyrosine hydroxylase antibody, TX 100, Affinity) immunohistochemistry is the same, differing only in the normal sera, primary and secondary antibodies used, their dilutions and incubation times. These are supplied in the table below.

Free floating sections were collected into 0.1 M phosphate buffered saline (PBS) and stored at 4°C for not more than 24 hours before reacting. Sections were rinsed twice for 15 minutes in PBS before incubation for 1 hour in a blocking serum, (to reduce the occurrence of non-specific antibody binding), consisting of 50mg bovine serum albumin (BSA; Sigma), 0.2ml normal serum (Sigma) and 5μl triton-X 100 (Sigma) in 10ml PBS at room temperature. The triton-X 100 was used to disrupt the cellular membranes of the tissue sections making them more permeable to the antibody solutions used. This was followed by incubation in the primary antibody specific for each protein diluted in 50mg BSA and 0.2ml normal serum in 10ml PBS. Sections were incubated for the time and temperatures shown below. After incubation with the primary antibody sections
were rinsed in PBS for half an hour changing the PBS three times. Sections were then incubated with a secondary antibody diluted in PBS for 2 hours at room temperature. The avidin-biotin signal amplification complex was prepared half an hour before use using a Vectastain Elite ABC kit at an A : B : PBS ratio of 1:1:48 and allowed to mix well.

Sections were incubated in this mixture for one and half hours at room temperature and then rinsed for half an hour in PBS changing twice. Sections were finally incubated in a 25mg 3,3'- diaminobenzidine tetrahydrochloride (DAB, Sigma) solution in 100mg 0.1M phosphate buffer and 1ml 33% hydrogen peroxide until the dark brown reaction product was visualised. DAB is a carcinogen and precautions were taken when handling and disposing of it i.e. gloves were always worn, the reaction took place under a fume hood and the solution was neutralised with sodium hypochloride (Sechelle) before disposal. The reaction was stopped in 0.1M phosphate buffer and the sections were rinsed for half an hour, changing the solutions three times, before being mounted onto clean gelatinised slides and left to air dry for 24 hours.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>NORMAL SERA</th>
<th>PRIMARY ANTIBODY</th>
<th>DILUTION</th>
<th>INCUBATION</th>
<th>SECONDARY ANTIBODY</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChAT</td>
<td>Horse</td>
<td>Monoclonal anti-ChAT</td>
<td>1:1000</td>
<td>64 hrs RT</td>
<td>Anti mouse - raised in horse</td>
</tr>
<tr>
<td>p75</td>
<td>Horse</td>
<td>Monoclonal anti-p75</td>
<td>1:3000</td>
<td>64 hrs 4°C or 8 hrs RT</td>
<td>Anti mouse - raised in horse</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Horse</td>
<td>Monoclonal anti-GAP-43</td>
<td>1:30,000</td>
<td>8 hrs RT</td>
<td>Anti mouse - raised in horse</td>
</tr>
<tr>
<td>TH</td>
<td>Goat</td>
<td>Polyclonal anti-TH</td>
<td>1:800</td>
<td>36 hrs 4°C</td>
<td>Anti rabbit - raised in goat</td>
</tr>
</tbody>
</table>

**Table 2.2** Table showing the appropriate solutions and incubation times for each antibody used for LM immunohistochemistry in this study.

8 - *In situ* hybridization using alkaline phosphatase conjugated probes

Gloves were worn at all times during this reaction. All equipment used in the pre-treatment and hybridization stages were autoclaved as above or sterilised with a mist of absolute ethanol before use.

**(8a) Pre-treatment**

All of the following steps were performed at room temperature. The prepared slides were rapidly immersed in 4% paraformaldehyde fixative/0.1M phosphate buffer, made with sterile DEPC water, for 30 minutes followed by 10 minute incubation in acetylation buffer (0.62ml acetic anhydride, Sigma, 3.92ml triethanolamine, BDH, and 2.25g sodium chloride, BDH, in 250ml DEPC water). Slides were then washed in
DEPC water for 10 minutes and dehydrated in 90% ethanol/DEPC water (10 minutes), 100% ethanol (10 minutes) and delipidated in chloroform (BDH, 10 minutes) before partial rehydration in 90% ethanol/DEPC water (10 minutes).

(8b) Hybridization

During the dehydration sequence described above the hybridization buffer was prepared using 2.5ml deionized formamide (Fluka), 125µl 50X Denhardts solution (Sigma), 200µl single stranded salmon testes DNA (Sigma), 0.5g dextran sulphate, sodium salt (Sigma), 1.25ml DEPC water and 1ml SSC. Approximately 2µl of an alkaline phosphatase linked c-DNA probe for GAP-43 mRNA (a gift from Dr. Piers Emson, Babraham Institute, Cambridge) was added to the buffer immediately before application to the slides. Slides were placed section side up in a humid sterile incubation chamber. A ring was drawn around the sections on each slide using a PAP pen (Dako) to prevent the buffer running off of the slide leaving the sections uncovered. The buffer was shared out equally between the slides, control slides receiving the same quantity of hybridization buffer only. The incubation chamber was sealed with tape and incubated at 37°C overnight.

(8c) Colour reaction

A stock solution of 20 x standard saline citrate (SSC) was prepared with 173.5g sodium chloride (BDH), 88.2g tri-sodium citrate (BDH) in 800ml DEPC water adjusted to pH 7.0 with 1M HCl (BDH). Slides were washed 3 times for 20 minutes in baths of 1X SSC diluted in distilled water at 45°C followed by a single one hour wash in 1X SSC at RT. Sections were then incubated in Buffer 1 for 30 minutes RT followed by buffer 2 for 10 minutes RT. All buffers were prepared fresh during the SSC washes, buffer 1: 25ml 2M Tris (BDH) and 4.38g sodium chloride (BDH) in 500ml distilled water, pH 7.4, buffer 2: 25ml 2M Tris (BDH), 2.95g sodium chloride (BDH) and 5.07g magnesium chloride hexahydrate (Sigma) in 250ml distilled water pH 9.5. The colour reaction buffer was then prepared from two stock solutions; solution A: 75mg 4-nitroblue-tetrazolium-chloride (NBT, Boehringer-Mannheim) dissolved in 1 ml 70% dimethylformamide/distilled water, solution B: 50mg 5-bromo-4-chloro-3-indoly-phosphate (BCIP, Boehringer-Mannheim) in 1ml dimethylformamide. Both solutions were stored at -20°C. The final colour reaction buffer, buffer 3, was prepared from 45µl solution A and 35µl solution B in 10ml buffer 2. The sections were ringed with the PAP pen and arranged in a humid incubation chamber as before. Each slide received an equal amount of colour reaction solution and the chamber was sealed with tape and covered with foil to prevent light affecting the photosensitive reaction. The sections were left to incubate overnight at RT or until the desired darkness of the reaction product was reached. To stop the colour reaction slides were immersed in buffer 3 for 10 minutes.
changing the buffer once (buffer 3: 2.5ml 2M Tris (BDH), 4.38g sodium chloride and 0.186g ethylenediaminetetra acetic acid (EDTA) in 500ml distilled water adjusted to pH 7.4 with concentrated HCl). The slides were left to air dry at least 24 hours, briefly immersed in Histoclear clearing agent and coverslipped using DPX mountant.

9 - In situ hybridization using digoxigenin labelled probes

(9a) Pre-treatment

The prepared slides were immersed in cooled 4% paraformaldehyde fixative/0.1M phosphate buffer, made with sterile DEPC water, pH 7.2 - 7.4, and left for at least 2 hours or overnight at 4°C. All of the following steps were performed at room temperature. The slides were washed in DEPC-treated 0.1M PBS 3 times, for approximately 5 minutes each wash, before being dehydrated in 70% ethanol/DEPC water for 5 minutes and washed twice in DEPC water (5 minutes). In this protocol PBS was made using 80g sodium chloride (BDH), 2 g potassium dihydrogen orthophosphate anhydrous (BDH), sodium dihydrogen orthophosphate anhydrous (BDH) and 2g potassium chloride (BDH) dissolved in 1 litre DEPC water and filtered. The sections were taken to a fume cupboard and immersed in 0.1M HCl (BDH) for 5 minutes and then washed in DEPC-treated 0.1M PBS twice for 10 minutes. The sections were permeabilized by immersing them in a solution of 1.5ml triethanolamine (BDH) and 250µl acetic acid anhydride (BDH) in 100ml DEPC water for 20 minutes at room temperature followed by 2x5 minute rinses in DEPC-treated 0.1M PBS. The sections were then dehydrated by being taken through a series of 70%, 80% and 90% ethanol (BDH) made with DEPC water for 5 minutes each solution.

(9b) Pre-hybridization

The prepared slides were air dried and the sections were ringed with a PAP-pen (Dako) before being placed in a humid (formamide/DEPC-treated 0.1M PBS) incubation chamber and covered with equal amounts of deionized formamide (Fluka) and pre-hybridization mix. 50ml of the pre-hybridization mix was made by mixing 5ml of 50x Denhardt's solution (Sigma), 5ml 0.5ml EDTA (Sigma), 5ml of 1M Tris-HCl (pH7.6), 2.5ml of tRNA (Yeast tRNA, 25mg/ml solution, Boehringer) and 2ml of 1M NaCl (BDH) in 30.5ml DEPC water. The incubation chamber was sealed and placed in an oven at 37°C for at least 3 hours.

(9c) Hybridization

The hybridization solution was prepared, applied to the slides as soon as the incubation chamber was removed from the oven. 10ml of the hybridization mix was made by mixing 5ml of deionized formamide (Fluka), 1ml of 10xGrundmix solution (to
make 10ml of 10xGrundmix add 200µl of 0.5M EDTA, 2ml of 1M Tris-HCl at pH 7.5, 2ml 50x Denhardt's solution, Sigma, 2ml of tRNA, (Yeast tRNA, 25mg/ml solution, Boehringer), and 1ml of 10mg/ml Poly-A-RNA, Sigma, to 2.8ml DEPC water), 667µl of 5M NaCl, 2ml of 50% Dextran sulphate (made with DEPC water), 500µl of 2M dithiothreitol (DDT, Sigma) and 754µl of DEPC water. 40µl of the digoxigenin-labelled antisense probe was added to this and mixed well. One at a time, the slides were removed from the incubation chamber and the pre-hybridization mixture was carefully blotted from them. The slides were returned to the humid incubation chamber and covered in hybridization mix, the chamber was sealed, returned to the oven and left overnight at 55°C.

(9d) Post-hybridization washing

The slides were removed from the incubation chambers and rinsed in prewarmed 1xSSC to remove the probe hybridization mixture before being washed twice, for 30 minutes each wash, in prewarmed 0.2xSSC in Coplin jars standing in a 55°C water bath. The SSC solutions were all prepared from a sterile DEPC-treated 20xSSC stock solution as used in the alkaline-phosphatase labelled probe in situ hybridization as described above and diluted in DEPC water. The sections were then washed 3 times in 50% formamide (Fluka) made with 0.1x SSC at 55°C for 1 hour each wash and finally rinsed for just 10 minutes in 0.2x SSC at room temperature. The following buffers were prepared during the above SSC washes; buffer 1: 100mM Tris-HCl at pH 7.5 (15.76g) and 150mM NaCl (8.8g) diluted with 1 litre DEPC water; buffer 3: 100mM Tris-HCl at pH 7.5 (15.76g), 150mM NaCl (5.8g) and 50mM MgCl₂ (10.16g) dissolved in 1 litre DEPC water; buffer 4: 10mM Tris-HCl at pH 7.5 (1.57g) and 1mM EDTA (375mg) dissolved in 1 litre DEPC water and adjusted to pH 8.0.

(9e) Visualization of the probe

Sections were washed in buffer 1 for 10 minutes and then immersed in modified blocking medium (1g Blocking Reagent, and 0.5g of BSA Fraction V, Boehringer, dissolved in 100ml buffer 1) for 30 minutes. The slides were quickly transferred to a humid incubation chamber, moistened with buffer 1, and covered with α-digoxigenin AP-coupled antibody (Fab fragments, Boehringer), made at a 1:350 dilution in the modified blocking medium. The incubation chamber was sealed and left at 4°C overnight. The following morning the slides were removed from the incubation chamber and washed twice for 15 minutes each wash in buffer 1, followed by one 2 minute wash in buffer 3. The slides were then returned to the incubation chamber and the sections were covered with AP-developer consisting of 10µl Levamisal (Sigma), 35µl of 50mg/ml x-Phosphate solution (Boehringer) and 33.7µl of 100mg/ml NBT solution (Boehringer) in 10ml of buffer 3. The chamber was sealed, placed in the dark and left at
room temperature until reaction product could be seen in the wet sections. The colour reaction was stopped by washing the slides twice for 10 minutes each wash in buffer 4 and the sections were air dried overnight at room temperature before being coverslipped with DPX mountant (BDH).

10 - Transmission electron microscopy

(10a) Preparation of tissue for general transmission electron microscopy

After collection, Vibratome sections were rinsed in 0.1 M phosphate buffer and osmicated for 1 hour in 1% O₃O₄ (Agar Scientific) in 0.1 M phosphate buffer and then block stained in 2% uranyl acetate (Agar Scientific) in sodium acetate buffer for 45 minutes at 4°C. Sections were dehydrated in alcohols and cleared in propylene oxide before being embedded in Araldite in the sequence shown in Table 2.3.

The processed sections were placed between two Melinex sheets (ICI) between glass sides. These were weighed down, to force out any excess resin and to produce flat sections, and placed in an oven for 16 hours at 60°C. The desired region of graft or brain tissue was cut from the section with a razor blade and fixed to the tip of a flat ended Araldite stub with cyanoacrylate glue (RS Components).

Semi-thin (1µm) sections were cut from these blocks with glass knives on a Reichert Ultracut ultramicrotome and stained with Toludine blue. Adjacent thin (70-100nm) sections were cut with a diamond knife and collected onto copper coated mesh and single slot grids covered with a thin formvar film (Agar Scientific). These sections were counterstained with 2% lead citrate in distilled water and viewed in a JEOL 1010 electron microscope.

(10b) Preparation of tissue for immunohistochemistry and transmission electron microscopy

50-80µm thick Vibratome sections were washed in 0.1M PBS three times for ten minutes each wash and rinsed in a blocking serum of 0.1% BSA (Sigma) in PBS overnight at 4°C before incubation in 0.1M sodium m-periodate (Sigma) in 0.1M PBS for 15 minutes. These sections were washed 3 times for 5 minutes in PBS and incubated in 1% sodium borohydride (Sigma) in PBS for 10 minutes. The wash cycle (3 x 5 minutes) was repeated and the sections were immersed for 15 minutes in a 20% solution of dimethyl sulfoxide (DMSO; Sigma) in PBS before undergoing 4 x 5-minute washes in PBS. The sections were placed in separate chambers of a Corning staining well with 200µl of the desired 1° antibody solution (see Table 2.4, gifts from Prof. M. Schachner, Institute of Neurobiology, ETH, Zurich, Switzerland) and left on a rocker table overnight at 4°C.
<table>
<thead>
<tr>
<th>STEP</th>
<th>TIME</th>
<th>SOLUTION</th>
<th>TEMPERATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 min</td>
<td>0.1M Millonig's Buffer* (MB)</td>
<td>Room Temperature (RT)</td>
</tr>
<tr>
<td>2</td>
<td>60 min</td>
<td>1% osmium tetroxide made in 0.1M MB</td>
<td>4°C</td>
</tr>
<tr>
<td>3</td>
<td>5 min</td>
<td>0.1M MB</td>
<td>RT</td>
</tr>
<tr>
<td>4</td>
<td>5 min</td>
<td>0.1M sodium acetate buffer</td>
<td>RT</td>
</tr>
<tr>
<td>5</td>
<td>45 min</td>
<td>2% uranyl acetate made in 0.1M sodium acetate buffer</td>
<td>4°C</td>
</tr>
<tr>
<td>6</td>
<td>5 min</td>
<td>0.1M sodium acetate buffer</td>
<td>RT</td>
</tr>
<tr>
<td>7</td>
<td>5 min</td>
<td>distilled water (DW)</td>
<td>RT</td>
</tr>
<tr>
<td>8</td>
<td>5 min</td>
<td>25% ethanol</td>
<td>RT</td>
</tr>
<tr>
<td>9</td>
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<td>RT</td>
</tr>
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<td>RT</td>
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</tr>
<tr>
<td>14</td>
<td>5 x 10 min</td>
<td>propylene oxide</td>
<td>RT</td>
</tr>
<tr>
<td>15</td>
<td>4 x 10 min</td>
<td>1:1 Araldite resin†: propylene oxide</td>
<td>RT</td>
</tr>
<tr>
<td>16</td>
<td>2 x 24 hours</td>
<td>Araldite resin</td>
<td>4°C</td>
</tr>
</tbody>
</table>

Table 2.3 The sequence of steps taken in the processing of Vibratome sections for general EM

* 1 litre of 0.1M Millonig's Buffer was made by adding 13.7g of sodium dihydrogen orthophosphate 1-hydrate (BDH) to 840ml distilled water and titrating to pH7.4 with approximately 160ml of 1M sodium hydroxide.

† 20ml of Araldite resin was made by mixing 10ml of dodecenyl succinic anhydride (Agar Scientific) with 10ml Araldyte CY212 (Taab) and 0.8ml dibutyl phthalate (Agar Scientific) for 5 minutes at 60°C. 0.4ml of benzyldimethylamine (Agar Scientific) was added and the mixture was stirred for a further 2 minutes.
<table>
<thead>
<tr>
<th>PROTEIN LABELLED</th>
<th>NORMAL SERA</th>
<th>PRIMARY ANTIBODY</th>
<th>DILUTION</th>
<th>PRIMARY INCUBATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI</td>
<td>0.1%BSA in 0.1M PBS</td>
<td>Polyclonal anti-L-1</td>
<td>1:100 (made in blocking serum)</td>
<td>24 hours at 4°C</td>
</tr>
<tr>
<td>N-CAM</td>
<td>0.1%BSA in 0.1M PBS</td>
<td>Polyclonal anti-N-CAM</td>
<td>1:50 (made in blocking serum)</td>
<td>24 hours at 4°C</td>
</tr>
<tr>
<td>TENASCIN - C</td>
<td>0.1%BSA in 0.1M PBS</td>
<td>Polyclonal anti-Tenascin-C</td>
<td>1:100 (made in blocking serum)</td>
<td>24 hours at 4°C</td>
</tr>
</tbody>
</table>

**Table 2.4** The antibodies used for immunohistochemical EM, their dilutions and incubation times.

The following morning the sections were returned to room temperature for a further 4 hours, or until the primary antibody incubation had lasted at least 20 hours in total, and then taken through the steps shown in Table 2.5.

The sections were then embedded between two Melinex sheets (ICI) between glass sides, pressed flat, and placed in an oven for 16 hours at 60°C. The desired tissue blocks were cut from the sections and mounted on Araldite stubs and semithin sections were cut for transmission electron microscopy described as above.
<table>
<thead>
<tr>
<th>STEP</th>
<th>TIME</th>
<th>SOLUTION</th>
<th>TEMPERATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 x 20 mins</td>
<td>2% Blotto (dried milk)</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>2</td>
<td>20 hours</td>
<td>Protein A conjugate peroxidase</td>
<td>4°C</td>
</tr>
<tr>
<td>3</td>
<td>10 mins</td>
<td>0.5% glutaraldehyde in 0.01M phosphate buffer (PB)</td>
<td>RT</td>
</tr>
<tr>
<td>4</td>
<td>10 mins</td>
<td>0.01M PB</td>
<td>RT</td>
</tr>
<tr>
<td>5</td>
<td>10 mins</td>
<td>0.06% DAB in 0.004M Tris buffer (TB)*</td>
<td>RT</td>
</tr>
<tr>
<td>6</td>
<td>20 mins</td>
<td>0.03% DAB in 0.004M Tris buffer (TB)†</td>
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</tr>
<tr>
<td>7</td>
<td>25 mins</td>
<td>0.01% hydrogen peroxide in 0.03% DAB/ 0.04M TB</td>
<td>RT</td>
</tr>
<tr>
<td>8</td>
<td>2 x 5 mins</td>
<td>0.01M PB</td>
<td>RT</td>
</tr>
<tr>
<td>9</td>
<td>30 mins</td>
<td>1% osmium tetroxide in 0.01M PB</td>
<td>RT</td>
</tr>
<tr>
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<td>2 x 5 mins</td>
<td>0.01M PB</td>
<td>RT</td>
</tr>
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<td>RT</td>
</tr>
<tr>
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</tr>
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<td>RT</td>
</tr>
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<td>100% acetone</td>
<td>RT</td>
</tr>
<tr>
<td>17</td>
<td>30 mins</td>
<td>2:1 100% acetone / Spurr resin</td>
<td>RT</td>
</tr>
<tr>
<td>18</td>
<td>30 mins</td>
<td>1:2 100% acetone / Spurr resin</td>
<td>RT</td>
</tr>
<tr>
<td>19</td>
<td>Overnight</td>
<td>100% Spurr resin</td>
<td>4°C</td>
</tr>
</tbody>
</table>

*Table 2.5  The sequence of steps taken in the processing of Vibratome sections for immunohistochemical EM

*0.06% DAB: 6mg of DAB (Sigma) dissolved in 10ml 0.04M Tris buffer at pH 7.6.
†0.03% DAB: 6mg of DAB (Sigma) dissolved in 20ml 0.04M Tris buffer at pH 7.6.
¶100% Spurr Resin: 10g of vinyl cyclohexene dioxide, 6g of resin, 26g of nonenyl succinic anhydride and 0.4g of dimethylaminoethanol (taken from a TAAB Spurr resin kit) mixed well.
11 - Enzyme linked immuno assay (ELISA) of NGF levels in graft and brain tissue

(11a) Preparation of samples

Tissue samples were kept at -70°C until the homogenisation buffer was prepared. 400ml of homogenisation buffer was freshly prepared using 9.35g sodium chloride (BDH), 200μl Tween 20 (RIA grade, Sigma), 2g bovine serum albumin (RIA grade, Sigma), 0.007g phenylmethyl-sulfonyl fluoride (Sigma), 0.02g benzethonium chloride (Sigma) and 1.49g EDTA mixed in 400ml of PBS. Once fully dissolved 0.004g of Aprotinin (Sigma) was added to the buffer. The tissue samples were individually placed in numbered centrifuge tubes placed in ice and 2ml of the buffer was added to each tube. Each tissue sample was homogenised for approximately 30 seconds at 27,000 RPM, the blade was removed and carefully washed with distilled water between each tube to prevent cross contamination between samples. The homogenates were then centrifuged for 60 minutes at 11,000 RPM at 4°C. Three 0.5ml samples of supernatant were taken from each tube and decanted into identical Eppendorf tubes which were sealed and labelled as per the tissue sample and the number of the centrifuge tube. The supernatant samples were stored at -70°C until assayed.

(11b) ELISA procedure for NGF

This protocol is based on that of Weskamp and Otten (1987). Microtiter plates were coated with 100μl per well of polyclonal anti-NGF antiserum (a gift form Dr. Janet Winter, Sandoz Institute, Gower Place, London) diluted to 1:30,000 in sodium carbonate/bicarbonate buffer, pH 9.6. The plates were covered and incubated overnight at 4°C. The following morning the coating solution was removed and the wells received 4 washes of 200ml of washing buffer consisting of PBS containing 0.4M sodium chloride and 0.05% Tween 20. 300μl of a blocking solution, made up of 5mg/ml BSA in PBS, was used to block any non-specific binding sites, and the plates were incubated in the blocking solution for 1 hour at 37°C before being rinsed 4 times in the washing buffer.

Each well was then incubated overnight at 4°C with 100μl of a standard solution of NGF, prepared from 1.56-200pg of NGF (Promega) dissolved in sample buffer (PBS containing 0.4M sodium chloride and 1mg/ml BSA), and 100μl of the samples prepared earlier for testing for NGF. Control wells were prepared in the same way but without the NGF solution. After incubation the wells were emptied and rinsed 4 times with the wash buffer and 100μl of monoclonal antibody raised against NGF (also a gift form Dr. Janet Winter, Sandoz Institute, Gower Place, London), isolated from the 23c4 cell line, diluted at 1:10,000 in PBS with 0.05% Tween 20 and 1mg/ml BSA was added to each well and the plates were incubated overnight at 4°C. The solutions were removed and the plates were washed 4 times as above. Each well then received 100μl of biotinylated rabbit anti-rat IgG secondary antibody (Zymed Laboratory) diluted 1:10,000
in PBS and 0.05% Tween 20 and incubated for 1 hour at 37°C. The wells were emptied, washed 4 times as above and 100μl of a 1:10,000 solution of Peroxidase conjugated Streptavidin (Dakopatts) diluted in PBS containing 0.05% Tween 20 was added to each well. The plates were then incubated for a further hour at 37°C. The wells were washed and the peroxidase activity was determined by adding 100μl of a substrate solution made up of 1ml acetate buffer, 100μl TMB, 2μl hydrogen peroxide in 9ml of distilled water and leaving for 30 minutes in the dark at RT until optimally developed. The reaction was stopped by adding 100μl of 2M sulphuric acid per well. The plates were then evaluated using an MR5000 ELISA reader using EIA CALC and a 4 parameter logistic curve fit. The change in optical density was measured at 450nm. The output reading indicated the concentration of NGF in pg per 100μl, the total amount of pg of NGF contained in 1 mg of the brain tissue sample was calculated and, from this, the amount of NGF contained in 1mg of the original wet brain tissue was determined. NGF levels were determined for external and internal portions, ipsilateral and contralateral striata, tibial nerve stump in leg, contralateral whole tibial nerve, tibial nerve grafts left in the leg; distal and proximal portions, tibial nerve cut and ligated and left in leg; distal and proximal portions, and samples of control tibial nerve and striata from unoperated animals.

12 - Dehydration and storage of slides

All tissue sections, apart from those processed for HRP retrograde labelling and non-radioactive in situ hybridisation, were dehydrated and cleared of lipids in the series of alcohols and Histoclear clearing agent shown below in Table 2.6.

Slides were coverslipped straight from the last bath of Histoclear using DPX mountant and left to air dry at RT before inspection. All slides were then stored at room temperature except those processed for HRP retrograde labelling and in situ hybridisation which were stored at 4°C between inspection as both reaction products are temperature sensitive and are known to fade at RT.
<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol/distilled water</td>
<td>10 seconds</td>
</tr>
<tr>
<td>95% ethanol/distilled water</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Histoclear</td>
<td>15 minutes</td>
</tr>
<tr>
<td>95% ethanol/distilled water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Histoclear</td>
<td>0 minutes</td>
</tr>
</tbody>
</table>

**Table 2.6** Dehydration sequence used for most of the histochemical and immunohistochemical procedures.
13 - Collection of data

(13a) Microscopy

Four different microscopes were used in this study; a Zeiss Axioplan photomicroscope, an Edge High Definition Stereo photomicroscope, a Zeiss SV8 dissection photomicroscope and a Zeiss transmitted-light Photomicroscope III. The majority of the bright field and dark field photomicrographs presented in the results were taken using the Zeiss Axioplan photomicroscope using x10, x20 and x40 Zeiss objectives and a Zeiss camera. The Edge photomicroscope was used in instances where high quality depth of field was required, for example in tissue processed for in situ hybridisation for GAP-43. The Edge microscope was used for bright field photography only, with a Nikon camera and Nikon objectives. The Zeiss transmitted-light Photomicroscope III was mainly used for the initial examination of slides with x2.5, x10, x20 and x40 objectives although it was used for some bright and dark field photography. The Zeiss dissection photomicroscope was used for bright and dark field photography at very low (< x16) magnification.

All black and white photomicrographs, bright and dark field, were taken on Ilford Pan F film. These films were developed in darkness for approximately 5 minutes, at 20°C in a 1 to 10 part solution of Acutol developer (Patterson) to cold tap water. The film was briefly rinsed in cold water before fixation for a further 5 minutes in darkness in a 1 to 3 part solution of Ilford Hypam fixative and tap water. After rinsing with running cold tap water for 10 minutes the film was hung in a Kodak warm air cabinet until dry. Black and white photomicrographs were printed on Ilford RC deluxe photographic paper, using grades 2 - 5 as appropriate, and developed by an Ilford 2150RCn printing machine. Colour photomicrographs were taken on Kodacolor Gold II film and printed by local commercial laboratories. Colour transparencies were taken on Ektachrome 64 T film (Kodak), which is adapted to the tungsten light generated by microscope bulbs, and printed by Colour Processing Laboratories of Warren St., London, WC1. These transparencies were converted into colour prints by the same company when required.

(13b) Measurement of cell area

For the cholinergic cell hypertrophy study

Five similar coronal sections through the graft within the striatum were selected from each animal. The graft and surrounding striatal hemisphere were first drawn, using a Zeiss Standard WL Research microscope with a camera lucida attachment, at low magnification. A border was traced around the graft, 400μm from the graft/striatum interface and the position of all AChE or ChAT positive perikarya visible at that
magnification within that area were then plotted in relation to the graft. This was used as a landmark reference map when locating perikarya at higher magnifications. All labelled perikarya which (a) were within this area, (b) had an unambiguous, complete cell outline and (c) had two or more distinct cell processes were then drawn at x100. A random sample of labelled perikarya within the corresponding area from the contralateral striatal hemisphere which matched criteria (b) and (c) were also drawn at x 100 for comparison.

The area of the cell profiles taken was measured using an Apple Mac Graphics tablet and collected into groups according to the survival time of the graft, whether the cell profiles were ipsilateral or contralateral to the graft and whether the graft was living or a control freeze-killed specimen. These results were analysed using 'Statview' and 'Deltagraft' statistic packages for the Macintosh IIIsi and presented in both tabular and graph form.

For the NADPH-diaphorase cell enlargement study

Five representative sections were selected and drawn at low power as described above. The position of all labelled perikarya within 400μm of the graft/brain interface was plotted as before to be used as a reference guide during further examination. As above, labelled perikarya with unambiguous, complete cell outlines and two or more distinct cell processes were analysed using the Seescan Image Analysis System (Seescan, Cambridge). Measurements of the perikaryal area of cell profiles ipsilateral to the graft taken at each survival time were compared to the areas of a corresponding population of NADPH-diaphorase positive perikarya contralateral to the graft and analysed using the same statistic packages as above. The results were presented in graph and table form.
Regeneration of the axons of neurons in the corpus striatum and substantia nigra into autologous peripheral nerve grafts

It is known that peripheral nerve grafts can promote the regeneration of axons from injured CNS neurons in a number of different nuclei of the adult brain. This chapter is concerned with the regenerative response of striatal neurons and neurons whose projections terminate within the corpus striatum of adult rats.

(a) Summary of methods used
See Chapter 2 for details:

Autologous tibial nerve grafts were implanted in the right striatum of adult Sprague Dawley rats. The distal end of these grafts was injected with HRP-conjugated retrograde tracers 3 days prior to sacrifice between 2 and 30 weeks po. Coronal freezing microtome sections were cut through the striatum and through the substantia nigra pars compacta and reacted for HRP. Sections were viewed under bright and dark field microscopy. The number and location of any retrogradely labelled neuronal perikarya were recorded.
(b) Criteria for the identification of retrogradely labelled neurons

A labelled structure was defined as a neuronal cell body if it:

a) had a distinct outline with no confusion between specific labelling and background levels of the reaction product,

b) had at least two labelled processes emanating from it.

Dense clusters of reaction product and reaction product crystals were not included in the neuron count if no clear cell outline was seen.

Labelled axons were identified as continuous, non-branching fine lines of reaction product running within the graft or towards the graft within the brain.

Dendritic processes were similar in appearance to axons but often tapered in width with distance from a labelled cell body within either the striatum, globus pallidus (GP) or substantia nigra and were often branched.

The position of neurons in consecutive sections was noted so that slices of the same neuron appearing in 2 neighbouring sections were only counted once and the neuron recorded only in the section in which the majority of the cell was seen. When large numbers of neurons were labelled, such as in the SNpc, only heavily labelled, well defined cell profiles were included in the count, reducing the confusion between lightly labelled cells and parts of cells which were more heavily labelled in other sections. Consequently the number of cells seen in these regions was probably underestimated.
(c) Results

At 2 weeks po a small number of retrogradely labelled axons were identified in the grafts of 2 of the 4 animals examined at this survival time (Figs. 3.1a and 3.2a and b) and approximately 2 labelled striatal neurons per animal were seen close to the graft tip (Figs. 3.2a and b). A few labelled axons and perikarya were also seen in the SNpc of these animals (Fig. 3.2c and 3.2d).

<table>
<thead>
<tr>
<th>SURVIVAL TIME (weeks po)</th>
<th>MEAN NUMBER OF LABELLED NEURONS PER ANIMAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Striatum</td>
</tr>
<tr>
<td>2 (n=4)</td>
<td>2</td>
</tr>
<tr>
<td>4 (n=4)</td>
<td>3</td>
</tr>
<tr>
<td>6 (n=6)</td>
<td>4</td>
</tr>
<tr>
<td>8 (n=2)</td>
<td>7</td>
</tr>
<tr>
<td>10 (n=2)</td>
<td>5</td>
</tr>
<tr>
<td>12 (n=2)</td>
<td>8</td>
</tr>
<tr>
<td>14 (n=2)</td>
<td>1</td>
</tr>
<tr>
<td>15 (n=3)</td>
<td>1</td>
</tr>
<tr>
<td>30 (n=1)</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of the number and location of retrogradely labelled neurons seen in the neostriatum, globus pallidus and substantia nigra pars compacta 2-30 weeks after implantation of a tibial nerve graft in the striatum of adult rats.

At 4 weeks po more axons were seen in the graft but the number of labelled striatal perikarya changed little (Fig. 3.3a and d). Many heavily labelled neurons were seen in the SNpc (Fig. 3.3c and f). By 8 weeks po the graft contained many labelled axons but the pattern of striatal and nigral perikaryal labelling was similar to that seen 4 weeks earlier (Fig. 4.5). No difference was seen in the distribution of labelled neurons in the nigra or striatum at any of the longer survival times examined (Fig. 3.5 and Chart 3.1). At survival times of 4 weeks po and later perikarya and axons from nigro-striatal neurons were seen to retrogradely label more consistently and in greater numbers than those from the striatum at each time examined (Table 3.1 and Chart 3.1).

In those experiments in which the graft tip impinged upon the globus pallidus there was a huge increase in the number of retrogradely labelled perikarya compared with
experiments in which the graft tip was located in the striatum (Fig. 3.6 and Chart 3.2). For example, in the 8 week survival group, experiment CW 259 with a graft in the globus pallidus had twice as many retrogradely labelled cells as animal 260 (Table 3.2). Labelled neurons were also seen in the ventral pallidum of this animal. In addition, two animals had labelled neurons in the region of the medial forebrain bundle (mfb; CW 259, 8 week survival; CW 256, 10 week survival). In both of these cases the lesion tract clearly transected this region (Chart 3.2).

Those retrogradely labelled neuronal perikarya which were clearly in the striatum showed some consistency in their morphological features (Fig.3.7). This helped to distinguish them from those neurons more frequently labelled in the globus pallidus. Labelled striatal neurons were of two types; most had large, rounded perikarya, approximately 25µm in diameter, and long processes, but others were smaller, spindle shaped neurons with extensive and highly branched dendritic fields. Pallidal neurons were typically smaller than either of these types of striatal neurons being approximately 15µm in diameter with triangular cell bodies. Neurons in the substantia nigra pars compacta were retrogradely labelled but apart from one case, where the graft terminated in the globus pallidus, none were seen in the substantia nigra pars reticulata.

Striatal perikarya were only retrogradely labelled when they were relatively close to the graft tip (mostly within 500µm of the graft/brain interface). Neuronal perikarya in the globus pallidus were able to extend axons into the graft from slightly greater distances, approximately 1mm, and the course of their retrogradely labelled axons could be followed through the brain parenchyma towards the grafts. In a small number of additional experiments where sections were processed to visualize both AChE and HRP approximately 50% of those striatal neurons which retrogradely labelled were also positive for AChE while almost all of the pallidal neurons which regenerated into more caudally placed grafts were also AChE positive.

Where both labelled perikarya and their axons were seen in the same section it was apparent that regenerating axons took direct paths into the graft and did not meander through the brain parenchyma before finally heading towards it. This was the case for both striatal and the pallidal neurons.
<table>
<thead>
<tr>
<th>TIME PO (WEEKS)</th>
<th>ANIMAL NUMBER</th>
<th>POSITION OF GRAFT TIP</th>
<th>NUMBER OF LABELLED PERIKARYA FOUND IN THE:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Corpus Striatum</td>
</tr>
<tr>
<td>2</td>
<td>263</td>
<td>C Str</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>264</td>
<td>C Str</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>296</td>
<td>L Cd Str</td>
<td>5</td>
</tr>
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<td></td>
<td>297</td>
<td>L Cd Str</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>274</td>
<td>M Cd Str</td>
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<tr>
<td></td>
<td>276</td>
<td>C Str</td>
<td>4</td>
</tr>
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<td></td>
<td>293</td>
<td>GP</td>
<td>7</td>
</tr>
<tr>
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<td>294</td>
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</tr>
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</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>279</td>
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<td>289</td>
<td>C Str</td>
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</tr>
<tr>
<td></td>
<td>290</td>
<td>L Cd Str</td>
<td>5</td>
</tr>
<tr>
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<td>291</td>
<td>M Cd Str</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>259</td>
<td>M Cd Str / GP</td>
<td>3</td>
</tr>
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<td>M Cd Str</td>
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<td>M Cd Str</td>
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</tr>
<tr>
<td>30</td>
<td>90</td>
<td>C Str</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.2  Graft tip position and the number and location of retrogradely labelled neuronal perikarya after application of tracers to the distal portion of these grafts. n = 26

Key to abbreviations

- C Str = central striatum,
- M R Str = medial rostral striatum,
- L R Str = lateral rostral striatum,
- M Cd Str = medial caudal striatum,
- L Cd Str = lateral caudal striatum,
- SNpc = Substantia nigra pars compacta,
- SNpr = Substantia nigra pars reticulata,
- mfb = median forebrain bundle,
- GP = Globus pallidus,
- V P = Ventral pallidum.

Diagramatic plan of the dorsal view of the striatum.
Abbreviations used:
G = PN graft,
S = ipsilateral striatum,
CS = contralateral striatum,
CC = cerebral cortex,
Cc = corpus callosum
V = lateral ventricle,
3V = third ventricle
ac = anterior commissure,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata,
GP = globus pallidus.

**Figure 3.0** Key to the small section diagrams accompanying each figure to show the position of the graft in the section being illustrated
3.1a (CW263) Appearance of a PN graft implanted into the striatum 3 days after application of HRP-conjugated retrograde tracers to the distal end. Although the graft appeared healthy, with good contact with the surrounding brain parenchyma, no retrogradely labelled axons or neuronal perikarya were seen in the striatum. Scale bar = 100\(\mu\)m.

3.1b (CW263) SNpc of the same animal; again labelled cell bodies were absent. Scale bar = 100\(\mu\)m.
Figure 3.2
Retrogradely labelled neurons at 2 weeks po.

G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrow = retrogradely labelled axons,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata.

3.2a (CW297) and b (CW296) Small numbers of retrogradely labelled axons were seen within the grafts and the few retrogradely labelled neurons found in the striatum (mean = 2 per animal) were located very close to the graft (within c.100µm of the graft/brain interface). Scale bar = 100µm.

3.2c (CW297) and d (CW297) Fewer labelled neuronal perikarya were found in the SNpc (mean = 1). Scale bar = 100µm.
Figure 3.3
Retrogradely labelled neurons at 4 weeks po.
G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrows = retrogradely labelled axons,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata.

3.3a (CW276) By 4 weeks po many more labelled axons were present within the grafts although the number of labelled striatal neurons remained low (mean = 3). Scale bar = 100μm.

3.3b Bright field detail of labelled neuron in 3a. Scale bar = 50μm.

3.3c (CW276) By 4 weeks po the number of retrogradely labelled neurons seen in the SNpc (mean = 34) was significantly greater than at 2 weeks po. Scale bar = 100μm.

3.3d (CW293) Retrogradely labelled striatal neuron at 4 weeks po. Scale bar = 100μm.

3.3e Detail of area boxed 3d showing the proximity of the labelled neuron to the graft. Scale bar = 100μm.

3.3f (CW294) Retrogradely labelled neurons of the SNpc at 4 weeks po. Scale bar = 100μm.
Figure 3.4
Retrogradely labelled neurons at 8 weeks po.

G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrows = retrogradely labelled axons,

3.4a (CW259). Although the graft appears to be entirely filled with retrogradely labelled axons by 8 weeks po the number of retrogradely labelled striatal perikarya remained low (mean = 7). Retrogradely label neuronal perikarya were found close to the graft, within 300μm of the graft/brain interface, and their regenerating axons appeared to project directly towards the graft rather than meandering through the brain parenchyma before eventually entering the graft tissue. Scale bar = 100μm.
Figure 3.4 - Continuation
Retrogradely labelled neurons at 8 weeks po.
G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrows = retrogradely labelled axons,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata.

3.4b and c (CW260) Bright field photomicrographs of retrogradely labelled striatal neurons showing their proximity to the graft. Note the large number of labelled axons visible within the graft. Scale bars = 100μm.

3.4d and e (CW260) By 8 weeks po the number of retrogradely labelled neurons in the SNpc had increased further (mean = 37). Scale bar = 100μm.
Figure 3.5
Retrogradely labelled neurons at 10 weeks po.
G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrows = retrogradely labelled axons,
SNpc = substantia nigra pars compacta,

3.5a (CW255) Retrogradely labelled striatal neuron positioned close to the graft/brain interface. Note the orientation of its axon, pointing directly towards the graft which contains many other labelled axons. Scale bar = 100μm.

3.5b (CW255) Bright-field detail of area boxed in 5a. Scale bar = 50μm.

3.5c (CW256) Detail of a graft at 10 weeks po demonstrating that even when the graft is heavily labelled it contains many discreet, single axons rather than diffuse tracer spreading down the graft from the site of injection. Scale bar = 100μm.

3.5d (CW255) By 10 weeks po the number of retrogradely labelled neurons seen in the SNpc changed little from that counted two weeks earlier (mean = 44). Scale bar = 100μm.
Figure 3.6
Neurons retrogradely labelled from grafts which terminate in the globus pallidus and ventral pallidum.

G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrows = retrogradely labelled axons,
GP = globus pallidus, VP = ventral pallidum

3.6a (CW259, 8 weeks po) In this animal the graft tip terminated at the medial caudal striatum/GP border resulting in a large number of retrogradely labelled neurons in the GP (n=37) and VP (n=72) but only 1 in the striatum. Scale bar = 1mm.

3.6b (CW259, 8 weeks po) Bright-field detail of the area boxed in 6a; retrogradely labelled pallidal neurons extending discreet axons into the tip of the graft. Scale bar = 100μm.
Figure 3.6 - Continuation

Neurons retrogradely labelled from grafts which terminate in the globus pallidus and ventral pallidum.

G = PN graft, arrow heads = retrogradely labelled neuronal perikarya,
S = striatum, arrows = retrogradely labelled axons,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata.
GP = globus pallidus, VP = ventral pallidum
mfb = median forebrain bundle

3.6c (CW293, 4 weeks po) The appearance of a number of retrogradely labelled pallidal neurons which extended axons into the nearby graft tip. Scale bar = 100μm.
3.6d (CW293, 4 weeks po) Bright-field detail of area boxed in 6c; note the difference in perikaryal size and morphology of these pallidal neurons compared to those retrogradely labelled in the striatum (Figs 2 - 6). Scale bar = 100μm.
3.6e (CW293, 4 weeks po) Another bright-field photomicrograph showing the large number of pallidal neurons which regenerated axons into grafts terminating in the GP. More pallidal neurons were retrogradely labelled from grafts ending in GP than striatal neurons from grafts placed in the striatum at the same survival times. Scale bar = 100μm.
3.6f (CW259, 8 weeks po) Retrogradely labelled neurons were also found in the ventral pallidum and the region occupied by the mfb bundle when the graft lesion tract also disrupted these structures during implantation even though the graft tip was found to terminate in the GP. Scale bar = 100μm.
3.6g (CW293, 4 weeks po) This animal, whose graft terminated in the GP, was the only one to have retrogradely labelled neurons in the SNpr. Scale bar = 100μm.
Figure 3.7

The morphological characteristics of retrogradely labelled neuronal perikarya found in the striatum.

G = graft,  arrow heads = retrogradely labelled axons.
S = striatum,  arrows = retrogradely labelled neuronal perikarya.

3.7a (CW296, 2 weeks po) Large, ovoid cell body (max. diameter c. 30μm) with many primary processes emerging from the poles of the perikaryon. Scale bar = 100μm.

3.7b (CW276, 4 weeks po) Slim, bipolar cell body (max. diameter c. 35μm) whose processes mainly emerge from the poles of the cell. Scale bar = 100μm.

3.7c (CW277, 6 weeks po) Large, ovoid cell body (max. diameter c. 35μm) with many primary processes projecting from all over the perikaryon. Scale bar = 100μm.

3.7d (CW277, 6 weeks po) Large, bipolar cell body (max. diameter c. 25μm) with 2 primary processes projecting from both poles of the perikaryon and another emerging from its side. Scale bar = 100μm.

3.7e (CW259, 8 weeks po) Smaller, round cell body (max. diameter c. 18μm) with many primary processes emerging from all over the perikaryon. Unlike the other cells described so far, the processes of this cell branch to form secondary processes close to its cell body small arrows projecting away from each forming a complex dendritic tree around the perikaryon. Scale bar = 50μm.

3.7f (CW256, 10 weeks po) Large, bipolar cell body (max. diameter c. 40μm) with long, unbranching primary processes emerging from the poles of the perikaryon only. Scale bar = 100μm.

3.7g (CW282, 12 weeks po) Large, bipolar cell body (max. diameter c. 38μm) with many long, primary processes emerging from both the poles and sides of the perikaryon. Scale bar = 100μm.

3.7h (CW263, 15 weeks po) Long, slim, bipolar cell body (max. diameter c. 35μm) with a few, heavily labelled primary processes emerging from both the poles and sides of the perikaryon. Scale bar = 100μm.

3.7i (CW90, 30 weeks po) Smaller cell body (max. diameter c. 20μm) with many long, primary processes emerging from both the poles and sides of the perikaryon. A few of these processes branch close to the cell body but continue to project in a similar direction, running almost parallel to each other unlike those of the cell described in Fig. 1.7e. Scale bar = 100μm.
Chart 3.1 The position of neuronal perikarya retrogradely labelled from an autologous peripheral nerve graft at 2 to 30 weeks post implantation into the corpus striatum of adult rats.

2 weeks survival
(CW297)

Composite of labelled nigral neurons taken from 17 sections.

Sections 1 - 8  9 - 16  17 - 24  25 - 32
Four sets of composite drawings taken from 32 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

4 weeks survival
(CW276)

Composite of labelled nigral neurons taken from 30 sections.

Sections 1 - 8  9 - 16  17 - 24  25 - 38
Four sets of composite drawings taken from 38 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

6 weeks survival
(CW291)

Composite of labelled nigral neurons taken from 18 sections.

Sections 1 - 8  9 - 16  17 - 24  25 - 32
Four sets of composite drawings taken from 27 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

Key:
S = Striatum, G = Graft, C = Cerebral cortex, V = Lateral ventricle, Ce = Corpus callosum, SNpc = Substantia nigra pars compacta, SNpr = Substantia nigra pars reticulata
Chart 3.1 Continuation

8 weeks survival
(CW260)

Composite of labelled nigral neurons taken from 12 sections.

Sections 1 - 5  2 - 10  11 - 18  19 - 28
Four sets of composite drawings taken from 27 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

10 weeks survival
(CW255)

Composite of labelled nigral neurons taken from 20 sections.

Sections 1 - 8  9 - 24  25 - 32  33 - 42
Four sets of composite drawings taken from 38 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

12 weeks survival
(CW51)

Composite of labelled nigral neurons taken from 24 sections.

Sections 1 - 8  9 - 16  17 - 24  25 - 32
Four sets of composite drawings taken from 27 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

Key
S = Striatum, G = Graft, C = Cerebral cortex, V = Lateral ventricle, Ce = Corpus callosum, SNpc = Substantia nigra pars compacta, SNpr = Substantia nigra pars reticulata
Chart 3.1 Continuation

**14 weeks survival**

(CW251)

Composite of labelled nigral neurons taken from 30 sections.

Sections 1 - 8  9 - 24  25 - 32  33 - 40

Four sets of composite drawings taken from 32 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

**15 weeks survival**

(CW261)

Composite of labelled nigral neurons taken from 25 sections.

Sections 1 - 16  17 - 24  25 - 32  35 - 40

Four sets of composite drawings taken from 38 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

**30 weeks survival**

(CW90)

Composite of labelled nigral neurons taken from 19 sections.

Sections 1 - 8  9 - 24  25 - 32  33 - 40

Four sets of composite drawings taken from 27 consecutive striatal sections showing the position of labelled perikarya in relation to the graft.

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Key

Chart 3.2 The distribution of retrogradely labelled neuronal perikarya when the graft impinges upon the globus pallidus during implantation.

**4 weeks survival**

(CW293)

Composite drawings taken from 26 striatal and 13 nigral consecutive sections showing increased numbers of retrogradely labelled perikarya when the graft enters the globus pallidus during implantation. This subject only, out of 26 animals, had labelled perikarya in the substantia nigra pars reticulata.

**8 weeks survival**

(CW259)

Composite drawings taken from 31 striatal and 12 nigral consecutive sections showing increased numbers of retrogradely labelled perikarya when the graft enters the globus pallidus during implantation. In this animal the graft tip terminates in the ventral pallidum and median forebrain regions.

Key

S = Striatum, G = Graft, C = Cerebral cortex, V = Lateral ventrical, Cc = Corpus callosum, SNpc = Substantia nigra pars compacta, SNpr = Substantia nigra pars reticulata
Composite drawings taken from 39 striatal and 15 nigral consecutive sections showing increased numbers of retrogradely labelled perikarya when the graft entered the globus pallidus during implantation. In this subject the lesion tract extends further into the ventral pallidum and median forebrain bundle regions.

Composite drawings taken from 39 striatal and 18 nigral consecutive sections showing increased numbers of retrogradely labelled perikarya when the graft entered the globus pallidus during implantation. In this subject the lesion tract transects both the ventral pallidum and the median forebrain bundle.

Key:
S = Striatum, G = Graft, C = Cerebral cortex, V = Lateral ventricle, Ce = Corpus callosum, SNpc = Substantia nigra pars compacta, SNpr = Substantia nigra pars reticulata
CHAPTER 4

Transmission electron microscopy of regenerating CNS axons in autologous peripheral nerve grafts implanted into the adult CNS

The ultrastructure of living and freeze-killed peripheral nerve grafts was examined using transmission electron microscopy (TEM), concentrating on the structure and cellular relationships of CNS axonal sprouts in the brain and in the implanted peripheral nervous tissue.

(a) Summary of methods used
See Chapter 2 for details:

Autologous tibial nerve grafts were implanted in the right striatum of adult Sprague Dawley rats. These animals were perfused 2, 4 and 8 weeks po and Vibratome sections of the graft and surrounding striatal tissue were processed for TEM. Sections through the graft tip, the proximal graft within the striatum and the distal graft within the cerebral cortex were compared. These experiments were repeated using freeze-killed acellular grafts. The appearance of axonal sprouts within and around the living and acellular graft was recorded and compared.
Figure 4.1 Schematic drawing illustrating the position of an implanted PN graft within the striatum and the zones and features of the graft/brain interface referred to during EM analysis.
(b) Terminology:

The terms used here to describe the distinct anatomical regions seen in this study in the adult rat striatum and in the peripheral nerve graft after graft implantation are taken from Campbell et al., (1992) and are as follows:

'The graft proper' - which contains clusters of Schwann cell columns and myelin debris.

'The junctional zone' (JZ) - which lies between the glia limitans and the graft proper.

'The brain parenchymal border zone' (PBZ) - this describes the area of degenerating axons and reactive axons and glia immediately surrounding the graft.

'Normal brain parenchyma' - this describes regions of brain tissue ipsilateral to the graft with few structural abnormalities, comparable in ultrastructure seen to that seen in the contralateral, control striatum.

'The graft tip' - the proximal region of the graft which usually tapers to end within the brain tissue.

'The proximal graft' - the portion of the graft surrounded by striatal tissue which is distal to the tip region.

'The distal graft' - which passes through the cerebral cortex.

Less graft-derived connective tissue was seen in this study than that of Campbell et al., (1992), owing to the stripping of the epineurium from the graft prior to implantation. Presumably as a consequence of this the perineurium was, in some places, re-arranged into small perineurial compartments around groups of Schwann cell columns.
(c) Results
Axonal sprouts in the corpus striatum

Living graft:

At 2 weeks po large numbers of small diameter axonal profiles were seen around the graft tip in the PBZ (Fig. 4.2), clustered into large bundles close to the glia limitans and around blood vessels. Similar bundles are not usually seen within normal brain parenchyma (Fig. 4.3a and b). The small nonmyelinated profiles found in bundles around the graft were mainly between 0.15μm and 0.4μm in diameter and were interpreted as being regenerating axonal sprouts. By 4 weeks po these bundles had changed little (Fig. 4.4), although some larger, and some myelinated, axon profiles were present. The majority of the axons in the bundles were only in contact with other similar axons. Some axons within these bundles contained aggregates of synaptic vesicles (Fig. 4.5) and, occasionally, exhibited fully formed synaptic contacts. The synaptic vesicles were round, flattened or pleomorphic and approximately 45nm in diameter, although smaller round vesicles, @ 30nm in diameter, were seen in a few profiles. In addition, axonal sprout profiles often contained a single dense-cored vesicle. The largest bundles of axonal sprouts were found around the graft.

Fibres on the periphery of these bundles were mostly impressed into the surface of reactive astrocytes (Fig. 4.4) which extended processes into the bundles of sprouts forming incomplete septa. These were more prominent at 4 than at 2 weeks po. Axonal sprouts were also found in contact with other structures within the PBZ; their ultrastructural characteristics seemed unaffected by whether they contacted oligodendrocytes, microglia, neuronal perikarya, myelinated axons or myelin debris. Some of the larger axons profiles displayed a different ultrastructure from the majority seen in the bundles: they had irregular profiles, many mitochondria and cisternae of smooth endoplasmic reticulum, large numbers of coated vesicles and a variety of synaptic-type vesicles but no presynaptic membrane specialization (Fig. 4.6). These were thought to be growth cones rather than axon terminals. Fewer axonal sprouts were seen in the brain at 8 weeks po than at the earlier survival times examined.

Freeze killed grafts:

Axonal sprout profiles, identical to those seen around living grafts, were found around the tip of freeze killed grafts (Figs 4.7 and 4.8) but in much smaller numbers than observed around living PN graft. The density of sprouts appeared to be little different around the graft tip and around neighbouring proximal graft regions.
**Axons within the grafts**

**Living grafts:**

At 2, 4 and 8 weeks po fewer axons were found in the JZ than in the PBZ. They mostly took the form of small fasicles of axons or isolated axon profiles around the graft tip (Fig. 4.9). Axons and fascicles were found in contact with a variety of other cell types such as Schwann cell processes, macrophages, astrocytes, meningeal fibroblasts (Fig. 4.10) or associated with Schwann cells within a basal lamina (Fig. 4.11). However, some were completely isolated with no other cell contact (Fig. 4.12a and b). No astrocyte cell bodies were found within the JZ, although reactive astrocyte processes were seen to have invaded the JZ at 4 and 8 weeks po and associations between axons and these processes were commonly seen (Fig. 4.13).

At 2, 4 and 8 weeks po the majority of axons found to have entered the graft were present in the graft tip; mostly within loosely fitting and folded, basal lamina-demarcated Schwann cell columns. The number of astrocyte and Schwann cell processes seen here increased over the 8 week period. Most axonal profiles were not in direct contact with the basal lamina but clustered within Schwann cell or astrocyte processes. At 2 weeks po, this was more apparent in the proximal graft (Fig. 4.14) than the graft tip due to the relative paucity of Schwann cell processes in some basal lamina tubes (Fig. 4.15). The size, 1.5 - 0.5μm in diameter, and vesicle content of these axons was similar to those seen in the brain PBZ. Many of the vesicles were pleomorphic or flattened (Fig. 4.14; 4.15; 4.16; 4.17) although axons containing rounded vesicles and single dense-core vesicles were also sometimes seen (Fig. 4.18; 4.19). At 2 weeks po (Fig. 4.20) axons were rarely seen in the distal graft although many, mostly small and nonmyelinated axons with rounded vesicles (30 - 50nm in diameter), were seen in Schwann cell columns in this region at 4 and 8 weeks po (Fig. 4.21a, b and c). A small number of large axonal profiles, resembling the putative growth cone structures seen in the PBZ, were identified in the graft tip and proximal graft at 2 and 4 weeks po (Fig. 4.22 and 4.23) but were not seen at 8 weeks po. By 4 and 8 weeks po, although many of the axon profiles remained in clusters within Schwann cell columns, some were seen to be invaginated into Schwann cells or their processes (Fig. 4.22). Myelinated axons were rarely seen; only 2 in one graft at 8 weeks po, and never at the earlier survival times studied. Many Schwann cell columns still did not contain axonal profiles at 4 (Fig. 4.23) and 8 weeks po. Contrary to the results of Campbell et al., (1992) in the thalamus, astrocyte processes were found in the distal portion of the graft. This may be related to the fact that the stripping of the epineurium from the graft prior to implantation was not carried out in the former study.
**Freeze-killed grafts**

A very small number of axonal profiles were identified close to, or in association with, the glia limitans in the JZ in freeze killed grafts. No axons were found deep in the grafts or within basal lamina tubes, even at the graft tip (Fig. 4.24).

**The structure of the junctional zone between the brain and the graft**

**Living grafts**

The composition of the JZ changed little between 2 and 4 weeks po. This area was particularly complex at the graft tip due to the integration of PNS tissue with the surrounding host brain. By 2 weeks an incomplete glia limitans, one or more layers of astrocyte processes covered by a basal lamina on the graft side, had formed. Although the basal lamina was still incomplete by 4 weeks po the number of gaps which could be found in it was much smaller. By 8 weeks po the glia limitans was much more complex than those seen at earlier survival times and was comprised of many layers of astrocytic processes. The large bundles of axonal sprouts seen in the PBZ at the earlier survival times were only present in regions where the glia limitans was simple. In some sections Schwann cells appeared to have invaded brain tissue, close to the glia limitans. The same cells were found to be surrounded by collagen fibres and basal lamina material. Astrocyte processes were seen to extend into Schwann cell derived basal lamina tubes within the grafts and regions of direct Schwann cell-astrocyte contact with no separating basal lamina were seen. However no Schwann cells were seen to invade deep into the brain tissue. Although the appearance of the graft/brain interface was more regular around the proximal graft region the thickness of the glia limitans was inconsistent. The perineurium of the graft had retracted from the graft tip after implantation but still covered most of the proximal graft region, although incompletely in places. However there was little evidence of invasion of the graft by axons through these discontinuities, axons mainly entering the graft through the exposed graft tip. Putative leptomeningeal cells (Fig. 4.13) were seen in several regions of the JZ, especially in the distal graft at 8 weeks po, occasionally forming compartments around Schwann cell columns or bundles of Schwann cell and astrocyte processes (Fig. 4.22).

**Freeze-killed grafts**

There was little difference between the JZ of freeze killed grafts and that of living grafts at the same time po.
Figure 4.2
Region around the tip of a PN graft, 2 weeks after implantation into the striatum.
(CW212)

JZ = Junctional zone, As = astrocyte processes,  
M = oligodendrocyte myelinated axons, S = synapses,  
Arrow heads = examples of putative axonal sprouts,  
Arrows = glia limitans.

Parenchymal border zone abutting the junctional zone around the tip of a PN graft 2 weeks after implantation into the striatum. Numerous small-diameter non-myelinated axonal profiles are present and contain relatively few organelles apart from a few neurofilaments and microtubules, and a small number of vesicles. Although some of these profiles may be pre-existing non-myelinated axons it is probable that most of these are newly formed axonal sprouts since they are not found in the same number in control striatal tissue (see Fig. 4.3a and b). These axonal profiles are mostly arranged in clusters although some do contact astrocyte processes or oligodendrocyte myelinated axons. Other larger profiles are present and contain vesicles of various kinds and some have fully formed synapses. Scale bar = 500nm.
Figure 4.3
Contralateral (unoperated) striatum in the equivalent position to the tissue shown in Fig. 4.2, 2 weeks po.

(CW212)
Arrow heads = small diameter axonal profiles,
S = Synapses,
M = oligodendrocyte myelinated axons.

4.3a Dense neuropil containing a mixture of neurites. A few small diameter profiles resembling the axonal sprouts shown in Fig.4. are present but large bundles of unmyelinated axons are not seen. Many synapses are present in this tissue. Scale bar = 500um.

4.3b The boundary between a cluster of myelinating axons and the striatal neuropil. Profiles similar to axonal sprouts are even less common amongst the myelinated axons. Scale bar = 500nm.
Figure 4.4
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228)
JZ = Junctional zone, As = astrocyte processes,
M = oligodendrocyte myelinated axons,
Small arrow heads = examples of putative axonal sprouts.
Arrows = glia limitans,
Large arrow heads = larger diameter axonal profiles,

At 4 weeks po presumptive axonal sprouts are still present forming large bundles close to the glia limitans which separates the graft junctional zone from the parenchymal border zone. Some larger diameter axonal profiles and two relatively normal oligodendrocyte-myelinated axons are present in the striatal tissue. At this survival time the compartmentalization of the axonal sprouts by astrocyte processes is more obvious than in Figure 4.1. Scale bar = 500nm.
**Figure 4.5**
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.

(CW228)
JZ = Junctional zone,  As = astrocyte processes,
PBZ = parenchymal border zone,
M = oligodendrocyte myelinated axons,
Arrow heads = examples of putative axonal sprouts,
Large arrow heads = larger diameter axonal profiles,
Arrows = glia limitans,  BV = blood vessel.

A large bundle of axonal sprouts and larger axonal profiles in the parenchymal border zone near the junctional zone. Some larger profiles form synaptic contacts. A cluster of sprouts can be clearly seen in the junctional zone close to a blood vessel and the astrocyte processes near the glia limitans are packed with intermediate filaments. Scale bar = 1μm.

**Figure 4.6**
Parenchymal border zone near the tip of a PN graft, 4 weeks after implantation into the striatum.

(CW228).
As = astrocyte processes,
Arrow heads = examples of putative axonal sprouts,
GC = presumptive growth cone,
Small arrows = coated vesicles.

A presumptive growth cone in a bundle of axonal sprouts in the parenchymal border zone containing a variety of membranous organelles but no synaptic membrane specialization. Coated vesicles are indicated by arrowheads. The growth-cone is in contact with an astrocyte processes as well as axonal sprouts. Scale bar = 500nm.
Figure 4.7
Region around the tip of a freeze-killed PN graft, 2 weeks after implantation into the striatum.
(CW286).
JZ = Junctional zone, As = astrocyte processes,
Arrow heads = presumptive axonal sprouts,
PBZ = parenchymal border zone,
Large arrows = disintegrating myelin around freeze-killed axons.

Degenerating Schwann cell debris found in the junctional zone of a freeze-killed graft. A small number of presumptive axonal sprouts are present in the junctional zone between myelin debris and astrocytic processes which have not yet formed a complete glial limitans of the parenchymal border zone. Scale bar = 500nm.

Figure 4.8
Parenchymal border zone near the tip of a freeze-killed PN graft, 2 weeks after implantation into the striatum.
(CW286).
As = astrocyte processes,
Arrow heads = presumptive axonal sprouts,
LC = unidentified living cell,
DC = dying cell.

Small bundle of non-myelinated presumptive axonal sprouts are present in the parenchymal border zone close to the junctional zone with a freeze-killed graft. An unidentified living cell, perhaps a macrophage, occupies most of this part of the periphery of the graft. A dying cell borders the bundle of sprouts within the PBZ. Scale bar = 1μm.
**Figure 4.9**

Region around the tip of a PN graft, 4 weeks after implantation into the striatum. 
(CW228).

*JZ* = Junctional zone,  *As* = astrocyte processes,  
*PBZ* = parenchymal border zone,  
Small arrow heads = presumptive axonal sprouts,  
Large arrow heads = large diameter, vesicle containing, axonal profiles among sprouts,  
Arrows = glia limitans,  
*BV* = blood vessel.

The junctional zone near the tip of a living graft contains a cluster of axonal sprouts in contact with unidentified cell processes (asterix) adjacent to blood vessel. The glia limitans is very thin where it separates the bundle of axonal sprouts in the parenchymal border zone from the graft tissue. Scale bar = 500nm.
Figure 4.10
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228),
Arrow head = axonal sprout,
LM = leptomeningeal cells.

At least one axon is directly in contact with, and surrounded by, leptomeningeal cells in the junctional zone. Scale bar = 250nm.

Figure 4.11
Region around the tip of a PN graft, 2 weeks after implantation into the striatum.
(CW212).
Arrow heads = axons,
Double arrows = basal lamina,
GC = growth cone,
SC = Schwann cell process.

A Schwann cell column in the JZ near the tip of a living graft containing many axons, most of which are in contact with Schwann cell processes but not the surrounding basal lamina. A large axonal profile resembling a growth cone (almost identical to the profile in Fig. 4.6) is also present within the column and is in contact with the basal lamina. Scale bar = 500nm.
Figure 4.12a
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228).
Arrow head = isolated axonal profile,
Arrows = collagen fibrils,
SCB = presumptive Schwann cell body,
As = astrocyte processes.
P = fibroblasts forming a perineurium-like structure.

An isolated axonal profile in the junctional zone near the tip of a living graft. The only identifiable structures with which it makes contact are collagen fibrils. Scale bar = 1μm.

Figure 4.12b
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228).
Detail of the lone axonal profile indicated in Fig. 4.12a.
Scale bar = 1μm
Figure 4.13
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228).
Arrow heads = axonal profiles,
Double arrows = basal lamina,
As = astrocyte processes,
LM = presumptive leptomeningeal cells.

Astrocyte processes (some of which enwrap axons) surrounded by basal lamina, can be seen within the tip of a living graft. Presumptive leptomeningeal cells are also present. Scale bar = 500nm.
Figure 4.14
Proximal region of a PN graft, 2 weeks after implantation into the striatum.  
(CW212).
Arrow heads = axonal profiles,
Arrows = pleomorphic vesicles,
Double arrows = basal lamina,
SC = Schwann cell processes,

A Schwann cell column in the proximal region of a living graft containing numerous small diameter axonal profiles, mainly separated from the basal lamina by Schwann cell cytoplasm. Several of the axonal profiles contain pleomorphic synaptic vesicles. Scale bar = 500nm.

Figure 4.15
Region around the tip of a PN graft, 2 weeks after implantation into the striatum.  
(CW212).
Arrow heads = axonal profiles,
Double arrows = basal lamina,
SC = Schwann cell processes,
SCB = Schwann cell body.

Basal lamina tubes containing Schwann cell processes and cell bodies and axonal profiles in the graft tip at 2 weeks po. Not all axons are separated from the basal lamina by Schwann cell cytoplasm, perhaps because of the relative paucity of Schwann cell processes in this region. None the less, most axons are either adherent to Schwann cell processes or are clustered together in the central parts of the tubes rather than contacting the basal lamina. Scale bar = 500nm.
Figure 4.16
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228)
Arrow heads = axonal profiles,
Double arrows = basal lamina,
As = astrocyte processes,
SC = Schwann cell processes.

Several axons within a loose basal lamina tube at 4 weeks po. In regions where the basal lamina tube is incomplete (asterisks), the axonal profiles make contact with astrocyte and Schwann cell processes. These profiles contain dense-cored, clear rounded and flattened vesicles. Scale bar = 500nm.

Figure 4.17
Proximal region of a PN graft, 4 weeks after implantation into the striatum.
(CW228).
Arrow heads = axonal profiles,
Double arrows = basal lamina,
SC = Schwann cell processes,
Arrows = cluster of flattened axonal vesicles.

Schwann cell columns in the proximal graft containing several axonal profiles, one with a cluster of flattened vesicles. Note the dense extracellular matrix in this region compared with the tip region of the same graft (Fig. 4.16). Scale bar = 200nm.
Figure 4.18
Region around the tip of a PN graft, 4 weeks after implantation into the striatum.
(CW228).
Arrow heads = axonal profiles,
As = astrocyte processes,
Double arrows = basal lamina,
Arrows = clear, rounded vesicles,
Small arrow heads = dense-cored vesicles,
Small arrows = pleomorphic vesicles.

A mixed column of astrocyte and Schwann cell processes near the tip of a graft containing four axonal profiles. These exhibit different types of synaptic vesicles including clear rounded, dense-cored and pleomorphic varieties. Scale bar = 500nm.

Figure 4.19
Proximal region of a PN graft, 4 weeks after implantation into the striatum.
(CW228).
Arrow heads = axonal profiles,
SC = Schwann cell processes,
Arrows = clear, rounded vesicles,
Small arrows = pleomorphic vesicles.
FB = fibroblast process.

Axonal profiles contacting Schwann cell and astrocyte processes within the proximal region of the graft. These axons contain a mixture of both clear-round and pleomorphic vesicles. Fibroblast processes can been seen within the dense extracellular matrix of the proximal graft. Scale bar = 500nm.
Figure 4.20
Distal region of a PN graft, 2 weeks after implantation into the striatum.
(CW212).
SC = Schwann cell processes,
Double arrows = basal lamina,
Small arrow heads = collagen fibrils.
Schwann cell processes forming Bands of Bungner within basal lamina tubes. No axonal profiles can be seen in this region at this survival time. Note the dense extracellular matrix and negatively stained appearance of the collagen fibrils surrounding the Bands of Bungner. Scale bar = 500nm.

Figure 4.21a
Distal region of a PN graft, 8 weeks after implantation into the striatum.
(CW288).
Large arrow heads = axonal sprouts,
SC = Schwann cell processes,
Double arrows = basal lamina,
Arrows = collagen fibrils,
FB = fibroblast processes,
Small arrow heads = dense-cored vesicles,
Small arrows = pleomorphic vesicles.
A Schwann cell column surrounded by a loose basal lamina at 8 weeks po. A small number of axonal sprouts (containing both pleomorphic and dense cored vesicles) can be seen within the column, most of which are enwrapped by Schwann cell processes although some are exposed to the basal lamina. Scale bar = 500nm.
**Figure 4.21b**
Distal region of a PN graft, 8 weeks after implantation into the striatum. (CW288).

Arrow heads = axonal sprouts,
SC = Schwann cell processes,
Double arrows = basal lamina,
Arrows = clear, rounded vesicles,
Small arrows = pleomorphic vesicles,
LM = leptomeningeal cell processes.

A single axonal sprout containing both round and pleomorphic vesicles surrounded by many Schwann cell processes within a basal lamina tube. Leptomeningeal cell processes can also be seen within the dense extracellular matrix. Scale bar = 500nm.

**Figure 4.21c**
Distal region of a PN graft, 8 weeks after implantation into the striatum. (CW288).

Arrow heads = axonal sprouts,
SC = Schwann cell processes,
Double arrows = basal lamina,
FB = fibroblast processes,
Small arrow heads = round vesicles,
Small arrows = pleomorphic vesicles.

Axonal sprouts in close contact with Schwann cell processes within a loose basal lamina tube. Round and pleomorphic vesicles can be identified within the axon profiles. Collagen fibrils and fibroblast processes can be seen within the extracellular matrix of the graft. Scale bar = 500nm.
A Schwann cell column in the proximal region of a living graft containing at least 3 axonal profiles, one of which (double arrowheads) has some characteristics of a growth cone and another of which (asterisk) is deeply invaginated into a Schwann cell process. The column is enclosed in a basal lamina and is surrounded by what are probably leptomeningeal cells. Scale bar = 500nm.
Figure 4.23
Proximal region of a PN graft, 4 weeks after implantation into the striatum. (CW229).
Arrow heads = axonal profiles,
Double arrows = basal lamina,
As = astrocyte processes,
SC = Schwann cell processes,
Mc = macrophage,
BV = blood vessel.

An anaxonal Schwann cell column surrounded by basal lamina. A Schwann cell process can be seen towards the top of this figure close to a macrophage. Astrocyte processes and a blood vessel can also be identified. Scale bar = 500nm.
Figure 4.24
Region around the tip of a freeze-killed PN graft, 2 weeks after implantation into the striatum.
(CW286).
Arrows = macrophage cytoplasm,
M = dead myelinated axons,
Arrow heads = Schwann cell debris.

Part of the endoneurium near the tip of a freeze-killed graft containing many dead myelinated axons, some surrounded by macrophage cytoplasm or disintegrating Schwann cell debris. Scale bar = 2μm.
CHAPTER 5

The site and level of NGF synthesis and the expression of p75NGFR neurotrophin receptor in the peripheral nerve graft and surrounding brain

Freeze killed grafts did not promote the same regenerative response as those containing living Schwann cells (see Chapter 4). This implies that the living cells in the graft produce growth promoting factors which can enhance axonal regeneration or affect neuronal size. Denervated Schwann cells are known to upregulate their production of NGF, which is one such factor. In this chapter (i) the synthesis of this neurotrophin by these grafts, once implanted into the adult rat corpus striatum, and (ii) the upregulation of receptors for this molecule by host neurons are investigated.

(a) Summary of methods used
See Chapter 2 for details:

Autologous tibial nerve grafts were implanted into the right striatum of adult Sprague Dawley rats. After 7 days fresh samples of graft and brain tissue from these and control unoperated rats were analysed for NGF content using a 2-site ELISA immunoassay.

The presence of p75NGFR low affinity NGF receptor protein in similar grafted striata was assessed at 14 days po using immunohistochemistry.
(b) Results

**Enzyme-linked immunoassay of NGF levels in the graft and surrounding brain tissue**

Tissue samples were taken from animals 7 days after surgery. Animal numbers indicate whether different tissues were taken from the same or different animals.

Table 5.1 shows the estimated level of NGF determined for each of the structures listed using a 2-site ELISA technique and measured in pg/ul. At 7 days po, sections of autologous tibial nerve graft implanted into the neostriatum of adult rats were found to contain much higher levels of NGF than the surrounding striatum. The highest concentration of NGF was found in the graft tissue with little difference between the levels of NGF found in either the portion of the graft within the brain or that which remained outside.

**The expression of low affinity NGF receptor p75^LN^GFR protein by adult striatal neurons in response to graft implantation**

Striatal neurons in the unoperated rat or contralateral to the grafts did not express immunohistochemically detectable levels of p75^LN^GFR. Up-regulation of p75^LN^GFR was not seen in any striatal perikarya in response to the implantation of a tibial nerve graft 14 days earlier (Fig. 5.1). The presence of immunoreactivity in cholinergic medial septal neurons acted as a positive control for this technique (Fig. 5.2b), confirming that the lack of immunopositive neurons in the striatum was not the result of a faulty visualisation procedure. The Schwann cells of the graft were also strongly immunopositive for this protein and this enabled the demonstration of the linear orientation of the Schwann cell columns within the graft (Fig. 5.1 and 5.2 a and c). There was no evidence of Schwann cell migration into the brain. p75^LN^GFR positive neuropil was seen 'lining' the graft/brain interface but no immunopositive neuronal cell bodies were identified in the striatal tissue around the graft. Some p75^LN^GFR immunopositive neurons were commonly seen in the more caudal sections (Fig. 5.3a). These neurons were always found in regions of the ventral pallidum and medial forebrain bundle and were present whether or not the graft had disturbed the area during implantation. In all such cases neurons with similar perikaryal size and morphology were found in the corresponding location in the contralateral hemisphere (Figs. 5.3b and c).
<table>
<thead>
<tr>
<th>TISSUE SPECIMEN</th>
<th>ANIMAL No.</th>
<th>WEIGHT (g)</th>
<th>NGF LEVEL (pg/mg)</th>
<th>MEAN NGF LEVEL (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXTERNAL GRAFT</td>
<td>CW106</td>
<td>0.0076</td>
<td>178</td>
<td>239</td>
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<tr>
<td></td>
<td>CW107</td>
<td>0.0072</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CW108</td>
<td>0.0058</td>
<td>266</td>
<td></td>
</tr>
<tr>
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<td>CW109</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CW210</td>
<td>0.004</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CW211</td>
<td>0.0029</td>
<td>296</td>
<td></td>
</tr>
<tr>
<td>INTERNAL GRAFT</td>
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<td>CW107</td>
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<td>CW211</td>
<td>0.0026</td>
<td>269</td>
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<td>23</td>
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<tr>
<td></td>
<td>CW107</td>
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<td></td>
<td>CW109</td>
<td>0.0274</td>
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<td></td>
<td>CW210</td>
<td>0.0318</td>
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<td></td>
<td>CW211</td>
<td>0.0332</td>
<td>14</td>
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<td>CONTRALATERAL STRIATUM</td>
<td>CW106</td>
<td>0.062</td>
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<td>12</td>
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<td>CW107</td>
<td>0.034</td>
<td>10</td>
<td></td>
</tr>
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<td></td>
<td>CW109</td>
<td>0.0302</td>
<td>18</td>
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<tr>
<td></td>
<td>CW210</td>
<td>0.031</td>
<td>9</td>
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<td></td>
<td>CW211</td>
<td>0.032</td>
<td>12</td>
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<td>ISOLATED SEGMENT OF PERIPHERAL NERVE LEFT IN SITU</td>
<td>CW111</td>
<td>0.0082</td>
<td>129</td>
<td>133</td>
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<td></td>
<td>CW112</td>
<td>0.0110.0</td>
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<td></td>
<td>CW113</td>
<td>0.081</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>TIBIAL NERVE CUT AND LIGATED; DISTAL SEGMENT</td>
<td>CW111</td>
<td>0.0164</td>
<td>143</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>CW112</td>
<td>0.0104</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CW113</td>
<td>0.0137</td>
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</tr>
<tr>
<td></td>
<td>CW114</td>
<td>0.0139</td>
<td>167</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 The level of NGF contained in graft and striatal tissue samples 7 days po as determined by enzyme linked immunoassay
Figure 5.1
(CW85)
Immunohistochemistry for p75NGFR low affinity NGF receptor.
G = graft, S = striatum, MS = medial septum,
Arrows = p75NGFR immunopositive Schwann cells,

At 14 days po no p75NGFR immunopositive neuronal perikarya were seen in the striatum surrounding the graft although high back ground staining was present. However, Schwann cells within the graft itself were strongly immunopositive for p75NGFR making their linear arrangement within the graft apparent. The Schwann cells at the proximal tip of this graft show very good contact with the striatal tissue but there was no evidence of Schwann cell migration out of the graft into the brain. Scale bar = 100μm.
Figure 5.2
Immunohistochemistry for p75<sup>NGFR</sup> low affinity NGF receptor.

G = graft, S = striatum,
Arrows = p75<sup>NGFR</sup> immunopositive Schwann cells,
Arrow heads = p75<sup>NGFR</sup> immunopositive neuronal perikarya,
MS = medial septum.

5.2a (CW85) Another example of immunonegative striatal neurons around strongly immunopositive PN graft at 14 days po. Scale bar = 100μm.

5.2b (CW86) Strongly immunopositive neuronal perikarya in the medial septum of the same animal acting as a positive control for the effectiveness of the antibody. Scale bar = 10μm.

5.2c (CW85) Detail of boxed area in Fig. 2a, discrete immunopositive Schwann cell columns can be seen within the graft, particularly at the graft/brain interface. Again, although background staining is high around the graft, no immunopositive neuronal perikarya are visible. Scale bar = 10μm.
Figure 5.3
Immunohistochemistry for p75\textsuperscript{NGFR} low affinity NGF receptor.
G = graft, S = striatum,
I = ipsilateral brain, C = contralateral brain,
Arrows = p75\textsuperscript{NGFR} immunopositive Schwann cells,
Arrow heads = p75\textsuperscript{NGFR} immunopositive neuronal perikarya.

5.3a (CW99) An example of a strongly p75\textsuperscript{NGFR} immunopositive neuron, with a large cell body and branching primary processes, in the caudal medial striatum a small distance from the graft. Due to its caudal medial location within the striatum this cell was thought to be one of the more rostral p75\textsuperscript{NGFR} immunopositive neurons of the ventral pallidum/basal forebrain areas. Scale bar = 100\textmu m.

5.3b and c (CW99) p75\textsuperscript{NGFR} immunopositive neurons of the ventral pallidum/basal forebrain areas which could be found in corresponding positions close to the caudal medial striatum with similar intensity of staining both ipsilateral (I) and contralateral (CS) to the graft. Scale bar = 100\textmu m.
CHAPTER 6

Hypertrophy of cholinergic striatal neurons ipsilateral to a peripheral nerve graft and the identification of AChE positive axons within the graft.

The large, aspiny, cholinergic interneurons of the adult rat corpus striatum have been shown to display perikaryal hypertrophy in response to infusions of exogenous NGF (Gage et al., 1985). This effect was examined in these cells after the implantation of either a living or freeze-killed peripheral nerve graft in the adult rat striatum. The presence of cholinergic axons within grafts of different survival times was also demonstrated.

(a) Summary of methods used:
See Chapter 2 details:

Live and freeze-killed autologous tibial nerve grafts were implanted into the right striatum of adult Sprague Dawley rats. At 1, 2, 3 and 4 weeks post sections were taken and histochemically processed for visualisation of AChE and at 2 and 4 weeks for ChAT immunohistochemistry. The perikaryal area of striatal AChE positive neurons located within 400μm of the graft/brain interface was measured and compared to that of a random sample of similar neurons from the contralateral striatum. Similar measurements were taken of ChAT immunopositive neurons in similar sections around the grafts. Both of these experiments were also repeated using freeze-killed PN grafts. The area of presumptive cholinergic neurons in Thionin stained striatal sections was also measured and compared to the previous results. The appearance of AChE positive axons in the grafts at different survival times was also described.
(b) Results

The effect of a peripheral nerve graft on the size of AChE positive striatal perikarya

No significant difference was found between the area of striatal AChE positive perikarya ipsilateral or contralateral to a living peripheral nerve graft at 1, 2, 3 or 4 weeks po (Table 6.1). In addition, no significant difference was found using control acellular, freeze-killed, grafts at 1, 2, or 4 weeks po (Table 6.2). From this we can conclude that AChE positive striatal perikarya show no hypertrophy in response to the implantation of a peripheral nerve graft regardless of its metabolic activity. The hypertrophy of AChE positive neurons ipsilateral to a freeze-killed graft at 3 weeks po may have been an artefact of the small sample number of neurons in that group (Table 6.2) as there is no consistency between any of perikaryal areas gathered either within or between different survival times in the freeze-killed samples.

Further analysis of the frequency of perikaryal sizes recorded in each sample resulted in similar unimodal curves for each histogram, covering the same range of cell sizes (Histograms 6.1 and 6.2). This was interpreted as further evidence that (a) a similar population of cells were sampled in each animal, and (b) no size fluctuations occurred in subsets of smaller neurons hidden by the overall results.

The effect of a peripheral nerve graft on the size of ChAT positive striatal perikarya

ChAT positive cells were examined at 2 and 4 weeks po only. ChAT positive cells were 19% larger in the grafted than the contralateral striatum at 2 weeks po and 22% larger at 4 weeks po. (Table 6.3). No single subset of ChAT immunopositive neurons were found to hypertrophy and the increases in perikaryal area were measured throughout the population of cholinergic neurons ipsilateral to the graft as shown in Histogram 6.3.

A repetition of this study using adjacent sections processed alternately for either AChE or ChAT, at 2 weeks po (Fig. 6.1), confirmed that ChAT positive neurons were larger on the operated side but the AChE positive neurons were not. It also highlighted some histological artefacts particular to each technique (Table 6.4). Surprisingly, the mean area of (hypertrophied) ChAT positive cells ipsilateral to the graft was smaller than that of AChE stained cells on either the grafted or control side. The coronal brain sections stained for AChE were also smaller than those processed for ChAT and had a greater number of positive perikarya per mm².
<table>
<thead>
<tr>
<th>SURVIVAL TIME (WEEKS)</th>
<th>ANIMAL ID NUMBER</th>
<th>MEAN CELL AREA PER ANIMAL GRAFTED STRIATUM (µm²)</th>
<th>MEAN CELL AREA PER ANIMAL CONTROL SIDE (µm²)</th>
<th>DIFFERENCE IN CELL SIZE PER ANIMAL</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>CW 61</td>
<td>245</td>
<td>228</td>
<td>+8%</td>
</tr>
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<td></td>
<td>CW 62</td>
<td>219</td>
<td>221</td>
<td>- 1%</td>
</tr>
<tr>
<td></td>
<td>CW 75</td>
<td>317</td>
<td>345</td>
<td>- 8%</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td></td>
<td></td>
<td>- 0.3%</td>
</tr>
<tr>
<td>2</td>
<td>CW 14</td>
<td>272</td>
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<td>+ 18%</td>
</tr>
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<td>CW 25</td>
<td>215</td>
<td>231</td>
<td>- 7%</td>
</tr>
<tr>
<td></td>
<td>CW 46</td>
<td>299</td>
<td>282</td>
<td>+ 6%</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td></td>
<td></td>
<td>+ 5%</td>
</tr>
<tr>
<td>3</td>
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<tr>
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<td>CW 70</td>
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<td>344</td>
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<tr>
<td></td>
<td>MEAN</td>
<td></td>
<td></td>
<td>- 2%</td>
</tr>
<tr>
<td>4</td>
<td>CW 2</td>
<td>301</td>
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</tr>
<tr>
<td></td>
<td>CW 15</td>
<td>249</td>
<td>289</td>
<td>- 14%</td>
</tr>
<tr>
<td></td>
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</tr>
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<td>CW 233</td>
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<td>185</td>
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<tr>
<td></td>
<td>MEAN</td>
<td></td>
<td></td>
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Table 6.1 The difference in mean cholinergic striatal perikaryal area ipsi and contralateral to a living peripheral nerve graft 1 - 4 weeks after implantation.

<table>
<thead>
<tr>
<th>SURVIVAL TIME (WEEKS)</th>
<th>ANIMAL ID NUMBER</th>
<th>MEAN CELL AREA PER ANIMAL GRAFTED STRIATUM (µm²)</th>
<th>MEAN CELL AREA PER ANIMAL CONTROL SIDE (µm²)</th>
<th>DIFFERENCE IN CELL SIZE PER ANIMAL</th>
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<tbody>
<tr>
<td>1</td>
<td>CW 63</td>
<td>307</td>
<td>314</td>
<td>- 2%</td>
</tr>
<tr>
<td></td>
<td>CW 64</td>
<td>274</td>
<td>375</td>
<td>- 27%</td>
</tr>
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<td></td>
<td>CW 74</td>
<td>358</td>
<td>339</td>
<td>+ 6%</td>
</tr>
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<td></td>
<td>MEAN</td>
<td></td>
<td></td>
<td>- 8%</td>
</tr>
<tr>
<td>2</td>
<td>CW 26</td>
<td>255</td>
<td>258</td>
<td>- 1%</td>
</tr>
<tr>
<td></td>
<td>CW 53</td>
<td>267</td>
<td>312</td>
<td>- 14%</td>
</tr>
<tr>
<td></td>
<td>CW 54</td>
<td>342</td>
<td>327</td>
<td>+ 5%</td>
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<td>MEAN</td>
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<td>- 4%</td>
</tr>
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<td>285</td>
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<td></td>
<td>CW 69</td>
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<td>4</td>
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<td>298</td>
<td>266</td>
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<td></td>
<td>CW 21</td>
<td>251</td>
<td>319</td>
<td>- 21%</td>
</tr>
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<td>MEAN</td>
<td></td>
<td></td>
<td>- 5%</td>
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Table 6.2 The difference in mean cholinergic striatal perikaryal area ipsi and contralateral to a freeze-killed peripheral nerve graft 1 - 4 weeks after implantation.
<table>
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<tr>
<th>2 WEEKS PO</th>
<th>4 WEEKS PO</th>
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<tr>
<td><strong>GRAFTED STRIATUM</strong></td>
<td><strong>CONTROL SIDE</strong></td>
</tr>
<tr>
<td>(μm²)</td>
<td>(μm²)</td>
</tr>
<tr>
<td><strong>MEAN CELL AREA (±S.E.)</strong></td>
<td>333 ± 6.6</td>
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<td><strong>NO. OF CELLS COUNTED</strong></td>
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</tr>
<tr>
<td><strong>RANGE (μm²)</strong></td>
<td>201 - 582</td>
</tr>
</tbody>
</table>

**Table 6.3** Comparison between the areas of ChAT positive striatal perikarya ipsilateral and contralateral to a living autologous tibial nerve 2 and 4 weeks post implantation

**Table 6.4.** The effect of different histological techniques on the appearance of control striatal sections.

In order to further clarify the effects of peripheral nerve grafts on striatal aspiny interneurons another set of sections from these animals was prepared using a Thionin stain and the size of presumptive large aspiny cholinergic perikarya were measured.

The identification of presumptive cholinergic neurons within a Thionin stained population was largely based on previous experience of the morphology of ChAT and

<table>
<thead>
<tr>
<th>ANIMAL ID NUMBER</th>
<th>MEAN STRIATAL AREA (mm²)</th>
<th>NO. OF CELLS PER SECTION</th>
<th>NO. OF CELLS PER SECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW190</td>
<td>5.85</td>
<td>207</td>
<td>35</td>
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<td>CW191</td>
<td>4.7</td>
<td>165</td>
<td>35</td>
</tr>
<tr>
<td>CW192</td>
<td>5.23</td>
<td>159</td>
<td>30</td>
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<td><strong>OVER-ALL MEAN</strong></td>
<td><strong>5.26</strong></td>
<td><strong>177</strong></td>
<td><strong>33</strong></td>
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<table>
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<tr>
<th>ANIMAL ID NUMBER</th>
<th>MEAN STRIATAL AREA (mm²)</th>
<th>NO. OF CELLS PER SECTION</th>
<th>NO. OF CELLS PER SECTION</th>
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<td>CW190</td>
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<td>21</td>
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<td>7.72</td>
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<td>19</td>
</tr>
<tr>
<td><strong>OVER-ALL MEAN</strong></td>
<td><strong>7.85</strong></td>
<td><strong>150</strong></td>
<td><strong>18</strong></td>
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</tbody>
</table>
AChE positive cells. The main criterion used was that of perikaryal size; cholinergic interneurons are the only striatal cell population classified as 'large', (Bolam et al., 1984) and comprise only 2% of the total striatal cell number (Phelps et al., 1985). All cells >143\(\mu\text{m}^2\), the smallest perikaryal size collected from either the AChE or ChAT positive control side data, were measured. By examining the slide initially at low power (Fig. 6.2a) the position of the sparsely distributed large cell profiles could be plotted in relation to the graft using the camera lucida and immediately distinguished from the other, more numerous, cell types of the striatum.

Closer examination of these cell profiles at higher magnification (Fig. 6.2b and c) allowed greater refinement of the sample taken. Cholinergic interneurons are typically large with bipolar perikarya and are very different from the medium spiny GABAergic projection neurons, which represent over 90% of the striatal population which have smaller, round cell bodies. Additionally, only perikarya with two or more obvious cell processes were included in the sample group. This increased the probability that the cross-sectional areas measured were taken from the central region of the cell body, not from its perimeter, giving a more uniform and representative measurement for each cell and a small, but highly selective, sample group. Cell profiles which did not fit the 'cholinergic' criteria, or did not have a complete outline were also excluded from the count.

<table>
<thead>
<tr>
<th>CELL MARKER</th>
<th>ANIMAL ID NUMBER</th>
<th>MEAN CELL AREA PER ANIMAL</th>
<th>DIFFERENCE IN CELL SIZE PER ANIMAL</th>
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<tr>
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<td>CW 190</td>
<td>337 (um(^2))</td>
<td>390 (um(^2))</td>
</tr>
<tr>
<td></td>
<td>CW 191</td>
<td>344 (um(^2))</td>
<td>336 (um(^2))</td>
</tr>
<tr>
<td></td>
<td>CW 192</td>
<td>400 (um(^2))</td>
<td>377 (um(^2))</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td>374 (um(^2))</td>
<td>377 (um(^2))</td>
</tr>
<tr>
<td>ChAT positive perikarya</td>
<td>CW 190</td>
<td>312 (um(^2))</td>
<td>273 (um(^2))</td>
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<tr>
<td></td>
<td>CW 191</td>
<td>337 (um(^2))</td>
<td>263 (um(^2))</td>
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<tr>
<td></td>
<td>CW 192</td>
<td>348 (um(^2))</td>
<td>300 (um(^2))</td>
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<tr>
<td></td>
<td>MEAN</td>
<td>333 (um(^2))</td>
<td>287 (um(^2))</td>
</tr>
<tr>
<td>Presumptive cholinergic perikarya (Thionin stained)</td>
<td>CW 190</td>
<td>307 (um(^2))</td>
<td>262 (um(^2))</td>
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<tr>
<td></td>
<td>CW 191</td>
<td>268 (um(^2))</td>
<td>303 (um(^2))</td>
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<tr>
<td></td>
<td>CW 192</td>
<td>322 (um(^2))</td>
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<td></td>
<td>MEAN</td>
<td>299 (um(^2))</td>
<td>281 (um(^2))</td>
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</table>

Table 6.5. Comparison of perikaryal area in adjacent serial striatal sections processed for AChE histochemistry, ChAT immunohistochemistry or thionin histochemistry at 2 weeks po.

The mean area of the Thionin stained perikarya sampled this way was smaller than in the AChE positive group but closely matched the ChAT immunostained group (Table 6.5). Neuronal hypertrophy was seen throughout the Thionin stained sample.
included in the count and was similar to the increase in size determined for the ChAT positive cells. Histogram 6.4 demonstrates that the increase in perikaryal area was seen throughout the population of cells sampled ipsilateral to the graft producing a similar unimodal curve as those on the contralateral side, only skewed to the right to indicate an increase in area.

The presence of AChE positive axons in the graft

A second group of grafted animals (n=14) were stained for AChE but without the DFP pretreatment. It was possible to see AChE positive striatal neuropil in these animals, and AChE positive axons in the grafts. Fig. 6.3a, b and c shows the presence of AChE positive axons within the graft at 2, 4 and 6 weeks po. In the longer survival time animals these fibres could be seen to extend up to 2mm into the graft. As the survival time increased the individual fibres became more distinct - compare fibres in Fig. 6.3a to those indicated in Fig. 6.3c. The majority of the stained axons entered the graft at the tip region, presumably because there is less of a mechanical barrier since no perineurium is present. Tyrosine hydroxylase immuno-positive axons were also seen in similar grafts at 2 weeks po (Fig. 6.3d) even in animals with bilateral superior cervical ganglionectionies. These axons are most probably of nigral origin and their presence demonstrates that not all of the axons seen in these grafts are from cholinergic neurons.
Figure 6.1
The appearance of AChE and ChAT positive striatal neurons both ipsilateral and contralateral to a PN graft at 2 weeks po.
G = graft, S = striatum, CS = contralateral striatum,

6.1a (CW190) AChE positive cholinergic striatal neurons ipsilateral to a PN graft at 2 weeks po. Scale bar = 100µm.
6.1b (CW190) AChE positive cholinergic striatal neurons contralateral to those in Fig. 6.1a. Scale bar = 100µm.
6.1c (CW190) ChAT immunopositive cholinergic striatal neurons ipsilateral to a PN graft at 2 weeks po. Scale bar = 100µm.
6.1d (CW190) ChAT immunopositive cholinergic striatal neurons contralateral to those in Fig. 6.1c. Scale bar = 100µm.
Figure 6.2
The appearance of thionin-stained striatal neurons both ipsilateral and contralateral to a PN graft at 2 weeks po.
G = graft, S = striatum,
CS = contralateral striatum,
Arrows = presumptive cholinergic neuronal perikarya,
Arrow heads = other neuronal perikarya.

6.2a (CW207) Low power photomicrograph of a thionin-stained striatal PN graft at 2 weeks po. Note that the surrounding striatal parenchyma consists of many small neuronal perikarya and very few, deeper stained neuronal perikarya which are thought to be cholinergic neurons. Scale bar = 100μm.

6.2b (CW207) High power photomicrograph of the same tissue showing the difference in perikaryal size and shape between presumptive cholinergic neurons and other neurons in the striatum. Scale bar = 100μm.

6.2c (CW207) High power photomicrograph of the striatal tissue contralateral to that in Fig. 6.2b. showing the difference in perikaryal size between presumptive cholinergic neurons on either side of the brain. Scale bar = 100μm.
Figure 6.3
Presumptive regenerating axons can be identified within the grafts using histochemistry for AChE and immunohistochemistry for tyrosine hydroxylase (TH).

G = graft, S = striatum,
Arrows = regenerating axons within the graft.

6.3a (CW171) A small number of diffuse presumptive AChE positive axons was identified within the graft at 2 weeks po. At this survival time axons were only seen close to the graft tip, extending for short distances into the graft. Scale bar = 100µm.

6.3b (CW241) By 4 weeks po the number of presumptive AChE positive axons which could be identified within the graft had increased and they were seen to extend for much longer distances along it following the same orientation as the Schwann cell columns identified in chapter 5. Scale bar = 100µm.

6.3c (CW248) At 6 weeks po many presumptive AChE positive axons were clearly visible along the entire graft. These axons appeared to be much darker, with more distinct outlines, than those seen at earlier survival times. Scale bar = 100µm.

6.3d (CW156) A very small number of TH positive axons were also identified within the grafts at 2 weeks po. As the animal had undergone an ipsilateral superior cervical ganglionectomy 3 days before perfusion it was presumed that these axons originated from dopaminergic neurons of the SNpc rather than from invading sympathetic fibres from the periphery. Scale bar = 100µm.
Figure 6.3
Presumptive regenerating axons can be identified within the grafts using histochemistry for AChE and immunohistochemistry for tyrosine hydroxylase (TH).
G = graft, S = striatum,
Arrows = regenerating axons within the graft.

6.3a (CW171) A small number of diffuse presumptive AChE positive axons was identified within the graft at 2 weeks po. At this survival time axons were only seen close to the graft tip, extending for short distances into the graft. Scale bar = 100μm.

6.3b (CW241) By 4 weeks po the number of presumptive AChE positive axons which could be identified within the graft had increased and they were seen to extend for much longer distances along it following the same orientation as the Schwann cell columns identified in chapter 5. Scale bar = 100μm.

6.3c (CW248) At 6 weeks po many presumptive AChE positive axons were clearly visible along the entire graft. These axons appeared to be much darker, with more distinct outlines, than those seen at earlier survival times. Scale bar = 100μm.

6.3d (CW156) A very small number of TH positive axons were also identified within the grafts at 2 weeks po. As the animal had undergone an ipsilateral superior cervical ganglionectomy 3 days before perfusion it was presumed that these axons originated from dopaminergic neurons of the SNpc rather than from invading sympathetic fibres from the periphery. Scale bar = 100μm.
Histogram 6.1 Comparison of the area of AChE positive perikarya ipsilateral and contralateral to a living autologous tibial nerve graft over time.
Histogram 6.2  Comparison of the area of AChE positive perikarya ipsilateral and contralateral to a freeze-killed autologous tibial nerve graft over time.
Histogram 6.3  Comparison of the area of AChE and Chat positive perikarya ipsilateral and contralateral to a living tibial nerve graft at 2 and 4 weeks post-operation.
Histogram 6.4. Comparison of the area of thionin stained presumptive cholinergic perikarya ipsilateral and contralateral to a living autologous tibial nerve graft 2 weeks po
CHAPTER 7

Response of a non NGF-sensitive population of striatal neurons and the projection of their axons into grafts

Denervated Schwann cells are able to synthesize other growth promoting factors besides NGF which include the neurotrophins BDNF and NT-3 and the cytokine CNTF. This chapter investigates the response to graft implantation of a population of striatal interneurons which are insensitive to NGF, the NADPH-diaphorase containing medium aspiny interneurons, and the extension of their axons into grafts at 2, 4, 6 and 8 weeks po. This population of striatal interneurons is quite distinct from the population of large, aspiny cholinergic interneurons examined in the preceding chapter.

(a) Summary of methods used
See Chapter 2 for details:

Autologous tibial nerve grafts were implanted into the right striatum of adult Sprague Dawley rats. At 2, 4, 6 and 8 weeks po striatal perikarya were histochemically stained for NADPH-diaphorase. The area of NADPH-diaphorase positive perikarya ipsilateral and contralateral to the tibial nerve graft were measured using a Seescan Image Analysis System (Seescan, Cambridge). Similar measurements were made using an acellular freeze-killed graft as a control. The appearance of NADPH-diaphorase positive axons within the graft was described for each survival time examined.
(b) Results

NADPH-diaphorase perikaryal enlargement

NADPH-diaphorase positive striatal perikarya within 400μm of the graft were seen to undergo hypertrophy in response to the implantation of an autologous tibial nerve graft. This hypertrophy was obvious up to 6 weeks po, and declined thereafter (Table 7.1b).

<table>
<thead>
<tr>
<th>SURVIVAL TIME (WEEKS)</th>
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<th>DIFFERENCE IN CELL SIZE (PER ANIMAL)</th>
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<td>CONTROL SIDE (μm²)</td>
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<td>MEAN</td>
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<td>2 (FREEZE-KILLED GRAFT)</td>
<td>CW 300</td>
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<tr>
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<td>117</td>
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Table 7.1. Perikaryal area of NADPH-diaphorase positive striatal neurons ipsilateral and contralateral to living or freeze-killed peripheral nerve grafts 2 - 8 weeks after implantation.

The greatest increase in cell area was seen at 2 weeks po. Visual inspection of the sections showed that the NADPH-diaphorase positive cells on the operated side were noticeably enlarged (Fig. 7.1a and b). Cells ipsilateral to the graft enlarged by approximately 28% when compared to those on the contralateral side. The level of hypertrophy subsequently decreased over the following weeks; by 8 weeks po these cells were enlarged in one of the three animals examined (Fig. 7.1c and d). The freeze-killed control grafts were examined at 2 weeks po, the time at which the greatest hypertrophy was seen amongst the living graft groups. Only a small increase of 2% was seen in the mean perikaryal area of NADPH-diaphorase positive neurons ipsilateral to a freeze-killed peripheral nerve graft compared to the area of the contralateral neurons.
(Table 7.1, Histogram 7.2). This supports the hypothesis that the hypertrophy seen in neurons ipsilateral to a living graft is caused by unidentified factors, released by the living cells in the graft rather than the mechanical damage done during its implantation.

The histograms in Histogram 7.1 show that the increase in cell size was seen across the whole range of cells sampled; there was no hypertrophy of any one particular subset within the NADPH-diaphorase positive population sampled; a smooth unimodal curve was produced for both the grafted and contralateral cell data. The mean cell area collected for the contralateral samples remained almost constant between the 4 time intervals studied.

**NADPH-diaphorase positive axons within the graft**

NADPH-diaphorase positive axons were seen in the grafts at all the time intervals examined. These axons were identified as fine lines of reaction product within the graft and were distinct, but few in number, at 2 weeks po (Fig. 7.2). These axons had a beaded morphology which was different from the slightly smoother processes seen to emanate from labelled perikarya within the striatum. The number of labelled axons seen within the grafts increased with time (Fig. 7.2 - 7.5) but was never as great as the number of HRP positive axons seen in the retrogradely labelled grafts at the same survival times (see chapter 3). Many NADPH-diaphorase positive sprouts were seen in the striatal tissue immediately surrounding the grafts. Examples of an axons crossing from the host brain into the graft were seen (Figs. 7.3b, 7.4a, 7.4c). Single axons were commonly seen, extending up to 200μm within the graft (Fig. 7.3b, c and e). Axons usually followed the dorsal ventral orientation of the Schwann cell columns within the graft and were often clustered together to form fascicles (Figs. 7.3a and d). Regenerating axons were more commonly seen running along the lateral edges of the proximal graft segment rather than deep within its central core. No examples of axonal branching were seen within the graft. As the striatal NADPH-diaphorase positive neurons examined in this study are interneurons with no efferent projection beyond the striatum, the graft appears to have promoted abnormally long axonal extension for this cell type. No examples of regenerating axons were seen within the freeze-killed grafts.
Figure 7.1
G = graft, S = striatum, CS = contralateral striatum
Arrow heads = NADPH-diaphorase positive neuronal perikarya

7.1a (CW137) NADPH-diaphorase positive striatal neurons surrounding a PN graft 2 weeks after implantation. Scale bar = 100 μm.
7.1b (CW137) NADPH-diaphorase positive striatal neurons contralateral to those shown in Fig. 7.1a. Scale bar = 100 μm.
7.1c (CW103) NADPH-diaphorase positive striatal neurons surrounding a PN graft 8 weeks after implantation. Scale bar = 100 μm.
7.1d (CW103) NADPH-diaphorase positive striatal neurons contralateral to those shown in Fig. 7.1c. Scale bar = 100 μm.
Figure 7.2
The appearance presumptive regenerating axons within the grafts.
G = graft,  S = striatum,
Arrows = presumptive regenerating NADPH-diaphorase positive axons.

7.2a (CW188) Short NADPH-diaphorase positive axons were identified within the grafts at 2 weeks po. These axons were short and usually found in the proximal graft, close to the edge of the graft. Scale bar = 100μm.

7.2b (CW188) The small number of regenerating NADPH-diaphorase positive axons seen in the graft at 2 weeks po also appeared to be closely associated with one another. Scale bar = 50μm.
Figure 7.3
More regenerating NADPH-diaphorase positive axons were seen within the graft at 4 weeks po.
$G =$ graft, $S =$ striatum,
Arrows = regenerating NADPH-diaphorase positive axons.

7.3a (CW131) By 4 weeks po tight fascicles of regenerating NADPH-diaphorase positive axons could be seen within the proximal tips of the graft. Scale bar = 100μm.
7.3b (CW131) Single regenerating NADPH-diaphorase positive axons were also seen within the graft. Scale bar = 100μm.
7.3c (CW131) Detail of the area boxed in Fig. 3b, note the darkly stained rounded end of the regenerating axon small arrow which could probably be interpreted as a growth cone-like structure. Scale bar = 100μm.
7.3d (CW131) Tight bundles of regenerating axons in the proximal tip of the graft. The majority of these axons are closely associated with one another although a small number of single axons can be seen breaking away from the bundle. Scale bar = 100μm.
7.3e (CW131) Single axon regenerating approximately 200μm along the side of the graft. Scale bar = 100μm.
7.3f (CW200) Non-fasciculated regenerating axons were frequently seen running along the sides of the grafts unlike the bundles of axons shown in Figs. 5.3a and 5.3d which were found within the central regions of the graft. Scale bar = 100μm.
Figure 7.4
Regenerating NADPH-diaphorase positive axons continued to invade the graft at 6 weeks po.
G = graft, S = striatum,
Arrow heads = NADPH-diaphorase positive neuronal perikarya,
Arrows = regenerating NADPH-diaphorase positive axons.

7.4a (CW199) Bundles of regenerating NADPH-diaphorase positive axons within the graft at 6 weeks po. Scale bar = 100μm.
7.4b (CW199) Detail of area boxed in Fig.4a showing two NADPH-diaphorase positive neuronal perikarya extending axons into the graft, the point of entry into the graft by one of the axons is indicated (small arrow). Scale bar = 100μm.
7.4c (CW199) Another example of a fascicle-like group of regenerating axons within the proximal tip of the graft and an NADPH-diaphorase positive neuron extending a regenerating axon towards the graft. Scale bar = 100μm.
7.4d (CW205) Many single axons running parallel to each other in the proximal tip of the graft following the same orientation as the graft Schwann cells demonstrated in chapter 3. Scale bar = 100μm.
Figure 7.5
By 8 weeks po the pattern of NADPH-diaphorase positive neuronal regeneration into the
grafts was similar to that seen at 6 weeks po.
G = graft, S = striatum,
Arrow heads = NADPH-diaphorase positive neuronal perikarya,
Arrows = regenerating NADPH-diaphorase positive axons.

7.5a (CW102) Appearance of an NADPH-diaphorase positive striatal neuron extending
a long axon parallel to the graft. Scale bar = 100\mu m.
7.5b (CW102) Detail of the boxed area in Fig. 7.5a showing the triangular cell body of
this neuron and its long primary processes. Scale bar = 100\mu m.
7.5c (CW204) Regenerating NADPH-diaphorase positive axons running close to the
sides of a graft at 8 weeks po. Scale bar = 100\mu m.
7.5d (CW103) Bundles of regenerating axons within the graft tip extending up into the
graft. Scale bar = 100\mu m.
7.5e (CW103) Another example of long NADPH-diaphorase positive axons within the
graft at 8 weeks po. One of the shorter regenerating axons is tipped by a dark bulb-like
structure (small arrow) which could be interpreted as a growth-cone. Scale bar =
100\mu m.
Histogram 7.1 Comparison of striatal NADPH-diaphorase positive perikaryal areas ipsilateral and contralateral to a living tibial nerve graft over time.
**Histogram 7.2**  Comparison of striatal NADPH-diaphorase positive perikaryal area ipsilateral and contralateral to a freeze-killed tibial nerve graft at 2 weeks po.
CHAPTER 8

Expression of GAP-43 mRNA and the synthesis of GAP-43 protein by adult striatal and nigral neurons ipsilateral and contralateral to an autologous peripheral nerve graft

Intrinsic differences between populations of CNS neurons may affect their ability to mount a regenerative response after injury either independently of, or in addition to, their ability to respond to any exogenous trophic support offered by peripheral nerve grafts. This chapter compares the expression of GAP-43, a growth associated protein, in neurons of the striatum and substantia nigra pars compacta of the adult rat, ipsilateral and contralateral to peripheral nerve grafts, two regions which display very different regenerative responses.

(a) Summary of methods used
See Chapter 2 for details:

Autologous tibial nerve grafts were implanted into the right striatum of adult Sprague Dawley rats. Sections of unfixed striatal and nigral tissue were processed for in situ hybridization using an oligonucleotide probe for mRNA for the growth associated protein GAP-43. The sites of mRNA synthesis were established 5 days, 10 days and 1 month after graft implantation. Similar observations were made using an immunohistochemical technique to visualize GAP-43 protein in sections from perfused animals at the same survival times. The position and appearance of any GAP-43 immunopositive sprouts and axons in the striatum and graft was recorded.
(b) Results

**In situ hybridization for GAP-43 mRNA**

In situ hybridization identified a population of GAP-43 mRNA containing neurons in both the corpus striatum and substantia nigra of the normal, unoperated adult rat and the control contralateral striatum. The striatal GAP-43 mRNA positive neurons were few in number, weakly labelled and scattered evenly throughout the body of the striatum; all had conspicuously large perikarya (Fig. 8.1a and b). Thus as already been shown by McKinney and Kent, (1994), the striatal GAP-43 mRNA positive cells correspond to the large striatal aspiny interneurons. The heavily labelled GAP-43 mRNA positive nigral neurons, which were found only in a diagonal swathe of cells corresponding closely with the position of the substantia nigra pars compacta (Fig. 8.2 a and b), match the perikarya of the dopaminergic nigro-striatal projection neurons described by Kruger et al. (1993).

The implantation of a tibial nerve graft into the corpus striatum of the adult rat seemed to have no modulatory effect on the expression of GAP-43 mRNA in either of the two regions at any of the survival times examined. Figure 8.1 a and b shows the appearance of these cells ipsilateral and contralateral to a tibial nerve graft at 10 days po. GAP-43 mRNA expression appeared to differ little either between survival times or between the grafted and control sides of the brain. However, it is probable that the heavy labelling characteristic of normal adult neurons of the SNpr may have masked upregulation of GAP-43 mRNA in response to implantation of the graft.

**Immunohistochemistry for GAP-43 protein**

No perikaryal GAP-43 protein could be detected in either the corpus striatum or substantia nigra when the above experiments were repeated using immunohistochemistry (Fig. 8.1c and d). GAP-43 positive sprouts were seen around the graft/brain interface at 10 days po, and within the graft itself (Fig. 8.3 c - f). Figures. 8.3 a and b show the course of an immunopositive axon, which can be traced for 160μm within the striatum, oriented towards the graft at 10 days po. This axon terminated in a strongly immunopositive fan-like structure (small arrow) which was interpreted as a growth cone due its morphology and the high level of GAP-43 protein visualised within it.

The only examples of up-regulation of either the protein or mRNA for GAP-43 were seen in a few isolated cortical cells located within 200μm of the injury tract caused by implantation of the graft. Fig. 8.4 a and b show the position and appearance of two distinctly immunopositive cortical cells found at 10 days po while figure 8.4c and d illustrates cortical up regulation of GAP-43 mRNA visualised by in situ hybridization at 5 days po.
Figure 8.1
No upregulation in GAP-43 mRNA or protein synthesis was seen in response to graft implantation by 10 days po.
G = graft, S = striatum, CS = contralateral striatum,
Arrow heads = GAP-43 mRNA containing or immunopositive neuronal perikarya.

8.1a (CW181) No GAP-43 immunopositive neuronal perikarya were found in the striatum ipsilateral to the graft at 10 days po. Scale bar = 100µm.
8.1b (CW181) Contralateral striatal sections showed no immunoreactivity for GAP-43 protein. Scale bar = 100µm.
8.1c (CW235) A small population of large striatal neurons are known to contain GAP-43 mRNA (McKinney and Kent, 1994) and were visible using in situ hybridisation, however no other population of striatal neurons were seen to up-regulate their synthesis of this molecule in response to graft implantation. Scale bar = 100µm.
8.1d (CW235) In the contralateral striatum, GAP-43 mRNA was only seen in a small population of large striatal neurons similar to those seen in Fig. 8.1c. Scale bar = 100µm.
Figure 8.2
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata,
Arrow heads = GAP-43 mRNA containing neuronal perikarya.

8.2a (CW204) At 5 days po the same pattern of GAP-43 mRNA containing neuronal perikarya were found in the SNpc on either side of the brain of these animals regardless of graft placement in the ipsilateral or contralateral striatum. Scale bar = 100μm.

8.2b (CW236) No difference was seen in nigral GAP-43 mRNA expression in similar grafted animals at 10 days po. Scale bar = 100μm.
Figure 8.3
GAP-43 immunopositive fibres, but no neuronal perikarya, were found within both the striatum and graft at 10 days po.
G = graft, S = striatum,
Arrows = GAP-43 immunopositive axons,
Small arrow head = GAP-43 immunopositive growth cone-like structure.

8.3a (CW181) A long GAP-43 immunopositive fibres was identified within the striatal parenchyma ventral to the graft at 10 days po. This fibre was tipped by a strongly immunoreactive, fan-shaped structure which was interpreted as a possible growth-cone. Scale bar = 100μm.
8.3b (CW181) Camera lucida drawing of the GAP-43 immunopositive regenerating fibre shown in Fig. 6.3a. Scale bar = 100μm.
8.3c (CW181) Many GAP-43 immunopositive fibres can be seen within the proximal tip of the graft at this survival time. Scale bar = 100μm.
8.3d (CW181) Long GAP-43 immunopositive fibres extending over 200μm from the tip of the graft. Scale bar = 100μm.
8.3e (CW183) Although GAP-43 immunopositive fibres are visible within the graft no GAP-43 immunopositive neurons can be seen in the surrounding striatal tissue. Scale bar = 100μm.
8.3f (CW183) Fine GAP-43 immunopositive fibres entering the graft from the striatum. Scale bar = 100μm.
Figure 8.4
Up-regulation of GAP-43 protein and mRNA was found in a very small number of cortical neurons found very close to the graft at 10 days po.
G = graft, S = striatum, CC = cerebral cortex, Cc = corpus callosum, LV = lateral ventricle, ac = anterior commissure.
Arrow heads = GAP-43 mRNA containing or immunopositive cortical neurons.

8.4a (CW181) Line drawing showing the position of the GAP-43 immunopositive cortical neurons. Scale bar = 100μm.
8.4b (CW181) Photomicrograph of GAP-43 immunopositive cortical neurons found in the boxed area in Fig. 8.4a. Scale bar = 100μm.
8.4c (CW182) Line drawing showing the position of cortical neurons found to have up-regulated GAP-43 mRNA expression. Scale bar = 100μm.
8.4d (CW182) Detail of GAP-43 mRNA containing cortical neurons found in the boxed area in Fig. 8.4c. Scale bar = 100μm.
Chapter 9

The effects of implanting PN grafts into the adult rat corpus striatum on the expression of the cell adhesion molecules L-1 and N-CAM and the extracellular matrix molecule tenascin-C

The ability of CNS neurons to regenerate axons after injury is dependent on the ability of the injured and regenerating axons to interact with their environment. This chapter examines the expression of mRNA of 3 molecules that are known to mediate axon-glia interactions during axonal regeneration and to influence axonal elongation, the cell adhesion molecules L-1 and N-CAM and the extracellular matrix molecule tenascin-C, in the adult rat brain after the striatal implantation of an autologous peripheral nerve graft. The expression of L-1 protein was also examined in the graft and surrounding striatum using immunocytochemistry at the ultrastructural level.

Summary of methods used:
See Chapter 2 for details:

Live and freeze killed autologous tibial nerve grafts were implanted into the right striatum of adult Sprague Dawley rats. At 2 weeks and 1 month po a small number of animals were either killed by exsanguination or perfused with fixative. Cryostat sections of unfixed brains were processed for in situ hybridisation using digoxigenin labelled probes made against L-1, N-CAM or tenascin-C, and Vibrotome sections of perfused brains were processed for TEM immuno-ultracytochemistry for L-1.
Results

This study was a preliminary investigation of the expression of selected cell surface and extracellular matrix molecules in and around PN grafts implanted into the striatum. It is incomplete because of the unexpected unavailability of further supplies of in situ hybridization probes for L-1 and N-CAM and an unexplained dramatic reduction in the sensitivity of the primary antibodies at EM levels only. It should be noted that these observations are taken from a very small sample of animals (n = 1) for each survival time studied for in situ hybridization and for EM immunocytochemistry.

In situ hybridization

N-CAM

At 2 weeks po, in situ hybridization identified a small population of N-CAM mRNA containing striatal neurons both ipsilateral (Fig. 9.1a) and contralateral (Fig. 9.1b) to the graft. The cells were few in number, and appeared to be larger than the majority of surrounding striatal cells (which could be seen owing to the relatively high level of background staining). These large N-CAM mRNA containing cells were scattered evenly throughout the striatum and were similar in perikaryal size and distribution to the large, aspiny, cholinergic striatal interneurons identified in chapter 6 and the GAP-43 mRNA containing striatal cells identified in Chapter 8 although there was no histochemical evidence to confirm this relationship. Additionally, there was no apparent difference between the visual intensity of mRNA staining for N-CAM in these cells ipsilateral or contralateral to the graft.

Another population of slightly smaller strongly positive N-CAM mRNA containing cells were seen around the graft as if 'lining' the graft/brain interface (Fig. 9.1a). This staining was particularly strong between the graft and cortical tissue but was not as wide spread in the striatum where these N-CAM positive cells were found much closer to the graft.

A large number of N-CAM mRNA containing cells were identified in the SNpc of the same animals at 2 weeks po (Figs. 9.1c and d). However, the number and distribution of these cells indicated that they did not represent the total population of neurons in the SNpc. The pattern and intensity of staining for this glycoprotein was similar in cells of the SNpc on both the side of the brain into which the graft was inserted and the contralateral side.

Two weeks later, at 1 month po, the population of slightly smaller strongly N-CAM positive cells lining the graft/brain interface was not as distinct as at the earlier survival time (Fig. 9.2a). However, a similar small population of large neurons containing N-CAM mRNA could be identified distributed throughout the striatum as in
the 2 week po samples (Figs. 9.2a and b; the levels of background staining was lower in these samples than in Figs 9.1a and b but the large, darkly stained cells can still be identified within the striatal tissue). The same pattern of staining for N-CAM mRNA was seen in the SNpc both ipsilateral and contralateral to the graft as at 2 weeks po (Fig 9.2c and d).

**L-1**

The pattern of striatal cell expression of mRNA for L-1 was similar to N-CAM at 2 weeks po; a small, widespread population of large striatal perikarya were strongly positive for L-1 mRNA in both the ipsilateral and contralateral striatum (Fig 9.3a and b) ie on grafted and contralateral sides. Unlike N-CAM, however, there was no evidence of up-regulated L-1 expression around the graft. Cells of the SNpc of these animals were strongly positive for L-1 mRNA on both sides of the brain (Fig. 9.3c and d). There was no apparent up-regulation of L-1 mRNA synthesis by striatal neurons in response to implantation of the graft. No examples of striatal L-1 mRNA synthesis were available for grafted tissue at 1 month po but the same pattern of neuronal N-CAM mRNA expression was seen in cells of the SNpc as at 2 weeks po (Figs. 9.5a and b).

**Tenascin-C**

At 2 weeks po synthesis of mRNA of the extracellular matrix molecule tenascin-C was very different from that of N-CAM or L-1 at the same survival time; it was expressed by cells both within, and immediately around the graft (Fig. 9.4a ). Most of the tenascin-C positive cells were located at the tip of the graft, extending a short distance into the parenchyma of the striatum along the lesion tract resembling fingers reaching out from the graft tip into the striatal parenchyma. There was no expression of this molecule by striatal perikarya either ipsilateral or contralateral to the graft (Fig. 9.4a and b), other than a small group of positive cells medial to the graft. It was not possible to determine whether these cells were neuronal or not. No examples of tenascin-C synthesis in the substantia nigra were available.

**EM immunohistochemistry**

The reaction product in all of the sections processed for EM immunohistochemistry is faint as a result of an unexplained reduction in the performance of all recent batches of the primary antibody at EM level.

**L-1**

At 6 days po faint L-1 immunoreactivity was seen on the surface of small axonal profiles enwrapped within the junctional zone of the graft (Figs 9.7 and 9.8). Relatively
strong L-1 reaction product was only seen on the surface of axons within the graft where they made contact with Schwann cell processes; Fig 9.9 demonstrates the selective immunoreactivity of an axonal profile which expresses L-1 on that part of its surface which abuts a Schwann cell body, but has no immunoreactivity on its opposite side which abuts basal lamina. Bundles of axonal profiles which were not closely associated with surrounding glial cells were also found in the junctional zone (Fig. 9.10). These profiles display only a faint immunoreactivity to L-1, on surfaces which contact other axonal profiles. At 17 days po the pattern of L-1 immunoreactivity had not changed: immunopositive reaction product was only visible on axonal profiles which were in contact with Schwann cell processes (Figs. 9.11).
Figure 9.1
At 14 days po a small population of N-CAM mRNA-containing neurons were identified around the graft and within the striatum on both sides of the brain. G = graft, S = striatum, CS = contralateral striatum, Arrow heads = N-CAM mRNA containing perikarya.

Figure 9.1a (CW226) A small population of sparsely distributed N-CAM mRNA-containing neuronal perikarya within the striatum. Additionally, N-CAM mRNA-containing cells are present around the graft/brain interface in both the striatum and cerebral cortex. Scale bar = 1mm.

Figure 9.1b (CW226) N-CAM mRNA-containing cells were also found in the striatum contralateral to the graft. Scale bar = 1mm.

Figure 9.1c and d (CW226) Identical diagonal swathes of strongly N-CAM mRNA-containing neurons were found in the SNpc of these animals both ipsilateral and contralateral to the graft. Scale bar = 1mm.
Figure 9.2
At 1 month po a similar small population of N-CAM mRNA-containing neurons were identified within the striatum on both sides of the brain to those seen at 14 days po. However at this survival time, no N-CAM mRNA-containing perikarya could be identified around the graft.

G = graft, S = striatum, CS = contralateral striatum,
Arrow heads = N-CAM mRNA containing perikarya,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata.

9.2a (CW227) N-CAM mRNA-containing striatal perikarya ipsilateral to the graft. Scale bar = 1mm.
9.2b (CW227) A similar population of N-CAM mRNA-containing neurons was also found in the striatum contralateral to the graft. Scale bar = 1mm.
9.2c and d (CW227) The pattern of N-CAM mRNA-containing cells in the SNpc ipsilateral and contralateral to the graft was no different at 1 month po from that seen at 14 days po (Fig. 9.1c and d). Scale bar = 1mm.
Figure 9.3
At 14 days po a small number of L1 mRNA-containing neurons were found within the striatum on both sides of the brain. No L1 mRNA staining was found to be associated with the graft.
G = graft,  S = striatum,  CS = contralateral striatum,
Arrow heads = L1 mRNA-containing perikarya,
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata.

9.3a (CW226)  L-1 mRNA-containing striatal perikarya ipsilateral to the graft distributed throughout the striatum. Scale bar = 1mm.
9.3b (CW226)  A similar population of L-1 mRNA-containing striatal neurons was also found contralateral to the graft. Scale bar = 1mm.
9.3c and d (CW226)  L1 mRNA-containing neurons were found in the SNpc of these animals. There appeared to be no difference in the distribution or visual intensity of reaction product in these cells between the nigra ipsilateral or contralateral to the graft. Scale bar = 1mm.
Figure 9.4
A mantle of tenascin-C mRNA-containing cells were identified around the grafts at 14 days po. However, no tenascin-C-mRNA containing neurons were found distributed throughout the striatum on either sides of the brain.
G = graft, S = striatum, CS = contralateral striatum,
Arrow heads = tenascin-C mRNA containing perikarya.

9.4a (CW168) Dark tenascin-C mRNA-containing perikarya were seen predominantly at the tip of the graft but could also be identified coating the graft/brain interface. A small isolated group of tenascin-C mRNA-containing perikarya were found within the striatum a short distance form the graft/brain interface, however the nature of these cells could not be identified. Scale bar = 1mm.
9.4b (CW168) No tenascin-C mRNA-containing striatal perikarya could be seen contralateral to the graft. Scale bar = 1mm.

Figure 9.5
At 1 month po the distribution of L1 positive nigral neuronal perikarya remained the same as that seen at 14 days po on both sides of the brain.
SNpc = substantia nigra pars compacta,
SNpr = substantia nigra pars reticulata,
Arrow heads = N-CAM mRNA-containing perikarya.
9.5a (CW227) L1 mRNA-containing neurons in the SNpc ipsilateral to the graft. Scale bar = 1mm.
9.5b (CW227) Similar L1-mRNA-containing neurons in the SNpc contralateral to the graft. Scale bar = 1mm.

9.6a (CW168) No reaction product can be seen on sections hybridised with L1/NCAM sense (control) probes. Scale bar = 1mm.
9.6b (CW168) No reaction product can be seen on sections hybridised with L1/NCAM sense (control) probes. Scale bar = 1mm.
Figure 9.7
Region around the tip of a PN graft, showing LI immunoreactivity 6 days after implantation into the striatum.
(CW281).
Arrows = L1 labelled axonal profiles,
JZ = junctional zone,
SC = Schwann cell processes,
SCB = Schwann cell body,
Double arrow heads = basal lamina.

A number of Schwann cell processes, and one cell body surrounded by a continuous basal lamina to form a Schwann cell column. One of the Schwann cell processes can be seen enwrapping a possible axonal profile and electron dense reaction product can be seen around the axon and to lesser extent in the mesaxon. Scale bar = 500nm.

Figure 9.8
Region around the tip of a PN graft, showing LI immunoreactivity 6 days after implantation into the striatum.
(CW 281).
JZ = junctional zone, SC = Schwann cell processes,
As = astrocyte processes, arrow heads = unlabelled axonal profiles,
Double arrows = basal lamina, arrows = L1 labelled axonal profiles.

Mixed cell column within the junctional zone comprising Schwann cell processes, astrocyte processes and axonal profiles surrounded by a loose basal lamina. Small axonal profiles within the column have weak reaction product on their surfaces which contact the surrounding Schwann cell process; however, the larger axonal profiles to the left of the picture which are not in contact with any other cells do not show immunoreactivity for L1. Scale bar = 500nm.
**Figure 9.9**  
Region around the tip of a PN graft, showing L1 immunoreactivity 6 days after implantation into the striatum.  
(CW 281).  
JZ = junctional zone, double arrows = basal lamina,  
As = astrocyte processes, SC = Schwann cell processes,  
Arrows = L1 labelled axonal profiles,  
SCB = Schwann cell body.  
S
chwann cell body and processes enwrapping small axonal profiles within a basal lamina tube. L1 reaction product can be seen around the axonal profiles especially where the Schwann cell processes make contact with one another. The axonal profile marked with an asterix only has L1 reaction product where it touches the Schwann cell surface and not where it makes contact with the basal lamina. Scale bar = 500nm.

**Figure 9.10**  
Region around the tip of a PN graft, showing L1 immunoreactivity 6 days after implantation into the striatum.  
(CW 281).  
JZ = junctional zone, double arrows = basal lamina,  
As = astrocyte processes, arrow heads = unlabelled axonal profiles,  
Arrows = L1 labelled axonal profiles.  
A bundle of small axonal profiles and astrocytic processes, within the junctional zone, not encapsulated by a basal lamina tube. A short discontinuous section of basal lamina is present next to an unidentified cell process at one side of the bundle. None of the axonal profiles are actually enwrapped by the other processes and only faint L1 reaction product can be seen where their surfaces meet. Scale bar = 200nm.
Figure 9.11
Region around the tip of a PN graft, showing L1 immunoreactivity 17 days after implantation into the striatum.
(CW 319).
Double arrows = basal lamina,
JZ = junctional zone,
SC = Schwann cell processes,
SCB = Schwann cell body,
Arrow heads = unlabelled axonal profiles,
Arrows = L1 labelled axonal profiles.

Schwann cell body and processes within a tightly fitting basal lamina tube at 17 days po. A number of small L-1 immunopositive axonal profiles are present within the tube, expressing L-1 on their surfaces which contact the Schwann cell processes, however, at least one axonal profile does not appear to express. Scale bar = 500nm.
DISCUSSION
Retrograde labelling of CNS neurons from PN grafts in the striatum

In this study I confirmed that CNS axons regenerate into PN grafts implanted into the corpus striatum of the adult rat (Benfey and Aguayo, 1982). At 2 weeks po, the shortest survival time studied, a small number of retrogradely labelled striatal perikarya were seen close to the graft tip and labelled axons were visible in the graft. Labelled nigral neurons were rarely seen. By 4 weeks po, although the pattern and frequency of striatal labelling remained unchanged, the number of labelled nigral neurons and axons in the graft had increased dramatically. At all subsequent time points studied the majority of labelled neurons were seen in the SNpc, not in striatal tissue. Retrogradely labelled striatal neurons were also seen in the globus pallidus and a few other nuclei, but only when the graft had impinged upon those sites during implantation. Retrogradely labelled striatal neurons were only found close to the graft tip and were conspicuously absent from that part of the striatum rostral and lateral to the graft. It is now widely accepted that living peripheral nerve tissue implanted into the brain, spinal cord or visual system can promote the regeneration of axons from CNS neurons (Aguayo, 1985; Berry et al. 1986). However, it is much less well appreciated that CNS neurons differ in their ability to respond to such grafts and not all populations of CNS neurons appear able to mount an effective regenerative response to injury in the presence of a PN graft. Neurons in the cerebellar cortex of adult rats cannot be retrogradely labelled from grafts in the cerebellum, unlike neurons in the deep cerebellar nuclei and those of the brainstem nuclei projecting to the cerebellum (Dooley and Aguayo, 1982, Vaudano et al., 1993 in abstract form). Grafts in the thalamus produce the best documented differential regenerative response: the majority of the neurons which regenerate axons into such grafts have cell bodies in the thalamic reticular nucleus; by comparison only a few thalamic projection neurons regenerate axons into the same grafts (Benfey et al., 1985; Morrow et al., 1993). The results of the present study mean that the striatum can now be added to the list of CNS regions the neurons of which show differential axonal regeneration into nerve grafts; specifically neurons in the SNpc and globus pallidus must be included with thalamic reticular neurons, deep cerebellar nucleus neurons and retinal ganglion cells, as populations of CNS neurons with a high potential for regeneration whereas striatal neurons show a much lesser capacity to regenerate into identical PN grafts.

Technical considerations

One of the main problems faced in gathering quantitative data concerning the regeneration of axons from striatal neurons was the difficulty in reliably discriminating between retrogradely labelled striatal and pallidal neurons. The division between caudal striatum and rostral pallidum is difficult to determine, especially when the histology of the...
area has been disrupted during graft implantation. In this study dark field microscopy, which highlights the white matter tracts and other landmarks not easily seen under bright field illumination, together with reference to the adult rat brain atlas by Paxinos and Watson (1986), was used to help distinguish between the two structures in order to correctly locate labelled cells, the morphologies of which are described in the Introduction. In some instances small quantities of tracer fluids spread onto the dorsal surface of the ipsilateral cortex following leakage from the graft. As there are no striato-cortical, nigro-cortical or pallido-cortical projections (Heimer et al., 1985), this should not affect the reliability of the retrogradely labelled striatal neuron counts. Furthermore, the results obtained from experiments in which the grafts were found to have died (for unknown reasons) act as controls. The viability of the grafts was evident under dark-field illumination; when graft tissue appeared to have little internal structure and the tip exhibited no continuity with the striatum at the graft/brain interface, the graft was assumed dead. These animals were not included in the table of results but it is significant that in these experiments no retrogradely labelled cell bodies or axons were seen in the striatum, SNpc or the globus pallidus, even when there was tracer leakage onto the surface of the brain. This shows that the retrograde labelling found in the present study was unlikely to have been the result of leakage of tracer onto the surface of the brain, or of the extracellular spread of tracer along the grafts.

The location and identity of the retrogradely labelled neurons

In this study few striatal neurons were seen to regenerate axons even though the proximal end of the graft, once implanted, must have been immediately adjacent to large numbers of injured striatal axons. Although is no definitive evidence for the neurochemical characteristics of the neurons which were retrogradely labelled in this study was not obtained, the size and shape of the most commonly labelled striatal neurons was very similar to that of the large aspiny cholinergic striatal interneurons, described as Type 1 striatal neurons by Bolam et al. (1984). The other type of striatal neuron which was retrogradely labelled resembled the medium spiny NADPH-diaphorase positive interneurons (Type II, Bolam et al., 1984). However, without additional immunohistochemical evidence this identification must remain tentative. When combined retrograde labelling and histochemical techniques were used to determine the phenotype of these neurons, no conclusive evidence was obtained for cholinesterase within retrogradely labelled cells. Neither the cholinergic or the type II cell normally has a projection outside the striatum. Striatal interneurons have extensive dendritic fields and axonal distribution within the striatum compared to projection neurons, which would increase the probability of their being damaged during graft implantation. Retinal ganglion cells have been shown to regenerate axons from dendrites into PN grafts in the retina, but only when their main
axons in the optic nerve are also damaged (So and Aguayo, 1985). However, a large
dendritic field could also provide better support to these cells when they are injured. The
same degree of damage to a neuron with a smaller dendritic field would possibly disable a
greater percentage of that cell’s total volume and significantly decrease its viability. Large
aspy cholinergic striatal interneurons comprise only 2% of the total striatal nerve cell
population (Phelps et al., 1985) while the diaphorase-positive medium spiny interneurons
are slightly more numerous at 7%. If these classes of interneurons are indeed the striatal
neurons which are most successful at growing axons into grafts, the relative sparseness of
these cell types may explain the infrequency of striatal cell labelling. In contrast, the
strongest and most consistent regenerative response was seen from perikarya in the SNpc,
most likely those of dopaminergic nigro-striatal projection neurons, whose axons were
probably interrupted during graft implantation. The retrogradely labelled cells in the SNpc
were found in clusters in positions consistent with the hypothesis that they were cells
whose axons had been injured near their terminal field. It is known that nigrostriatal
projection fibres show a topographical organization with dopaminergic neurons of the
ventro-medial SNpc mainly extending fibres to the anterior and dorso-medial striatum and
the majority of dorso-lateral nigral neurons projecting to the caudal and ventro-lateral
region of the striatum (Veening et al., 1980). However, this relationship was difficult to
recognize in the present study. The one instance of neurons in the substantia nigra pars
reticulata being retrogradely labelled out of 26 animals in the present study is difficult to
explain. This graft (animal CW293) impinged upon the globus pallidus, although much of
the graft tip remained in the striatum. However since there are no known SNpr afferents to
the pallidus or striatum, the most likely explanation is that the graft or injury tract must
have interrupted the SNpr projection to the thalamus.

Comparison with previous studies

The present results are apparently quite different from those described by Benfey
and Aguayo (1982). They reported finding over 200 striatal neurons retrogradely labelled
through PN grafts implanted into the corpus striatum of 8 adult rats but only 22 of nigral
origin. Their results were obtained using HRP (Type VI, Sigma) -soaked gelfoam pads
which would be expected to reduce the extent of retrograde labelling compared with the
HRP conjugates used in the present study. These tracers have been shown to be 20-50
time more effective and consistent than HRP alone for retrograde and orthograde labelling
of CNS neurons even at small doses (Trojanowski et al., 1981, 1982). One explanation
for the apparent disparity between the present results and those of Benfey and Aguayo
(1982) lies in the specificity of the uptake of the different tracers. The HRP-conjugated
ligands used in the present study have high affinity for receptors on axonal membranes,
the GM1 monosialoganglioside for cholera toxin conjugated HRP, and N-
acetylglucosamine for WGA. The ligands and conjugated HRP are internalized into the neuron by absorptive endocytosis which is more sensitive than the bulk endocytosis of free HRP (Trojanowski et al., 1981), but, conceivably, could be selective for different neurons. Indeed, in peripheral nerves, cholera toxin subunit-B conjugated HRP has been found to preferentially label large diameter myelinated fibres while wheat germ agglutinin conjugated HRP labels unmyelinated and small calibre myelinated peripheral fibres (Robertson and Grant, 1985; LaMotte et al., 1991). On the other hand, Ericson and Blomquist (1988) using cholera toxin conjugated HRP visualized with monoclonal antibodies, found no preferential uptake of the ligand by any CNS neuronal population.

Another explanation of the apparent discrepancy with regard to the extent of regeneration of striatal neurons lies in the size of the graft used: Benfey and Aguayo (1982) used grafts comprising the entire sciatic nerve trunk while in this study only the tibial nerve was implanted into the striatum. Morrow et al. (1993), found that grafts of peroneal nerve into the thalamus stimulated few TRN neurons to regenerate (approximately 1.1 neurons per animal). However by implanting 2 peroneal nerves sutured together to form one graft, or a single tibial nerve (approximately twice the size of a peroneal nerve), the number of labelled neurons increased dramatically to an average of 163 and 272 neurons per animal respectively. Consequently the greater number of retrogradely labelled striatal neurons found in the Benfey and Aguayo study may have resulted from the use of larger grafts. However the tibial nerve is the major component of the sciatic, and tibial nerves used as grafts in the thalamus, induce the regeneration of large numbers of neurons (Morrow et al., 1993) comparable with the numbers induced to regenerate by entire sciatic nerve grafts (Benfey et al., 1985).

The injury tract and the position of the graft tip influence regeneration

After PN grafts are placed in the brain some retraction of the grafted nerve commonly takes place, leading to the formation of an injury tract between the graft tip and the site to where it originally extended. The position of this tract of damaged tissue had a considerable influence on which neurons regenerated into the grafts. For example, if the injury tract reached the globus pallidus, many neurons in that structure were retrogradely labelled. Similarly, retrogradely labelled neurons were found in the ventral pallidum and medial forebrain bundle in 4 animals out of the 26 examined and in all 4 there was evidence that the injury tract had impinged directly on these regions; in each case the lesion tract extended beyond the graft below the anterior commissure and into these nuclei. Pallidal neurons would have remained uninjured when the grafts were initially placed entirely in the striatum and it is not surprising that under those circumstances they did not regenerate axons into the grafts. However, even if the graft tip was found, at the termination of the experiment, to reside in the striatum, pallidal neurons regenerated axons
into the grafts in cases in which the injury tract reached the globus pallidus. Furthermore, it is also unlikely that those labelled neurons found in the region of the medial forebrain bundle and ventral pallidum seen in a small number of animals were the result of tracer taken up within the striatum by axotomized fibres of passage as they were only seen in isolated cases in which the injury tract reached those nuclei and not in every animal in which the graft entered the striatum. It is not clear whether axons grow into the tip of a graft which subsequently retracts, or whether axons from injured neurons grow along the tract, perhaps attracted by factors from the graft.

**The distance between the site of axotomy and the graft or injury tract is critical for axonal regeneration**

The distance between the site of axotomy and the perikaryon of an injured neuron has been shown to have some bearing on its subsequent regenerative ability (see section 3b in Introduction). It has been observed that neurons injured closest to the graft regenerate more consistently than those whose perikarya are further than 2-3 mm from the implanted graft (Benfey and Aguayo, 1985). Short axotomy-soma distances have long been known to result in more profound chromatolytic changes (Lieberman, 1974). Many of the changes which constitute the cell body response to axotomy are likely to play a role in subsequent regenerative events. These changes include the expression of growth associated molecules, including GAP-43 (Skene, 1989). GAP-43 upregulation by CNS neurons in response to axotomy has been shown to be greater following a proximal axotomy than following a distal axotomy (Doster et al., 1992; Weise et al., 1992). Consequently one reason for the proximity to the graft (or injury tract) of the retrogradely labelled striatal and ventral pallidal perikarya is that only those neurons were subjected to a sufficiently proximal axotomy to initiate the changes in gene expression. This would not be of such importance in the neurons of the substantia nigra pars compacta which, even in control animals contain high levels of the mRNA for this protein (Kruger et al., 1993). Since GAP-43 expression correlates well with ability to regenerate axons into grafts (Vaudano et al., 1995) the presence of GAP-43 may permit a rapid regenerative response stimulating early extension of axons into the graft before the formation of a complete glia limitans.

**How do PN grafts promote CNS axonal regeneration?**

The living cells of peripheral grafts have a direct influence on the regeneration of surrounding CNS tissue. Freeze-killed peripheral nerve segments retain physical structures such as basal laminae and extra cellular matrix components which are advantageous for axonal elongation but are metabolically inactive. These grafts do not promote the same regenerative response as living grafts, (Smith and Stevenson, 1988;
This implies that some active mechanism of support is involved in CNS regeneration rather than merely the provision of a substrate lacking inhibitory factors (Caroni and Schwab, 1988b). One of the most well documented properties of PN grafts which may affect CNS regeneration is the synthesis of neurotrophins by Schwann cells in response to loss of axonal contact following axotomy. After lesion the distal parts of peripheral nerves undergo a series of changes classified as Wallerian degeneration: transected axons and myelin normally deteriorate rapidly and their debris is phagocytosed by macrophages; Schwann cells undergo mitosis and put out long processes within the existing basal lamina tubes forming Bands of Büngner. Schwann cells in segments of transected peripheral nerve have been shown to up-regulate the synthesis of many different growth promoting molecules in addition to NGF in response to a peripheral nerve lesion. These include BDNF (Meyer et al., 1992), NT-3 (Maisonpierre et al., 1990) and NT-4/5 (Hallböök et al., 1991; Ip et al., 1992) and, although the level of expression of CNTF is down-regulated to 30% of that of uninjured nerves, the synthesis of this cytokine is also continued in injured peripheral nerves (Sendtner et al., 1992). Receptors for all of these factors are found within the CNS. A similar process of degeneration and Schwann cell dedifferentiation occurs in peripheral nerve tissue once implanted into the adult CNS (Aguayo et al., 1981; Campbell et al., 1992). Presumably injured CNS neurons close to these grafts are exposed to graft-derived neurotrophins and the closer to the graft, the higher will be the concentration to which they are exposed.

The mechanisms of differential regenerative axonal growth

The expression of receptors for the trophic factors by different populations of neurons and the time course of neurotrophin synthesis relative to the time of graft implantation are important factors which may affect the ingrowth of axons under the influence of graft-derived trophic factors. Cholinergic interneurons in the corpus striatum, about 2% of the total population of striatal neurons (Phelps et al., 1985), have been shown to be strongly influenced by exogenous NGF; although they normally express the high affinity TrkA receptor (Steininger et al., 1993) intraventricular infusion of neurotrophic factors such as NGF and CNTF, has been found to stimulate the perikaryal hypertrophy and upregulation of the low affinity receptor, p75\textsuperscript{NGFR} by these neurons (Fischer et al., 1994; Hagg et al., 1989; 1992). Dopaminergic neurons of the SNpc are sensitive to BDNF (Hyman et al., 1991). These two neurotrophins are regulated by different mechanisms within injured peripheral nerve and are secreted at very different times: a 15 fold upregulation in NGF mRNA is detectable from 6 hours to 2 weeks post transection (Heumann et al, 1987), while BDNF upregulation takes much longer, starting at 3 days post lesion and not peaking until 3 weeks later (Meyer et al., 1992). If these
specific neurotrophins play important roles in regeneration it is possible that the regenerative response would be dictated by the time course of neurotrophin upregulation. This would lead to the early regeneration of striatal neurons and the relatively late regeneration of axons from the SNpc, the result observed in the present study. Furthermore neurons expressing mRNAs for Trk B and Trk C are widespread in the striatum, implying that many other striatal neurons should have been able to respond to neurotrophins from the grafts. It is unlikely that neurotrophin availability is the sole determinant of differential axonal regeneration into grafts.

Other possible causes for the differential regenerative responses of adult mammalian CNS neurons to peripheral nerve grafts could include differences in the microenvironment surrounding the injured axons; nonmyelinated axons might be expected to have a greater chance of regenerating than myelinated fibres. Oligodendrocyte membranes and CNS myelin have been shown to express molecules which inhibit neurite outgrowth and cause the collapse of growth cones in vitro, and inhibit axonal regeneration in vivo (see Introduction, section 4). Recently it has been shown that MAG a cell surface molecule found in myelinated tracts in the CNS, is also inhibitory to the outgrowth of axons from CNS neurons in vitro (McKerracher et al., 1994). It is reasonable to hypothesize that regenerative sprouts from injured nonmyelinated axons might have less chance of encountering CNS myelin during the initial stages of their elongation. Evidence from studies using chemical lesioning of monoaminergic fibres showed that, under those circumstances, at least some populations of unmyelinated axons have considerable powers of regeneration in vivo (Aguayo et al., 1984; Gage et al., 1985). Nigrostriatal axons are nonmyelinated (Hattori et al., 1973) and were the most consistently successful axons at regenerating in the present study. However, although it is unclear whether or not all TRN axons are myelinated, the axons of retinal ganglion cells are almost entirely myelinated within the optic nerve yet they produce many regenerative axonal sprouts after injury (Hall and Berry, 1989; Zeng et al., 1994) and regenerate vigorously into PN grafts (Berry et al., 1985; So and Aguayo, 1986).

Another explanation of the differential regeneration of CNS axons is that there are differences between neurons in the intrinsic determinants of axonal growth. One probability is that some neurons may not be capable of undergoing the changes in metabolic activity and gene expression which are presumably necessary for the elongation of an axon over long distances. The growth of TRN axons into PN grafts is accompanied by the expression of c-jun, (Vaudano et al., 1992, abstract form only), GAP-43 (Vaudano et al., 1995) and the adhesion molecule L1 (Zhang et al., 1995a) by their parent cell bodies. In contrast, most thalamic projection neurons, which do not regenerate axons into grafts, do not increase their expression of these molecules. Neurons of the SNpc contain high levels of mRNA for GAP-43 (Kruger et al., 1993) and L1 (unpublished) even in the
unoperated animal, and these levels remain high in animals with a PN graft in the corpus striatum. Consequently, neurons in the SNpc may be constantly expressing some of the genes whose products are necessary for axonal regeneration, whereas most striatal projection neurons appear not to be capable of expressing those genes even after axotomy.

Some CNS neurons have been shown to undergo morphological and physiological changes in response to specific neurotrophins regardless of whether they are injured or not, (Hagg et al., 1989; Fischer et al., 1994; Hagg et al., 1992). This trophic effect may promote the collateral sprouting of processes from neurons left intact by graft implantation possibly extending as far as, and into, the graft.

**Ultrastructural observations of PN grafts implanted into the striatum**

This ultrastructural study describes the regenerative events which take place when a tibial nerve autograft is implanted into the corpus striatum of adult rats, and focuses on the formation of regenerative axonal sprouts and their growth into and within the grafts. By 2 weeks after operation very large bundles of regenerative CNS axonal sprouts had formed around the graft tip. Similar bundles were still present at 4 weeks. A tiny proportion of the sprouts grew into the grafts, apparently without using astrocyte processes or any other obligatory cellular "bridge". The growth and maturation of the regenerating axons in the grafts took place deep within Schwann cell columns, i.e. not in contact with the basal lamina. These events were fundamentally similar to those occurring following implantation of a nerve graft into the thalamus (Campbell et al., 1992) or after attaching a segment of a peripheral nerve to the retinal stump of the severed optic nerve (Hall & Berry, 1989), but regeneration was a slow process compared to peripheral nerve regeneration or the growth of other types of CNS axons in PN grafts.

**The identity of the small nonmyelinated axonal profiles**

The axon-like profiles in the PBZ around the graft tip were identified as regenerative axonal sprouts for several reasons. They were similar to developing CNS axons (Henrikson & Vaughn, 1974; Peters et al., 1991) and to regenerating CNS axons identified in other studies, both involving PN grafts (Hall & Berry, 1989; Campbell et al., 1992) and following simple injuries (Dyson et al., 1988). In some experiments involving the visual system similar profiles have been unambiguously identified as regenerating sprouts by anterograde labelling (Richardson et al., 1982; Taylor et al., 1989; Zeng et al., 1994). The sprouts in the present study were far more numerous than were small, nonmyelinated axonal profiles in the contralateral, undamaged striatum and were found in large, distinctive bundles. They were also quite different from
regenerating PNS axons, which under some circumstances can penetrate short distances into CNS tissue forming large organelle-packed varicosities (Reier et al., 1983; Carlstedt, 1986; Anderson et al., 1989).

The sprouting response to the implantation of PN grafts into the CNS

Of particular significance is the observation that the axonal sprouts found around the tip of tibial nerve autografts in the striatum were almost identical in structure and arrangement to axonal sprouts found around similar grafts in the dorsal thalamus (Campbell et al., 1992). This suggests that the formation of large numbers of small axonal sprouts within c. 150mm of the graft and their collection into large bundles, mainly found just beneath the glia limitans or around blood vessels, may be the typical response of CNS gray matter to the implantation of a nerve graft. The main differences between the present observations and those of Campbell et al. (1992) were that graft implantation appears to induce the formation of larger numbers of regenerative sprouts in the striatum than in the dorsal thalamus, the striatal sprouts were longer lasting and they had a vesicle content somewhat different from those found in the thalamus. It is not clear why more sprouts should be produced in response to a graft in the striatum. Clearly a different population of neurons is axotomized by the implantation procedure, which probably explains the differing vesicle content of the sprouts. The neuronal perikarya which can be most frequently retrogradely labelled from the grafts in the striatum are found in the substantia nigra (see Chapter 3) but since only a tiny proportion of the sprouts grow into the grafts and we have not identified the neurons which give rise to the remaining sprouts we can only speculate that these neurons may have intrinsic characteristics which predispose them to sprout formation, if not to substantial elongation (see below). Tew et al. (1992) found similar large, persistent, bundles of axonal sprouts around grafts of pieces of muscularis externa from the gut implanted into the striatum but far fewer sprouts around similar tissue which had been freeze-killed, indicating that the sprouting response may not be a non-specific response to injury. The hypothesis that sprout formation is stimulated by factors emanating from the grafts is also supported by the observation in the present study that the largest bundles of sprouts were consistently found near the tip of living grafts, where there was no perineurium, in regions where the glia limitans was thin. Clearly, however, not all axonal sprouting in the striatum is the result of factors produced by the living Schwann cells etc. in the grafts since some bundles of sprouts were found around freeze-killed grafts and a simple mechanical lesion (stabwound) has been shown to elicit a noticeable, although transient, sprouting response from injured neurons of the thalamic reticular nucleus (Vaudano et al., 1995). Retinal ganglion cell axons also respond to optic nerve injury and/or PN grafting by the production of fasciculated axonal sprouts but in apparently smaller numbers than are
produced following nerve implantation in the corpus striatum (Richardson et al., 1982b; Hall & Berry, 1989; Zeng et al. 1994). As in the thalamus many of the sprouts disappear over the course of a few weeks. The most likely explanation of the loss of sprouts in the thalamus around a graft or in the injured optic nerve is that many of the perikarya giving rise to the sprouting axons die relatively soon after axotomy. More than 90% of axotomized retinal ganglion cells die in the first few weeks after injury and only a small proportion is salvaged by PN grafting (Berry et al., 1987; Villegas-Perez et al., 1988). Thalamic projection neurons are also known to die in large numbers when their target territories are damaged (Jones, 1985) but it is not known if projection neurons are a major source of axonal sprouts around grafts in the thalamus. The neurons that produce axonal sprouts near grafts in the striatum may be more resistant to axotomy-induced cell death.

The growth of regenerating CNS axons from the brain into the graft

Within the brain most axonal sprouts were within large bundles so that their nearest neighbours were other axonal sprouts, but they were also associated with astrocyte processes, myelinated axons, oligodendrocytes and microglia, in descending order of frequency. Apart from the obvious homophilic association there was little to suggest that the axons were using any specific type of cell as a substrate for elongation. The absence of ultrastructural abnormalities in axons with any of these contacts provides no evidence that they possessed repulsive or growth-inhibiting properties. These observations do not differ significantly from those made for nerve grafts in the thalamus (Campbell et al., 1992).

By 2 weeks after operation axonal sprouts had already penetrated into the grafts, but they constituted only a very small proportion of those present in the brain. There was no evidence that the sprouts followed astrocyte "bridges" into the grafts; axons in the JZ were often lacking in cellular contacts at this stage, and were otherwise as often in contact with fibroblasts and macrophages as astrocyte processes, whose ingrowth into the grafts only became extensive at 4 weeks. Astrocyte bridges (Weinberg & Raine, 1980) were formed eventually but the time course of their development seems more compatible with them having a role in stabilizing the brain/graft axonal pathways than a role in guiding the axons into the graft. These events were very similar to those observed following implanting a nerve graft into the thalamus (Campbell et al., 1992). The sequence of cellular events is also basically similar to that seen following the attachment of a peripheral nerve to the severed optic nerve in adult rats (Hall & Berry, 1989), i.e. the outgrowth of astrocyte processes followed axonal outgrowth. Curiously, although the numbers of axonal sprouts produced following grafting onto the injured optic nerve or into the thalamus appear to be lower, the number of axons regenerating into and through the grafts are greater than found in the present study, an observation
which has been confirmed by retrograde labelling studies (Berry et al., 1987; Morrow et al., 1992; Chapter 3; unpublished observations by Hossein & Campbell in this laboratory).

Within the grafts the regenerating CNS axons were found to become associated with Schwann cell processes at an early stage and the subsequent elongation of the axons through the grafts was exclusively along basal lamina covered Schwann cell columns. Many of the axons in the columns resembled those in the PBZ around the graft tip. The elongating axons were characteristically found clustered together, separated from the basal lamina by Schwann cell processes. This is also the case for regenerating thalamic axons, and contrasts with what is widely believed to occur during peripheral nerve regeneration when the elongating axons are usually described as being situated between the basal lamina and the Schwann cell surfaces (e.g. Martini, 1994). These deep parts of the bands of Büngner are regions where the Schwann cell surfaces are most strongly immunoreactive for the cell adhesion molecules L1 and N-CAM (Zhang et al., 1993, 1995a). This may be significant because L1 in particular has been shown to be important for supporting axonal elongation on Schwann cell surfaces (Seilheimer & Schachner, 1988). However, the putative growth cone profiles identified in the grafts were usually, although not always, in contact with the basal lamina at some part of their surface and the relative importance of interactions at these sites remains unclear.

The rate of growth and maturation of the regenerating striatal axons within the graft seem slow compared with that of regenerating peripheral nerve fibres (Anderson et al., 1983; reviewed by Lundborg, 1988), CNS axons regenerating into grafts in the thalamus (Campbell et al., 1992) or optic axons regenerating into grafts in hamsters (Cho & So 1987; Hall & Berry, 1989). Most axons in the proximal graft at 4 weeks in the present study remained in an immature relationship with their associated Schwann cells, i.e. they were not invaginated into the Schwann cell cytoplasm and none was myelinated. Many more axons were present at the graft tip than in the proximal graft even though these points were separated by only c. 1.5mm. Optic axons regenerate into grafts at a rate of c. 2mm/day (Cho & So, 1987) and become myelinated by 4 weeks (Hall & Berry, 1989). In this laboratory it has been found that there are commonly myelinated axons in grafts in the thalamus by 4 weeks, although their numbers are variable (Campbell et al., 1992; Zhang et al. unpublished). Most of the neuronal perikarya which can be retrogradely labelled from grafts in the striatum are found in the substantia nigra, or the globus pallidus if the graft touches that structure (see Chapter 3), but retrograde labelling of the nigra from the graft is meagre (<5 cells) before 4 weeks. The nigrostriatal tract is believed to be nonmyelinated, although there is little published information on the matter, and would consequently not be expected to become myelinated in a graft. The rate of elongation of axons is determined in part by the
intrinsic growth potential of the neurons (Fawcett, 1992), a good marker for which is the expression of the growth-associated protein, GAP-43 (Skene, 1989). There is evidence for the increased expression of GAP-43 following optic nerve injury (Doster et al., 1991; Zeng et al 1992), and after the implantation of a PN graft into the thalamus (Vaudano et al., 1995) but there is little evidence for the upregulation of GAP-43 following the implantation of a graft into the striatum (see Chapter 8). However nigral neurons contain high levels of GAP-43 mRNA even in the unoperated animal (see Chapter 8).

Because of the similarities between these observations on the effects of PN grafts in the corpus striatum and those made in previous studies of grafts in the thalamus (Campbell et al., 1992) and, to some extent, studies of grafts attached to severed optic nerves (Hall & Berry, 1989) it is now possible to begin to make generalized statements about the responses of CNS tissue to nerve grafts. It appears that neurons in quite different regions of the CNS respond to the grafts by producing large numbers of axonal sprouts, a variable proportion of which grow into the peripheral nervous tissue, apparently without guidance from Schwann cells extending into the CNS or astrocytes extending into the grafts. The regenerating CNS axons initially grow in clusters along the central parts of Schwann cell columns, away from the basal lamina. Astrocyte processes grow into the Schwann cell columns more slowly than axons. None the less, only selected populations of CNS neurons are capable of regenerating many of their axons through the grafts and discovering the reasons for this differential regenerative ability may prove crucial for the development of strategies to repair the injured brain or spinal cord.

**NGF synthesis by PN grafts implanted into the striatum and subsequent neurotrophin receptor expression**

Sections of peripheral nerve implanted into the striatum were found to contain much higher levels of NGF protein at 7 days po than the surrounding CNS tissue. Additionally, twice as much NGF protein was present in striatal tissue ipsilateral to the graft than in contralateral striatal samples, which had no contact with the graft. In light of these results it was assumed that some of the NGF synthesized by the graft diffused into the surrounding brain tissue and was available to any injured NGF sensitive striatal neurons. Surprisingly then, in view of the study by Gage et al. (1989) no striatal neurons were seen to re-express p75<sub>NGFR</sub>, the low affinity receptor for NGF and other neurotrophins, after graft implantation (see below). The production of NGF by the grafts together with the direct course of axons from striatal neurons towards the grafts, suggests that there may be a tropic response by the regenerating axons to substances emanating from the grafts.
NGF expression in the grafts

This study presents the first evidence that PN grafts continue to produce NGF after implantation into the CNS, as transected segments of peripheral nerve are known to do when left in situ. However, it is not known whether graft NGF production follows the same time course as NGF production in transected peripheral nerves as described by Heumann et al., (1987; see Introduction) which could only be established by performing a series of assays of grafts and striatal tissue at different survival times. However, the same bi-phasic modulation of NGF production by transected peripheral nerves would not be expected from PN grafts implanted into the CNS. In the distal stumps of transected peripheral nerve the first peak in NGF levels (a 15-fold increase measured at 6 hours after transection), was attributed to the accumulation of protein retrogradely transported from the target tissue. This cannot take place in PN graft tissue detached from the target tissue although NGF in the graft might be expected to accumulate near its proximal end. No increase in the synthesis of NGF mRNA was measured within the transected peripheral nerve until 3 days post transection (Heumann et al., 1987) and its eventual upregulation was closely associated with the time of macrophage invasion into the transected nerve segment. Further work in this laboratory has shown that the dynamics of macrophage recruitment into PN grafts implanted into the thalamus is broadly similar to that in transected peripheral nerves in situ (H. McCarthy et al., personal communication). The sequence of morphological changes in PN grafts in brain and the formation of bands of Büngner is also similar to that in injured nerves in situ (see Chapter 4). However, c-jun expression by Schwann cells in grafts implanted into the thalamus was found to be down regulated by 7 days po (Vaudano et al., 1992), although cultures of dissociated Schwann cells, taken from transected peripheral nerve, expressed high levels of c-jun up to 30 days po, (the longest experimental time examined; DeFelipe and Hunt, 1994) regardless of whether they were in contact with regenerating axonal profiles or not. This may indicate that the programme of expression of some molecules in these grafts is altered by the CNS environment and is different from that in peripheral nerve transection experiments.

It is still not known whether other neurotrophins known to be produced by the distal stumps of transected peripheral nerve, such as BDNF, NT-3 and NT-4/5, are also synthesized by PN grafts after their implantation into the CNS (as no assays were available to me to test this). As discussed earlier, the up-regulation of BDNF mRNA synthesis in transected peripheral nerve left in situ does not begin until 3 days after axotomy, takes up to 3-4 weeks to reach maximum levels in and is thought to be regulated by different mechanisms from those governing NGF mRNA upregulation. If grafts were to cease neurotrophin production within a few weeks after implantation,
perhaps under the influence of factors from the brain, then the later expressed neurotrophins (such as BDNF) may not be produced in high enough levels to offer neurotrophic support to any injured CNS neurons which are sensitive to their effects. Consequently, those neurons able to respond to the earlier expressed neurotrophins (such as NGF) would have a distinct regenerative advantage.

**Gradients in neurotrophin concentrations**

So far, the free availability of the neurotrophic factors produced by the graft to the injured surrounding brain parenchyma has been assumed. However, the difference between NGF levels in the graft and the ipsilateral striatum suggests that a concentration gradient exists between the two tissues. Furthermore, if BDNF is released from the graft it is likely that a more dramatic concentration gradient for this molecule would develop. Studies examining the dynamics of neurotrophic factors entering the brain via intraventricular infusions have shown that NGF was able to diffuse easily through both the walls of the ventricles and through the brain tissue (Kordower et al., 1993), but BDNF was only found to penetrate a few mm into the striatal tissue bordering the ventricle, consequently the diffusion of other neurotrophins from the graft through the striatum may be hindered. Further examination (Kordower et al., 1993) determined that the ependymal cells, which line the lateral ventricles, are particularly rich in truncated trkB receptors, as are astrocytes, and these probably bind much of the free protein, preventing it from diffusing further into the tissue and limiting the amount left to do so.

However, it may be that the existence of such concentration gradients of neurotrophins is advantageous in promoting axonal regeneration into the grafts. Gundersen (1985) established that only the growth cones of neurites emerging from explanted chick DRGs *in vitro* are sensitive to NGF, as withdrawal of NGF from their growth cone stopped elongation but its withdrawal from other regions of the cell such as its soma or axon had no effect on neurite outgrowth. NGF-sensitive axons have been shown to elongate towards high concentrations of that molecule. Gunderson and Barrett (1979) showed that elongating chick DRG neurites *in vitro* preferentially grew towards micropipettes containing high concentrations of NGF, turning 180° from their original course, but showed no preferential orientation towards micropipettes containing control solutions. Additionally, no concurrent effects were found on the initiation of neurites from the explant, neurite survival or the speed of neurite elongation. Further investigation showed that even when the culture medium contained NGF neurites preferentially extended towards the highest concentration of NGF available and grew in the direction of any source of NGF above that of the background level (Gundersen and Barrett, 1980). In light of these results, the NGF concentration gradient which exists between the graft and the striatum (as established in this study) may be advantageous in
attracting NGF sensitive regenerating neurons into the graft in vivo. Indeed, when both the axon and cell body of a regenerating striatal neuron were visualised within the same striatal section (see Chapters 3 and 7), the regenerating axons clearly followed a direct route into the graft and did not appear to meander through the striatal parenchyma until coming across the graft by chance. Instead, some active property of graft seemed to attract the regenerating axons directly towards it.

Ostensibly, it would appear that BDNF sensitive neurons injured by graft implantation have a much reduced chance of regenerating axons into the graft; it is likely that maximal synthesis occurs long after graft implantation when influences from the brain have reduced Schwann cell c-jun expression (Vaudano et al., 1992, in abstract form only) and may be dependent on the long-term viability of the graft. Additionally, the diffusion of the neurotrophin, once synthesized, from the graft may be limited to within a narrow zone around the graft because of the large number of truncated trkB receptors present within the striatum (see Lindsay et al., 1994). Together, these factors limit the trophic support available to BDNF sensitive neurons in the period immediately after injury. However, a large number of axons originating from SNpc neurons (the nigrostriatal projection neurons) have been identified within PN grafts implanted into the striatum (see Chapter 3). Significantly, the majority of these axons were only identified within the grafts at survival times of 4 weeks and more, which coincides with the known time of peak BDNF production in transected peripheral nerve left in situ discussed earlier. Although we have no experimental evidence to confirm that BDNF produced by the graft mediates the regeneration of SNpc axons found in the present study (indeed Knüsel et al., 1992, found that intraventricular infusions of BDNF were insufficient to prevent the retrograde cell death of nigrostriatal neurons after axotomy), the chronological coincidence of these events is compatible with such a relationship. However, in contrast to the situation with NGF, there is no direct evidence that axons show tropic responses to BDNF. However, it remains possible that this regenerative ingrowth of nigro-striatal fibres is stimulated by any number of other graft-produced factors which have neurotropic properties and are yet to be identified. For example, mRNA for GDNF, which has been shown to have neuro-protective effects on injured nigrostriatal neurons in vivo (Beck et al., 1994), has been isolated in Schwann cells in the PNS (Henderson et al., 1994) but it is not yet known whether PN grafts are able to synthesize GDNF or whether injured dopaminergic neurons of the SNpc could benefit from this during axonal regeneration.

The tropic effect of NGF on injured CNS neurons

In vivo, injured NGF sensitive, cholinergic neurons will also extend axons towards high concentrations of NGF within the CNS. This was unequivocally
demonstrated by Hagg et al. (1991) who implanted freeze-killed acellular PN grafts into the medial septum of adult rats; CNS fibre ingrowth was only found when the graft tissue was treated with NGF. Additionally, regenerating NGF-sensitive CNS nerve fibres were found preferentially to orientate towards the highest available concentration of NGF as seen with peripheral fibres in vitro. Varon and Hagg (1993) found that the number of medial septal neurons which extended axons into PN graft bridges implanted between the hippocampus and axotomised septum decreased by two thirds if NGF was infused into the nearby lateral ventricle distal to the graft. Furthermore, no fibres were found to invade the graft if the infusion cannula was placed proximal to it, presumably the concentration of NGF infused into the brain was greater than that produced by the graft, causing the regenerating fibres to grow into the region with the greatest level of NGF. The intracerebral infusion of NGF has also been shown to affect sympathetic nerve fibres innervating the cerebral blood vessels. Following fimbria/fornix transection sympathetic fibres have been identified growing into the hippocampus surgically deprived of its cholinergic input (Isaacson et al., 1992; Isaacson and Crutcher, 1995). This has been correlated with an increase in NGF concentration in the hippocampus, perhaps bought about by the cessation of NGF retrograde transport to the septal nuclei. Intraventricular infusion of NGF, however, increased the concentration of NGF in the CSF surrounding the cerebral arteries and decreased the extent of sympathetic invasion of the hippocampus following lesion (Saffron and Crutcher, 1990). In the latter experiment sympathetic fibres showed an inability to grow from a region of relatively high NGF concentration (the cerebral blood vessels) to a region with a relatively low concentration (the hippocampus) even though the hippocampal NGF levels were sufficient to attract sympathetic fibres in the absence of NGF infusion. Although PN grafts appear to produce less NGF than the amount administered during infusion studies, the results of the present study have shown, in the striatum at least, that their NGF content is above the normal endogenous levels found in this region of the brain. Therefore, it is highly possible that some CNS axons may have received a tropic influence from the graft. A similar consideration would appear to apply to peripheral fibres.

This is especially the case when the position of the external portion of the graft is considered. After graft implantation the distal end of the graft was positioned to lie across the exposed skull with the distal tip sutured to the fascia of the ipsilateral temporalis muscle. Although little angiogenesis was seen around the nerve tissue located on the muscle, the exposed skull and overlying nerve graft quickly became covered with newly formed vascular tissue. In long survival time grafts the angiogenesis in this region was so dense that it sometimes became difficult to discriminate between the graft and regenerated vascular periosteum. In view of this close association between the newly
forming, presumably innervated, blood vessels and the external portion of the graft, which was shown to synthesize NGF (and had been stripped of its epineurium before implantation) it would seem inevitable that, for example, sympathetic perivascular fibres would also be tropically attracted into the graft. However, bilateral superior cervical ganglionectomy had no obvious effect on the density of TH positive fibres in the grafts, suggesting that the numbers of sympathetic fibres which extend into the graft was low.

**p75NGFR up-regulation by the graft and surrounding striatum**

The present study has established that PN grafts continue to synthesize NGF after their implantation into the striatum and that NGF produced by the graft is able to diffuse into the surrounding striatal tissue. In addition to this, a population of known NGF-sensitive striatal neurons was found to undergo perikaryal hypertrophy after graft implantation (see Chapter 6), a response which is characteristically induced in adult medial septal neurons (which are also cholinergic and NGF-sensitive) by intraventricular infusion of NGF (Hagg et al., 1989; Vahlsing et al., 1991). However, PN graft implantation into the striatum failed to induce the re-expression of p75NGFR by any adult striatal neurons. This result was unexpected in light of the results of Gage et al. (1989) who reported the re-expression of p75NGFR by striatal cholinergic interneurons after chronic infusion of NGF directly into the striatum. However, the Gage investigation into striatal p75NGFR expression differed in a number of ways from the present study. Instead of PN grafts, Gage et al. implanted the cannulae attached to Azlet osmotic minipumps directly into the striatum and delivered a regular dose of 25mg/ml 2.5S NGF directly into the brain continuously for 2 weeks. As discussed earlier, the exact rate and concentration of PN graft NGF synthesis is still unknown but it is reasonable to assume it is much lower than the chronic infusion which was administered during the Gage study. This presumption is in agreement with the observation by Hagg and Varon (1993, discussed above), that regenerating medial septal neurons preferentially chose to orientate their axons towards sites of NGF infusion rather than a PN bridge graft. This effect was attributed to the elongating neurites being attracted to the greatest available source of NGF, in that instance the site of infusion rather than the PN graft. Another variant which may have led to different results between the present study and that of Gage et al., (1989) was the sensitivity of the antibody used to visualise p75NGFR. Although both studies used the same monoclonal antibody, clone 192-IgG, specific for p75NGFR, the much smaller amount of NGF secreted by the graft in the present study may have only stimulated a very weak up-regulation of p75NGFR which may have been difficult to visualise. However, despite the use of the Vectastain Elite kit avidin-biotin complex in this study (which is approximately 9 times more sensitive than that used by Gage et al., 1989 to intensify the immunoreaction product), no re-expression of p75NGFR was seen in
any of the striatal neurons surrounding the graft. Other laboratories have also found the 
NGF induced re-expression of p75^NGFR by striatal neurons difficult to reproduce, even 
when the original protocol of Gage et al. (1989) has been repeated using p75^NGFR 
antibodies from the laboratory of Gage et al., (1989) (Abelleira et al., 1994, in abstract 
form only, and personal communication).

Interestingly, another neurotrophic factor, CNTF, which is a cytokine rather than 
a neurotrophin, has been shown to effect p75^NGFR re-expression by striatal cholinergic 
terneurons both in vitro and in vivo. Magal et al. (1991) found that although CNTF 
did not significantly modify the number of striatal neurons taken from E17 foetal rats 
surviving in culture, the number which expressed p75^NGFR did increase after application 
of the cytokine. In the adult brain intraventricular infusions of CNTF stimulated p75^NGFR 
re-expression by striatal cholinergic interneurons but failed to induce the concurrent 
perikaryal hypertrophy reported by Gage which appears to be a characteristic effect of 
NGF on cholinergic forebrain neurons (Hagg et al., 1991).

Recently, injury alone (the implantation of a sterile strip of gelfoam into a 
preformed biopsy cavity) has been found to increase levels of CNTF in the adult rat 
striatum detectable by Northern blot analysis (Asada et al., 1995). The time of peak 
CNTF levels, 7 days po, was also found to correspond with the presence of the greatest 
number of type 1 astrocytes around the implant which, significantly, have been shown 
to produce CNTF when grown in culture (Rudge et al., 1985). However, CNTF does 
not possess a secretory sequence (Stokli et al., 1989) and is it not clear whether much of 
this molecule is actually available to the injured striatal tissue or how much is retained 
within the astrocytes. Asada et al. (1995) hypothesized that CNTF could be liberated as 
a result of cell rupture but, although it is possible, it is not clear why this should occur. 
The retention of CNTF within astrocytes around sites of striatal injury, such as PN graft 
implantation which has been shown to induce rapid astrogliosis (see Chapter 5), may 
explain why striatal neurons expressing p75 surrounding the graft did not become 
immunoreactive, as they may not have been exposed to the free cytokine. Additionally, 
the astrocytic reaction in the present study may have been less extensive than that 
observed by Asada et al., (1995) where a sample of striatal tissue was first removed 
before implantation of the gelfoam. The implantation of autologous graft tissue rather 
than a synthetic 'foreign' body may also have reduced the resultant level of astrocyte 
reaction in the present study.

Striatal levels of NGF and expression of p75^NGFR

The present study has demonstrated that at least two different populations of 
striatal neurons are able to undergo hypertrophy and regenerate long axons into living 
PN grafts implanted into the striatum (see Chapters 6 and 7) without undergoing re-
expression of the low affinity neurotrophin receptor p75\textsuperscript{NGFR}. That this regenerative response was mediated by graft-derived factors was illustrated by the inability of these neurons to mount the same regenerative response into freeze-killed, metabolically inactive, PN grafts. Originally thought to bind NGF with low affinity, p75\textsuperscript{NGFR} is now known to bind to all of the neurotrophin family (Rodriguez-Tebar et al., 1990; 1992). The binding affinity of this receptor is different for each neurotrophin; NGF binds most readily with p75\textsuperscript{NGFR} while BDNF has a much slower association rate (for review see Choa and Hempsted, 1995). However, higher affinity receptors for neurotrophins also exist within the nervous system which are more selective and, unlike p75\textsuperscript{NGFR}, possess intracellular domains which allow them to transduce a signal from their specific neurotrophin. As described above trkA functions as the high affinity receptor for NGF (Klein et al., 1991a); additionally trkB transduces both BDNF (Klein et al., 1991b; Glass et al., 1991) and NT-4 (Berkermeier et al., 1991; Ip et al., 1992) and trkC mediates the neurotrophic effects of NT-3 (Lamballe et al., 1991). The widespread distribution of the low affinity neurotrophin receptor throughout the nervous system is indicative of a more specific role for the high affinity receptors which appear to confined within particular neuronal populations. That p75\textsuperscript{NGFR} is found on cells in a number of different tissues outside the nervous system and most of which are, as far as is known, unresponsive to NGF (Thomson et al., 1988), implies that this molecule has a much more general role in the adult body than just the low affinity binding of neurotrophins by neurons. Common co-localization, of mRNA for the p75\textsuperscript{NGFR} with trkA high affinity neurotrophin receptor mRNA has been identified within the adult CNS, suggesting that a significant functional relationship must exist between these two molecules. However, although mRNA for both the high and low affinity NGF receptors are co-localized within some populations of cholinergic neurons within the brain (such as those of the medial septum) only mRNA for trkA, not p75\textsuperscript{NGFR}, has been identified within the striatum (Vazquez and Ebendal, 1991), significantly within the population of large cholinergic striatal neurons which, as reported in Chapter 3, regenerate very well after graft implantation. It is possible then that NGF produced by PN grafts implanted into the striatum does exert a neurotrophic effect on the large aspiny cholinergic interneurons but this effect is mediated via the high affinity trkA receptor independently of p75\textsuperscript{NGFR}.

A number of models have been proposed in an attempt to explain how the low and high affinity neurotrophin receptors interact to transduce neurotrophin signals and why this partnership is advantageous. At first it was thought that the high affinity receptor was in fact a heterodimeric complex of p75\textsuperscript{NGFR} and each neurotrophin specific trk subunit (Hempted et al., 1991). However, antibodies directed against p75\textsuperscript{NGFR} do not stop trkA from binding with NGF and, most significantly, a strain of p75\textsuperscript{NGFR} deficient mice have been bred which are fertile, viable and have apparently near normal
nervous system apart from decreased sensory innervation of their footpads (Lee et al., 1992). This is powerful evidence that \( p75^{NGFR} \) plays an auxiliary or complimentary role in NGF transduction and is not essential for this process (for reviews see Barbacid, 1993 and Choa and Hempstead, 1995). Although \( trkA \) has a very high affinity for NGF the kinetics of its binding to this molecule is surprisingly slow (Mahadeo et al., 1994) and much lower than that for \( p75^{NGFR} \). Interestingly, when these two receptors are co-expressed the rate of high affinity receptor NGF binding was found to increase 25 fold (Mahadeo et al., 1994) and this has lead to the ligand-presentation model of receptor interaction. This model proposes that the auxiliary function of \( p75^{NGFR} \) is to rapidly bind to NGF to either increase the local concentration of the neurotrophin around the high affinity \( trk \) receptor, or to collect the neurotrophin and then transfer it directly over to the waiting \( trk \) receptor (for review see Choa and Hempsted, 1995).

However, whatever the normal role of \( p75^{NGFR} \), it appears that there is no \( p75^{NGFR} \) re-expression by neurons in the striatum when an axonal regenerative response is clearly taking place and it is therefore difficult to believe that this molecule is essential for successful regeneration of axons from CNS cholinergic neurons. It has already been established that the large, aspiny cholinergic interneurons of the striatum express the high affinity NGF receptor \( trkA \) (Steininger et al., 1993.) which may be sufficient to mediate their tropic interactions with PN grafts.

**The morphological effect of PN graft implantation on striatal cholinergic interneurons**

Implantation of a living tibial nerve graft was found to induce an increase in the size of ipsilateral ChAT immunopositive striatal perikarya at 2 and 4 weeks po. The perikaryal areas of AChE positive striatal neurons showed no consistent change ipsilateral to either a living or freeze-killed graft at any survival time. AChE positive axons were seen in the graft at all the time points examined, but there was evidence to suggest that these axons originated from cholinergic cells.

**The effect of a PN graft on the size of AChE positive striatal perikarya**

The results from the study of AChE positive striatal perikarya were disappointingly inconsistent for both the living and freeze-killed grafts. There was no compatibility of measurements between animals within the same experimental groups for graft type or survival time po. In all cases, except for the 3 week po freeze-killed graft group, there were animals showing an apparent increase in cell size and animals showing an apparent decrease in cell size within the same experimental group. The best example of this variance is the 4 week po living graft group, which had the largest sample number of
animals (n=5); the estimates of the mean perikaryal area of AChE positive neurons ipsilateral to the graft ranged from between 30% greater to 19% smaller than that of contralateral AChE positive perikarya. The strongly positive result of the 3 week freeze-killed group is so uncharacteristic of the general trend of the freeze-killed grafted animals, being the only increase in cell size seen overall and such an extreme one, that this result too has been disregarded as another unreliable chance result.

AChE has been successfully used as a marker for cholinergic neurons in other cell size studies, giving reliable and consistent results (Fischer et al., 1987; Hagg et al., 1991; Fischer et al., 1994). Why should the results gained in this set of experiments be so variable? Clearly, the experimental techniques used in this study should be examined. Initially, AChE histochemistry was used to allow the dual identification of AChE positive perikarya and HRP retrogradely labelling within the same sections visualised using a concurrent HRP-AChE method (Mesulam, 1982). The sections processed for the AChE cell size study were also stained using the same method, but without the steps relevant to the visualization of HRP, with the intention to use these as a histological reference for the dual labelled cells. However, retrogradely labelled cells were very rare using the combined method and this branch of the study was discontinued, by which time the AChE positive cell size investigation was almost complete. Although many studies have demonstrated the enlargement of the large cholinergic striatal interneurons under various experimental conditions, none of those identified AChE-positive cells with the Mesulam method of AChE visualization.

All animals used for AChE histochemistry in this study were treated with 1.8mg/kg DFP 4 hours prior to sacrifice. This compound destroys AChE activity, rapidly leaving the intercellular reserves of this enzyme exhausted (Butcher and Woolf., 1982). It is important to note that all animals received an injection of atropine sulphate concurrent with the administration of DFP to maintain the animals alive by blocking ACh receptors. During the 4 hour interval before sacrifice de novo synthesis of AChE begins, most rapidly in the perikarya of cholinergic neurons (Lehmann and Fibiger, 1979), and if perfusion takes place at the correct time after administration of the DFP the distribution of this enzyme is limited to the perikarya of cholinergic cells, with no spread into their processes. DFP treatment is essential when trying to clearly visualize the perikarya of the large aspiny cholinergic striatal interneurons without masking by the dense network of AChE positive processes within the striatal neuropil.

Although this is now a standard histochemical procedure in studies of cholinergic neuronal perikarya (Fischer et al., 1987, Fischer et al., 1994), biochemical assay has shown that the AChE level of DFP treated mice may not fully recover after the 4 hour interval, and may not return to control levels even 40 days post injection in some animals (Smolen et al., 1987).
The fixative used in the preparation of tissue can also effect the final AChE activity. McGeer and McGeer, (1989), found that 4% paraformaldehyde or 4% paraformaldehyde with glutaraldehyde can reduce striatal AChE activity by 28% of the control levels, compared to controls perfused with saline and cold-stored until the assay. It is possible that the quantity and distribution of AChE within a cell may determine its real or apparent size when visualized in this way, particularly if there is insufficient enzymic activity to fill the cells with reaction product. Since paraformaldehyde reduces the level of AChE measured in tissue by biochemical assay it is possible that some of the AChE in these cholinergic cells was denatured during perfusion; probably in deposits close to the external surface of the cell (ie from the 'edges' of the cell when seen in cross section). As the outline of the cells measured in this study was determined by the AChE colour reaction, denaturing part of the enzyme content of the cells could reduce the accuracy of this technique for estimating the size of cholinergic neurons. Inspection of AChE stained sections, however, showed that these AChE positive cells appeared to be filled with reaction product and had distinct, regular boundaries consistent with those seen on similar ChAT immunopositive neurons. Any AChE positive perikarya with obviously abnormal morphologies would not have been included in the study.

As one of the functions of AChE is to break down any ACh remaining in the post-synaptic cleft after neural transmission this enzyme is also manufactured by non-cholinergic neurons which synapse with ACh transmitting cells. Although only the large aspiny interneurons of the striatum are actually cholinergic, up to six different types of striatal neurons have been classified as containing AChE (eg Kaiya et al., 1980). Therefore, AChE may not be as specific a marker for the large aspiny cholinergic striatal interneurons as originally hoped. Comparison of the density of AChE or ChAT positive cells in adjacent sections stained for either AChE or ChAT showed that the population of neurons positive for ChAT was smaller than that for AChE (18 cells per mm² compared to 33 cells per mm² respectively or 150 and 177 cells per section). Although up to 6 types of striatal neurons synthesize AChE (Kaiya et al., 1979), only the large aspiny interneurons of the striatum use ACh as their transmitter and express ChAT. It is therefore possible that a sub-population of smaller, non-cholinergic neurons were included in the AChE count masking any hypertrophic response of the cholinergic interneurons. However, as shown in the results, the shape of the histograms for neuron in the side of the brain containing the graft at each survival time did not indicate that any sub-group of ipsilateral cholinergic neurons was behaving conspicuously different from the overall trend.

The AChE positive neuronal perikarya in these sections were also larger than those in ChAT preparations. As in the present study, Hagg et al. (1987), found discrepancies between the size of cholinergic striatal perikarya when processed for either ChAT or
AChE; the mean perikaryal area for ChAT immunopositive cells was approximately 100mm² smaller than that of similar cells visualized using AChE in a previous study (Fischer et al., 1987). The use of the different histochemical procedures was cited as one possible explanation for the difference in size between the two groups of neurons.

However, hypertrophy of striatal cholinergic neurons has been observed in response to intraventricular infusions of neurotrophins such as NGF or even just phosphate buffered saline (Gage et al., 1989). In view of these past findings, this study was repeated using choline acetyl transferase as a more specific marker for the large aspiny cholinergic striatal interneurons which did not necessitate pre-treatment with DFP.

The effect of a PN graft on the size of ChAT-positive striatal perikarya

At 2 weeks po the perikaryal size of ChAT immunopositive striatal interneurons ipsilateral to a PN graft was found to be 19% larger than similar striatal neurons on the contralateral side of the brain. This perikaryal hypertrophy was sustained and still detectable at 4 weeks po and appeared to include all subsets of CHAT immunopositive neurons.

While AChE mRNA is found in up to 6 different types of striatal neurons (Bernard et al., 1995) ChAT is only found in the large aspiny interneurons and so can be considered a much more specific marker for this study. The morphology, location and distribution the large, aspiny striatal AChE and ChAT positive neurons is so similar that it is highly likely that they constitute the same population of cells (Satoh et al., 1983; Lehmann and Fibiger, 1979). It is therefore surprising that perikaryal enlargement was seen in ChAT immunopositive neurons ipsilateral to the graft at 2 and 4 weeks po while AChE containing-neurons showed no consistent effect.

The dynamics of the synthesis of the many different growth promoting molecules in addition to NGF produced by Schwann cells in response to a peripheral nerve exision is unknown once the graft has been implanted into the adult CNS. However, the continued hypertrophy of some classes of striatal neurons ipsilateral to living and not freeze-killed grafts up to 8 weeks po indicates that some active property of the graft, such as neurotrophin synthesis, is instrumental in stimulating cell enlargement.

As discussed previously elsewhere, PN grafts are now known to continue synthesizing NGF once transplanted into the adult rat striatum. However, nearby striatal neurons showed no upregulation of the p75 LNGFR in response to graft implantation. The assumption that this response would occur was based on the results of studies which showed that intraventricular infusion of NGF induced concurrent cellular hypertrophy and up-regulation of the p75 LNGFR by cholinergic striatal interneurons (Gage et al., 1989) and by cholinergic medial septal neurons (Hagg et al., 1989; Fischer and Björklund, 1991). It is possible that cellular hypertrophy and the onset of expression of
p75 LNGFR by striatal cholinergic neurons are mediated by separate mechanisms. The increase in cell size could have been stimulated by another factor, or combination of factors, produced by the graft rather than by NGF alone. This is particularly obvious in the case of striatal NADPH-diaphorase containing neurons which do not apparently express a receptor for NGF. Further evidence that factors other than NGF could influence striatal cholinergic neurons was established when Hagg et al., (1992) found that a 2 week infusion of human recombinant CNTF caused the upregulation of LNGFR expression in these cells without promoting any increase in cell size. Gage et al. (1989) reported a 15% hypertrophy of large striatal cholinergic neurons even when infused with a serum directed against NGF. In contrast the implantation of freeze-killed grafts was, in the main, ineffective at causing the hypertrophy of striatal cholinergic neurons.

Separate intraventricular infusions of NGF, NT-3 and NT-4/5 have been found to induce significant increase (20-40%) in the size of rat striatal cholinergic neuronal perikarya when compared to contralateral cholinergic cells (Fischer et al., 1994). However, these results were taken from aged rats where the existence of age dependent memory impairments had already been established. Such impairments have been correlated with the atrophy of cholinergic forebrain neurons (Fischer et al., 1991). As the original size of the cholinergic neurons measured for these animals is unknown, it is not possible to determine whether such increase in somal size reflect a genuine enlargement or recovery to a previously larger size. Infusions of BDNF had no effect on the size of striatal cholinergic neurons (Fischer et al., 1994). However mRNA for trkB and autoradiographically determined BDNF binding sites were present in the striatum, although not associated with cholinergic perikarya (Altar et al., 1994) and weaker than in surrounding brain areas. As BDNF has been shown to support the survival of striatal GABAergic neurons in culture (Hyman et al., 1991) it is possible that this neurotrophin derived from the grafts may have some, as yet undetermined, effect on at least one population of striatal cells.

Injury induced neuronal hypertrophy

Injury to the brain has been shown to stimulate the enlargement of some CNS neuronal perikarya. Pearson et al. (1987) reported a 25% hypertrophy of cholinergic neurons of the basal forebrain at 3 weeks after contralateral neocortical ablation. This perikaryal enlargement persisted for at least 372 days post lesion (the longest survival time studied), much longer than the enlargement of striatal NADPH-diaphorase neurons examined in Chapter 7 of the present study. Medial septal neurons also underwent perikaryal enlargement after the unilateral removal of the contralateral hippocampus or section of the ventral hippocampal commissure. It was thought unlikely that the stimulus for cell enlargement was a diffusible factor released from the site of injury. Instead, it

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was concluded that the hypertrophy of cholinergic neurons in the basal nucleus contralateral to cortical injury was in fact due to axotomy of basal nucleus neurons ipsilateral to the injury which normally project through the corpus callosum. This, in turn, probably produced a compensatory sprouting response from some (undamaged) basal nucleus neurons which project to the undamaged ipsilateral neocortex. Similar mechanisms were invoked to explain the enlargement of septal neurons following contralateral hippocampal injury (Pearson et al., 1987). The cellular mechanisms of such hypertrophic responses to injury are unclear, but may involve an increased neurotrophin supply to the undamaged neurons, or it could be that perikaryal hypertrophy is a biproduct of axonal growth and/or regeneration.

The presence of AChE positive fibres in the graft

AChE histochemistry showed a different pattern of axonal staining within the grafts at two weeks po than that shown by retrograde labelling (see Chapter 3). Retrograde labelling techniques will only visualize regenerating axons if they extend as far as the site of tracer application, leaving any shorter axons unlabelled. The histochemical technique used in this chapter will stain AChE containing-axons regardless of how far they extend into the grafts. It is not surprising then that a greater number of AChE positive axons were seen in the graft tip than were seen in the retrograde labelling study (see Chapter 3).

The AChE positive axons in the grafts appeared to become more distinct with time; at two weeks po axons in the graft had a slightly diffuse form, but with time their profiles became sharper and more intensely stained. One possible explanation for this is that with time these sprouts extend into the existing basal lamina tubes within the graft and begin to associate with Schwann cells. In the early stages of regeneration into grafts, several unmyelinated axons will be ensheathed by one Schwann cell, drawing them closer together forming fascicles of axons running up the Schwann cell column; this would give rise to a broader and more intensely stained AChE positive structures within the graft.

As already discussed, non cholinergic cells can also synthesize and contain AChE. At 4 weeks po there is a perceptible increase in the number of AChE positive axons seen in the graft by comparison with 2 weeks po coinciding with the time at which greater numbers of regenerating axons from the SNpc are revealed by retrograde labelling (see Chapter 3). The nigro-striatal fibres of dopaminergic SNpc neurons are known to contain AChE (Henderson, 1981) and it is possible that some of the AChE positive axons present in the graft at 4 weeks and later are nigral in origin.

In view of the up-regulation of BDNF synthesis by transected peripheral nerve, PN grafts are a possible source of this neurotrophin to surrounding striatal tissue.
However, no striatal perikarya were seen to retrogradely transport recombinant human BDNF infused directly into the striatum. On the other hand, approximately 28% of the dopaminergic neurons of the SNpc, the TH immunopositive cells of the nigrostriatal pathway, were found to be immunopositive for retrogradely transported BDNF (Mufson et al., 1994).

BDNF, GDNF and NT-3 can support the survival and differentiation of dopaminergic fetal mesencephalic neurons in vitro and in vivo striatal infusions of BDNF can augment nigro-striatal dopamine turnover in the adult rat (Seroogy and Gall, 1993; Lin et al., 1993). However, BDNF does not offer the same neuroprotection to striatal neurons against excitotoxic MPTP lesion as NGF does and so is therefore not comparable to NGF in all of its neurotrophic effects (Frim et al., 1993). GDNF, injected into the striatum or substantia nigra, is able to either protect the nigro-striatal projection neurons, if injected before chemical insult with MPTP, or promote their regeneration afterwards (Frim et al., 1993).

Schwann cells can act as a source of neurotrophic activity for foetal and adult dopaminergic neurons (Collier and Martin, 1993). E14 foetal ventral mesencephalic neurons co-cultured with a 10mm segment of adult sciatic nerve, which was kept physically separate from the foetal cells to allow only the exchange of diffusible factors between the two cultures, showed a 375% increase in the number of TH positive cells compared to co-cultures of foetal neurons grown without the nerve segment (Collier and Martin, 1993). A significant increase was also seen in the size of the perikarya and the lengths of the neurites of these cells. The same experiment was repeated using varying numbers of primary Schwann cells cultured from neonatal sciatic nerve and the response was dose dependent; the greater the number of primary Schwann cells introduced into the culture, increasing the amount of diffusible factors available to the foetal cells, the greater the neurotrophic response seen. This response was gained by infusing the foetal cells with primary Schwann cell conditioned medium reinforcing the hypothesis that this effect was the result of some diffusible factor released by the Schwann cells. Martin and Collier (1993) did not go as far as to identify the compound(s) produced by the Schwann cells which was or were responsible for this effect but BDNF is obviously a strong candidate. However, although many double labelled cells were found in the SNpc when the retrograde tracer fluorogold was infused into the striatum simultaneously with BDNF (Mufson et al., 1994), single labelled neurons were seen for both tracers suggesting that not all of the TH immunopositive neurons of the SNpc which project fibres to the striatum are sensitive to BDNF.

It is therefore possible that the regenerative response of the cholinergic striatal interneurons is not as great as would otherwise appear from the initial AChE positive axonal staining.
The morphological effects of PN graft implantation on medium aspiny striatal interneurons

The implantation of a PN graft into the striatum was found to induce perikaryal hypertrophy in a population of NADPH-diaphorase positive neurons ipsilateral to the graft. The degree of perikaryal enlargement (when compared to the same population of cells contralateral to the graft) was greatest at 2 weeks po, the earliest survival time studied, but steadily declined over the following 6 weeks until there was little difference between the perikaryal size of NADPH-diaphorase positive striatal neurons ipsi or contralateral to the graft. No change in perikaryal size was found in striatal NADPH-diaphorase positive neurons ipsilateral to freeze-killed PN grafts at 2 weeks po, the time when the greatest perikaryal enlargement was found in these neurons ipsilateral to a living PN graft. A small number of NADPH-diaphorase positive axons was identified within living grafts at 2 weeks po and their numbers thereafter steadily increased over time. However, although the number of NADPH-diaphorase positive axons found within the graft had very much increased by 8 weeks po, they were still much less numerous than the axons filled with HRP reaction product in the retrograde labelling experiments at the same survival time (described in Chapter 3).

The hypertrophy of NADPH-diaphorase positive striatal neurons

The normal response of intrinsic CNS neurons to injury is perikaryal shrinkage rather than enlargement (Barron 1989). It can readily be presumed that the perikaryal enlargement of NADPH-diaphorase positive striatal neurons reported here was induced, directly or indirectly, by factors actively synthesized by the graft, (as opposed to mechanical damage caused during graft implantation), as control experiments, which freeze-killed, acellular grafts were implanted into the striatum, did not induce perikaryal hypertrophy in this population of neurons. Similarly, it appears that the hypertrophic response of cholinergic striatal neurons in Chapter 6 was stimulated by graft-derived factors, possibly NGF, as no hypertrophy was seen when freeze-killed grafts were used. In the case of CNS cholinergic interneurons the cells are known to express trkA and a direct relationship has already been established between the intraventricular infusion of NGF and the subsequent perikaryal hypertrophy of cholinergic neurons (Hagg et al., 1989; Fischer et al., 1994). However, as yet it is not known which receptors for trophic factors and cytokines are expressed by NADPH-diaphorase positive striatal neurons and it is consequently unclear which, if any, of the neurotrophic factors known to be produced by injured segments of peripheral nerve might have brought about the hypertrophy by direct interactions with the neurons. In view of this, it remains entirely
credible that the perikaryal enlargement of NADPH-diaphorase positive neurons after graft implantation recorded in Chapter 7 was mediated indirectly by graft-derived factors which interacted with other brain neurons or glial cells. Nonetheless, the clear implication of the hypertrophy of NADPH-diaphorase positive neurons is that factors other than NGF are involved in mediating the response of brain neurons to PN grafts.

**Neurotrophin induced hypertrophy**

As yet, however, the only neurotrophic relationship that has been established between NADPH-diaphorase positive striatal neurons and any one of these factors was made *in vitro* using organotypic slices of mouse striatum and different neurotrophin containing media. Ardelt et al. (1994) found that after one week, slices treated with NT-4/5 contained significantly more surviving neurons than those cultured with NGF, BDNF or NT-3. They also showed that somatostatin immunoreactive cells (which also contain NADPH-diaphorase) were well represented amongst the surviving cultured neurons although they only comprise 1-2% of the total population of the striatum in the normal rat (Scherer-Singler et al., 1983). However, as the slices were taken from 3 or 4 day post-natal mice it may be possible that the apparent increase in the number of these cells was due to the differentiation of precursor cells still present within the slice rather than the neurotrophin-induced survival of these cells. Furthermore, there is no evidence that NT-4 caused the hypertrophy of the cultured neurons.

It is also possible that the perikaryal hypertrophy response may be a by-product of another process they stimulate such as axonal regeneration. Such a mechanism would resemble that discussed in the cortical ablation experiment of Pearson et al. (1987) described above. In the present study maximal neuronal hypertrophy coincided with a period of increased axonal elongation, and by the time that many long NADPH-diaphorase positive axons had been established within the graft neuronal hypertrophy had abated. Similar relationships between somal enlargement axonal regeneration have been recorded in injured neurons of both the PNS and CNS (Goldschmidt and Stewart, 1980; Hendrickson and Dineen, 1982).

**The origin of NADPH-diaphorase positive fibres within the graft**

It is possible that not all of the NADPH-diaphorase positive axons observed within striatal PN grafts originate from within the striatum. Just as dopaminergic neurons of the SNpc are able to extend TH-positive axons into striatal PN grafts after injury, it is possible that other, non-striatal NADPH-diaphorase containing axons axotomised by graft implantation may do the same. The striatum contains fibres from many regions of the CNS, both as direct afferents to striatal neurons and fibres of passage, but it is not clear whether any of these are NADPH-positive. Indirect evidence
from excitotoxin studies suggests that the striatum receives no NADPH-diaphorase positive input; quinolinic acid has been shown to selectively destroy intrinsic NADPH-diaphorase positive neurons and no NADPH-diaphorase axons are seen to re-invade the striatal tissue after injury (Davies and Roberts, 1987; Tew et al., 1994). However, controversy exists over the sensitivity of NADPH-diaphorase positive striatal neurons to quinolinic acid and Beal and colleagues report that this kind of excitotoxic lesion mimics the cellular pathology of Huntington’s disease and results in only the partial decrease of NADPH-diaphorase levels within the striatum implying that some NADPH-diaphorase positive projections either extend to or through the striatum (see Beal et al. 1986; 1989; Roberts et al., 1993).

One likely source of NADPH-diaphorase axons within the grafts are the fibres which accompany cerebral blood vessels, fibres which originate from peripheral parasympathetic and sensory ganglia. NADPH-diaphorase positive nerve fibres have been identified innervating the circle of Willis and the basilar artery (Kadota et al., 1994). Similarly, the proximal anterior and middle cerebral arteries receive nitric oxide synthase positive innervation from the parasympathetic sphenopalatine ganglia (Nozaki et al., 1993), and nitric oxide synthase shows a high degree of co-localization with NADPH-diaphorase in the nervous system (Dawson et al., 1991) NADPH-positive neurons are found within the trigeminal ganglion and nodose ganglion (Aimi et al., 1991) and although superior cervical ganglion neurons are NADPH-diaphorase negative they are surrounded by a fine network of NADPH-positive fibres which extend away from the ganglion (Morris et al., 1993).

However, there is evidence to suggest that not all of the NADPH-diaphorase axons seen within the grafts are extra-striatal in origin; in a small number of sections regenerating axons could be traced from striatal NADPH-diaphorase positive perikarya into the graft and given that the chances of sectioning the tissue in the right plane to see this occurrence must be very slim it could be assumed that many more such axons make their way into the grafts from the brain parenchyma than are actually seen to do so. Furthermore unpublished observations from this laboratory suggest that very few trigeminal neurons can be retrogradely labelled from grafts in the striatum (L. Harker, personal communication).

**The effect of PN graft implantation into the striatum on GAP-43 expression in the adult CNS**

The effect of PN graft implantation on GAP-43 levels within the striatum and SNpc

A small population of GAP-43 mRNA-containing neurons was found to be present within the striatum and graft implantation had no obvious effect on their
distribution or on the intensity of their staining at either 5 or 10 days po. Neuronal perikarya immunoreactive for GAP-43 protein were not identified within the striatum and, apart from some dark neuropil immunoreactivity along the graft/brain interface, no GAP-43 immunoreactivity was seen above the background level of staining ipsilateral or contralateral to the graft at either 5 or 10 days po. Many neurons of the SNpc were found to contain GAP-43 mRNA, but no obvious difference was seen in the number, distribution or intensity of GAP-43 mRNA expression in the SNpc ipsilateral or contralateral to the graft at either survival time after grafting or in unoperated animals. Despite the high levels of neuronal GAP-43 mRNA within the SNpc, no GAP-43 immunoreactivity could be identified in SNpc perikarya in unoperated animals or at 5 or 10 days after the insertion of a PN graft. In summary, the implantation of a PN graft into the striatum appeared to have no effect on the expression of GAP-43 by the neuronal populations examined. However, the neurons which regenerated most axons into the grafts, those of the SNpc, always expressed high levels of GAP-43 mRNA.

Neuronal expression of GAP-43 in the corpus striatum

The presence of GAP-43 protein within the uninjured adult striatum is well documented; Neve (1987) and Benowitz et al. (1988) found evidence of GAP-43 protein in striatal tissue but only in the neuropil and not in the neuronal perikarya. Additionally, at the EM level, DiFighia et al. (1990) were able to show that although GAP-43 immunoreactivity was found, on rare occasions, in dendrites, most of the reaction product was placed in discrete patches along the shafts of, and at the preterminal sites of, small unmyelinated axons. They also identified another subset of structures within the striatal neuropil which contained GAP-43 immunoreactivity; these were a small number of axon terminals which formed asymmetric synapses and contained round vesicles. By lesioning the cerebral cortex they were able to determine that although cortico-striatal projection fibres, which characteristically form asymmetric synapses and contain round vesicles, form one population of striatal terminals containing GAP-43 protein, others must include those of intrinsic striatal axons and those of afferent axons such as those of the nigro-striatal pathway (which is thought to be unmyelinated).

In situ hybridisation studies using probes made against GAP-43 mRNA have confirmed that both intrinsic striatal neurons and the dopaminergic nigro-striatal projection neurons of the SNpc express the GAP-43 gene and might, therefore, give rise to GAP-43 containing axons. However, reports of the distribution of GAP-43 mRNA within the striatum and its afferent neurons in the adult rat brain have differed dependent on the different in situ hybridisation methods used. Kruger et al. (1993), using radio-labelled probes, demonstrated that many neurons in the SNpc contained very high levels of GAP-43 mRNA (as did Bendotti et al., 1991, in a study confined to the brain stem),
but failed to detect any GAP-43 mRNA containing neurons in the striatum. McKinney and Kent (1994), however, also using radio-labelled labelled probes, could not only detect GAP-43 mRNA in histochemically co-localized ChAT positive striatal neurons but were also able to discriminate different levels of its expression within these cells. Despite the high levels of GAP-43 mRNA found within sections of the SNpc in the present study, GAP-43 immunoreactivity in the neuronal perikarya was indistinguishable from the background staining levels. This is consistent with the hypothesis that the protein is rapidly exported from its site of synthesis in the perikaryon into the axon where it is rapidly transported towards the axon terminal (Benowitz et al., 1981). In summary, most uninjured striatal neurons do not express detectable levels of GAP-43 mRNA or protein in their perikarya. Striatal cholinergic neurons contain detectable levels of GAP-43 mRNA, and SNpc neurons also contain high levels but neither neuronal type accumulates the protein within their perikarya. Other classes of striatal neurons do not appear to express GAP-43 mRNA even though a few such neurons probably regenerate axons into PN grafts. In the striatum GAP-43 protein is found in axons probably derived from some intrinsic striatal neurons and various afferent fibres.

GAP-43 expression following graft implantation

The alkaline phosphatase-conjugated oligonucleotide probe used in this study was able to visualise the populations of GAP-43 mRNA-containing neurons described above in both the striatum and SNpc but did not detect any modulation in GAP-43 mRNA expression in response to graft implantation. There are two possible explanations for this: 1) that the type of probe and/or the method of in situ hybridisation used were insensitive to any variations in GAP-43 mRNA levels which took place in the neurons and 2) that no modulation actually took place, and the GAP-43 mRNA content of striatal and SNpc neurons was indeed unaffected by graft implantation.

The same method of in situ hybridisation used in this study and identical probes have successfully been used to demonstrate both the up-regulation (Vaudano et al., 1995; Chong et al., 1994a) and later down-regulation (Chong et al., 1994b) of GAP-43 mRNA expression after injury in axotomised neurons which are normally GAP-43 mRNA negative. In the present study, GAP-43 mRNA was detected in a small number of cortical cells found close to the graft which do not normally express mRNA for GAP-43. In view of this, it is reasonable to conclude that this method is sensitive enough to successfully detect the up-regulation of mRNA in cells which are normally GAP-43 mRNA negative. Consequently, it seems likely that most striatal neurons do not increase their expression of GAP-43 mRNA following the implantation of a PN graft. However, the discrimination of increased levels of GAP-43 mRNA within cells which already express considerable amounts of this molecule in the control state is more problematic.
Changes in the expression of GAP-43 mRNA in the SNpc or, perhaps, even striatal cholinergic interneurons may have been impossible to detect. A more quantifiable method, such as silver grain counting after radio-labelled probe in situ hybridisation, might have been able to detect subtle changes in GAP-43 mRNA levels. It is also possible that in areas of the brain such as the SNpc, where there are many closely packed GAP-43 mRNA expressing cells, up-regulation of GAP-43 mRNA by previously negative cells may have been masked. To detect this a stereological counting method would have to have been employed. None the less, it is unlikely that a major up-regulation of GAP-43 by all the SNpc neurons which regenerated into the grafts would have gone undetected in the present study.

Although Basi et al. (1987) presented evidence to suggest that the rate of gene transcription regulated the expression of GAP-43, Perrone-Bizzozero et al. (1991) reported that injury had no effect on the rate of GAP-43 protein transcription and that the number of nascent transcripts from the GAP-43 gene produced was the same in developing rat cortical neurons, NGF- treated PC 12 cells and regenerating optic nerves in goldfish, regardless of whether they were extending/regenerating axons or not. If the modulation of GAP-43 protein levels within injured striatal and SNpc neurons was also regulated at post-transcriptional levels then no increase in GAP-43 mRNA levels (as identified by in situ hybridisation) would be expected after injury, which would be in keeping with the results of the present study. Unfortunately it is still not clear whether GAP-43 gene expression is regulated at the transcriptional or post-transcriptional levels in regenerating neurons.

It was perhaps surprising not to find an increase in the perikaryal content of GAP-43 protein in striatal and SNpc neurons after graft implantation despite the high levels of GAP-43 mRNA they contain. The sensitivity of the antibody against GAP-43 protein used in this study was demonstrated by its ability to visualise GAP-43 immunoreactive neuropil around the graft, the presence of GAP-43 immunopositive axons within the grafts and newly expressed GAP-43 protein within a small number of cortical neurons which do not normally contain GAP-43 in the adult brain.

GAP-43 protein expression in the perikarya of axotomised CNS neurons

The results of this study have confirmed that the perikaryal accumulation of GAP-43 protein is not a prerequisite of axonal regeneration in the CNS. It has been shown that GAP-43 immunoreactivity is present in the axons of CNS neurons which regenerate into PN grafts (Campbell et al., 1991; Vaudano et al., 1995), and in the axonal sprouts produced after optic nerve injury, even without the presence of a graft (Doster et al., 1991). However, there was no evidence for the accumulation of GAP-43 protein in the perikarya of regenerating TRN neurons following graft implantation in the
thalamus (Vaudano et al., 1995). Similarly there was no perikaryal accumulation of GAP-43 protein in injured neurons in the present study. In contrast, however, Doster et al., (1991) demonstrated that RGCs axotomized close to their perikarya (within 3mm) became GAP-43 immunopositive after optic nerve transection in the absence of a PN graft (although those lesioned more distally did). Similarly, Schaden et al. (1994) found a very small number of GAP-43 immunopositive RGCs in the retinae of adult rats at 21 days after optic nerve transection but many more were found in the retinae of identical animals which had received PN grafts anastomosed onto the ocular stump of their optic nerves immediately after transection. All of the GAP-43 positive RGCs had also regenerated long axons into the PN grafts.

Two interesting points arise from the reports on RGC perikaryal GAP-43 immunostaining that are in contrast to the results of the present study: 1) that the distance of the optic nerve lesion from the RGC perikarya had some effect on its accumulation of GAP-43 and 2) the 1:1 relationship between GAP-43 immunoreactivity of RGC perikarya and the regeneration of their axons into a PN graft. Although both striatal neurons and neurons of the SNpc are known to contain high levels of GAP-43 mRNA in their normal state, neither were found to accumulate GAP-43 protein in their perikarya after graft implantation into the striatum, even though axons from both neuronal populations were present within the grafts. The relationship that Doster and colleagues identified between the distance of axonal injury from the neuronal perikaryon and GAP-43 immunoreactivity in the perikaryon was not found in the present study; although neurons of the SNpc were axotomized far from their perikaryon and did not show a subsequent up-regulation of perikaryal GAP-43 protein (in keeping with the results of Doster et al., 1991), neither did any of the striatal neurons which were lesioned very close to their perikarya. (For example, Chapter 3 demonstrated that all of the striatal neurons retrogradely labelled in this study could be found well within the 3mm limit observed for RGC injury-induced GAP-43 accumulation).

Why should GAP-43 protein accumulate in the perikarya of CNS neurons regenerating axons?

GAP-43 accumulation in the cell body of neurons has no known function. Most of the postulated functions of GAP-43 involve organelles in growth cones, nerve terminals or axons (see introduction). GAP-43 accumulation in perikarya could theoretically be the result of increased synthesis or reduced export or degradation of the protein. Jones and Aguayo (1991) reported an in situ hybridisation study that showed similar increased levels of GAP-43 mRNA in RGC following both intraorbital axotomy, which produces perikaryal immunostaining and intracranial axotomy, which does not produce perikaryal GAP-43 accumulation. Any increased GAP-43 synthesis in RGCs
following intracranial injury would therefore have to involve post-transcriptional mechanisms. If the RGC perikaryal immunoreactivity for GAP-43 was purely the result of protein build-up starting at the site of injury, filling up the axon and eventually accumulating in the cell body, then in the present experiments the injured striatal cholinergic neurons, or even SNpc neurons, which are high in GAP-43 mRNA, would have been expected to become immunoreactive in the long term experiments. This did not occur. However, Doster et al. (1991) did not find any preliminary increase in GAP-43 protein levels at the site of interorbital lesion which would also be expected if the gradual retrograde build-up of GAP-43 protein was taking place. In the normal animal GAP-43 protein is exported away from its site of synthesis within the perikaryon by fast axonal transport (Benowitz et al., 1981). If axonal injury was to disrupt this mechanism of axonal transport from the cell body into the axon, a build up in GAP-43 protein levels within the perikaryon might be expected. However, this does not seem to be the case, because in the experiments of Doster et al., (1991) the export of GAP-43 into the optic nerve was apparently greater following proximal axotomy than following distal axotomy.

The most simple explanation of the accumulation of GAP-43 protein in RGC perikarya following intraorbital axotomy is therefore that its rate of synthesis is increased by post-transcriptional mechanisms to an extent sufficient to overload the export machinery of the cells. This does not happen in regenerating TRN neurons or striatal or SNpc neurons.

The relationship between GAP-43 expression and axonal regeneration

The neurons which most successfully regenerated axons into PN grafts in the present study were those of the SNpc and, to a lesser extent, presumptive striatal cholinergic interneurons, both of which constitutively express high levels of GAP-43 mRNA. This adds to the evidence that expression of, or ability to re-express, GAP-43 mRNA may be an important prerequisite for axonal regeneration. Neither injury alone, nor the presence of a living PN graft, seems to stimulate GAP-43 mRNA re-expression in adult striatal neurons, unlike the TRN neurons (Vaudano et al., 1993 and 1995) or RGCs (Doster et al., 1991) both of which regenerate vigorously. This may be one reason for the poor axonal regeneration into grafts of most striatal neurons, which do not have high levels of GAP-43 mRNA in their perikarya. In vitro, a direct relationship has been found between the expression GAP-43 and neurite outgrowth in PC12 cells and the availability of the neurotrophic factor NGF. Van Hooff et al. (1986) found that exposure to NGF increased the level of B50/GAP-43 in PC12 cells to over double that normally found in both undifferentiated and differentiated control cultures, and a later study determined that treatment with NGF also stimulated the relocation of immunogold labelled B50/GAP-43 from lysosomal structures, such as multivesicular bodies, secondary lysosomes and Golgi apparatus, to the plasma membrane where B50/GAP-43
labelling was most pronounced on the lamellipodia and filopodia of neurite growth cones (Van Hoof et al., 1989). In PC12 cell lines at least, the role of NGF and GAP-43 in promoting neurite outgrowth seem to be closely related: viral transfection of human GAP-43 cDNA into PC12 cells had the effect of not only accelerating initial neurite outgrowth but also increased their sensitivity to NGF 10 fold (Yankner et al., 1990). Similarly, application of GAP-43 antisense oligonucleotides to cultures of PC12 cells blocked the normal NGF-induced neurite outgrowth from those cells (Jap-Tjoen-San et al., 1992).

In the striatum, however, the close relationship between GAP-43 and NGF does not seem to be as easy to demonstrate. As already discussed above, the large aspiny cholinergic interneurons of the striatum contain varying levels of GAP-43 mRNA ranging from very high to very low levels (McKinney and Kent, 1994). Interestingly, it has also been shown that over 99% of the same population of cholinergic neurons express the trkA high affinity receptor for NGF (Sobreviela et al., 1994). It is at first sight surprising then that the implantation of PN grafts (which synthesize NGF) into the striatum does not stimulate a detectable increase in GAP-43 mRNA or the accumulation of GAP-43 protein in striatal cholinergic perikarya, nor the up-regulation of the p75 low affinity NGF receptor (see Chapter 5) within these cells. However, it is not clear that exposure to neurotrophins is always a stimulus for GAP-43 expression. The morphological characteristics of the retrogradely labelled neurons described in Chapter 3 more often fitted those of the NGF sensitive and GAP-43 containing large striatal cholinergic interneurons than any other striatal cell type but the results of Chapter 7 show that at least one other population of striatal neurons was able to regenerate long axons into the graft. These neurons, the medium aspiny interneurons, do not contain mRNA for GAP-43 or express either the high or low affinity receptors for NGF, nor do they appear to accumulate GAP-43 protein after injury, but a few of them are still able to mount a successful regenerative response when compared to the majority of striatal neurons. Although it is possible that the grafts may have been invaded by NADPH-diaphorase positive axons from the periphery, a small number of NADPH-diaphorase positive perikarya were seen to extend axons directly into the graft and, just as Strittmatter et al. (1995) were able to demonstrate by the almost normal development of the nervous system in GAP-43 knockout mice, it is possible then that GAP-43 is not essential for axonal regeneration into PN grafts from these cells.

Vaudano et al. (1995) noted that the majority of dorsal thalamic neurons which re-expressed GAP-43 mRNA after PN graft implantation into the thalamus were concentrated near the graft, and concluded that they were damaged, presumably with a very proximal axotomy, during graft implantation, and that the injury stimulated the up-regulation of GAP-43. Further investigation showed that although mechanical injury

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alone was a sufficient stimulus to induce the transient expression of GAP-43 mRNA in thalamic neurons, particularly in the TRN, prolonged synthesis of GAP-43 occurs only in the presence of a graft (Vaudano et al., 1995). These authors also showed a direct correlation between the capacity of thalamic neurons to up-regulate and maintain synthesis of GAP-43 mRNA expression and the ability of axons to extend long distances within the graft; they demonstrated this using immediately adjacent sections of grafted thalamus taken 2 days after the application of the same mix of HRP-conjugated retrograde tracers to the distal end of the graft as used in the present study. Alternate sections were reacted for either radio isotope-labelled in situ hybridization for GAP-43 mRNA or retrogradely transported HRP and compared. In the majority of cases, high levels of in situ hybridization and HRP reaction product appeared to be localised within the same positions in the adjacent sections and, presumably, the same thalamic neurons. A similar relationship was also seen amongst specific populations of cerebellar cells - neurons of the deep cerebellar nuclei exhibited prolonged up-regulation of GAP-43 mRNA synthesis after implantation of a PN graft and were able to regenerate many axons into the grafts. However, Purkinje cells of the cerebellar cortex, which did not up-regulate GAP-43 expression in response to graft implantation, did not show any regenerative ability (Vaudano et al., 1993). Strittmatter et al. (1995) suggested that rather than being essential for axonal elongation during development, GAP-43 plays a complementary role, enhancing the pathfinding ability of developing neurons and hence enabling them to find their way to their correct target areas. That such a role exists for GAP-43 during the regeneration of injured adult CNS axons may be feasible, and in the light of the GAP-43 knock-out mouse study this may be an area of much research in the future.

It is likely that another role for GAP-43 exists within the striatum other than promoting regeneration after injury. It has been suggested that GAP-43 is a marker for synaptic plasticity within the adult brain and that those regions which play a part in the acquisition of new skills and memories are rich in this molecule (reviewed by Benowitz et al., 1990). Neve et al., (1988, using human tissue) found very high levels of GAP-43 mRNA in cortical areas involved with the initial processing of sensory information, such as the hippocampus and a number of integrative areas of the neocortex, and concluded that this was related to the synaptic plasticity characteristic of these regions. Only low levels of GAP-43 mRNA are found in the striatum. However, it must be remembered that only the large, aspiny, cholinergic interneurons of the striatum contain GAP-43 mRNA. These cells comprise only 2% of the total number of striatal neurons, (Phelps et al., 1985, explaining why the overall level of striatal GAP-43 mRNA is so low), and form synaptic connections with both incoming nigral and cortical fibres and all other types of striatal neuron, including other cholinergic interneurons. As the striatum forms
an important part of the sensory motor processing pathways and the limbic system, it is feasible that the GAP-43 mRNA containing intrinsic cholinergic neurons are involved in the integration of neuronal information and any growth promoting properties which may be conferred on striatal neurons may, in reality, just be a beneficial side effect of its presence.

The significance of GAP-43 gene deletion experiments

Strittmatter et al. (1994), created GAP-43 knock-out mice, which demonstrated unequivocally that GAP-43 is not essential for growth cone formation or neurite extension in the developing nervous system (see Introduction). However, given the prevalence of GAP-43 at sites of neuronal development and regeneration, its role is likely to be closely associated with these two processes. Through a series of experiments Strittmatter and colleagues have shown that GAP-43 stimulates the binding of GTP-g-S protein to Gβ, a signal transducing G-protein which is a major component of growth cone membranes (Strittmatter et al., 1990; Strittmatter and Fishman, 1991). The inhibitory effect of CNS myelin on growth cone formation in vitro, has been associated with G protein activation (Igarashi et al., 1993) which, in turn, can be effected by GAP-43 (Strittmatter et al., 1994b). Additionally, Doherty et al. (1991) found that the growth cone enhancing effects of NCAM and N-cadherin on PC12 cell line neurite extension were mediated via G-proteins, further emphasizing the importance of their signal transducing role in growth cone guidance. It seems likely that, as concluded by Strittmatter et al. (1995), that the lack of GAP-43 in GAP-43 deficient mutant mice may result in a reduction in the ability of growth cones to distinguish between subtle changes in growth guidance cues due to changes in G protein signal transduction mechanisms.

Expression of the cell adhesion molecules L-1 and N-CAM and the extracellular matrix molecule tenascin-C in striatal PN grafts and brain of adult rats

Small widely distributed populations of L-1 and N-CAM mRNA containing neurons were identified within the grafted striata of adult rats at 7 days and 1 month po. However, as an identical population of striatal neurons, matching these cells in number, size and intensity of mRNA expression, was also found at both survival times in the contralateral, unoperated side of the brain it was surmised that the expression of L-1 and N-CAM mRNA by this population of cells is unaffected by PN graft implantation into the striatum. Additionally, N-CAM mRNA expression was up-regulated by a limited number of smaller cells located very close to the graft at 7 days po but this effect was undetectable 3 weeks later at 1 month po. Striatal graft implantation did not affect the
expression of mRNA for L-1 or N-CAM by neurons in the SNpc which was high on both sides of the brain regardless of PN graft implantation. L-1 reaction product was found within the graft and JZ at 6 and 17 days po, mostly on the surfaces of regenerating axons where they made contact with Schwann cell processes in the bands of Büngner. Additionally, some weak reaction product was seen between a small number of axon profiles not surrounded by a basal lamina within the JZ where they made contact with each other. Strong up-regulation of tenascin-C mRNA expression was seen at 7 days po in a discreet population of unidentified cells lining the graft/brain interface, which were thought to be reactive astrocytes, and the lateral ventricle ipsilateral to the graft which was not seen on the contralateral side of the brain.

However, these are only preliminary observations gained from a very small sample of subjects and, in the case of the EM immunocytochemistry study, using very weak antibodies. In order to make any firm statement concerning the synthesis and expression of cell adhesion and extracellular matrix molecules at molecular and ultrastructural levels in the rat brain after PN graft implantation into the striatum, these experiments would have to be repeated a number of times. This will be dependent on the supply of reliable probes and antibodies.

Cell adhesion molecule expression in the injured adult striatum

Surprisingly, PN graft implantation into the striatum had no apparent up-regulatory affect on the level of CAM mRNA expression by the majority of striatal or nigral neurons. Given the relatively large amount of NGF PN grafts implanted into the striatum are known to synthesize (see Chapter 5) this result was quite unexpected as, in vitro at least, NGF has been shown to up-regulate the expression of both L-1 and N-CAM in PC12 cells (Doherty et al., 1987, 1988; Mann et al., 1989) and Zhang et al, (1995) have shown that PN grafts cause the upregulation of L-1 by some CNS neurons. Similarly, NILE, which is immunochemically identical to L-1, was originally identified as an NGF-inducible large external glycoprotein (Bock et al., 1985). This effect need not be confined to neurons which express the high affinity receptor for NGF; the induction of L-1 mRNA by PC12 cells has recently been found to be modulated by cell-cell interactions but not mediated via trkA (Itoh et al., 1995). How these results relate to the present study is unclear; EM examination showed that many regenerating axons expressed L-1 reaction product where they contacted other L-1 containing glial structures within the graft but this does not seem to have been sufficient to induce up-regulation of L-1, even in the large cholinergic striatal interneurons - a population of cells, known to contain mRNA for trkA and, under certain circumstances, to also express the low affinity NGF receptor p75 (Hagg et al., 1991).
Interestingly, the distribution and size of the N-CAM and L-1 mRNA containing striatal and nigral neurons found on both sides of the brain corresponded closely to that of the GAP-43 mRNA containing neurons observed in Chapter 8. It has already been established that the striatal GAP-43 mRNA containing cells are the large aspiny cholinergic interneurons (McKinney and Kent, 1994) and the majority of the small population of striatal cells retrogradely labelled in Chapter 3 displayed many of the morphological characteristics of these interneurons (ie large bipolar perikarya and long, unbranching processes) although this relationship was not confirmed histochemically. DiFiglia et al. (1989) also demonstrated that the large striatal cholinergic interneurons are immunoreactive for N-CAM protein and the present study suggests that they additionally express L-1 mRNA. Furthermore, DiFiglia et al. (1989) also identified N-CAM immunoreactivity within the other population of striatal interneurons, the medium aspiny interneurons, which were also shown to regenerate long axons into striatal PN grafts (see Chapter 7). However, in the present study this relationship was not obvious at the mRNA level but in light of the smaller perikaryal size of this population of interneurons any up-regulation of mRNA synthesis of this molecule may have been masked by the high background levels seen in the 7 day sample examined here. Additionally, given the low frequency of both the large cholinergic and medium diaphorase-positive aspiny striatal interneurons within the striatum (approximately 2% each, Phelps et al., 1985 and Scherer-Singler, 1983 respectively), the small number of such cells which could be retrogradely labelled from the grafts, and the small number of sections examined in this preliminary investigation the chances of seeing one of these cells would have been very low. The possibility of this error occurring could be greatly reduced by increasing the number of animals used and the number of sections examined from each.

Cell adhesion molecule expression in the SNpc after striatal PN graft implantation

The expression of N-CAM and L-1 mRNA by neurons in the SNpc is particularly salient with reference to the strong regenerative response of these neurons identified in Chapter 3 by retrograde labelling. The perikarya of the retrogradely labelled nigrostriatal projection neurons observed in the present study were located within the same region of the SNpc as those nigral neurons shown to express mRNA for GAP-43 and the cell adhesion molecules L-1 and N-CAM.

As discussed earlier, the concentration of these particular molecules within the neuronal populations described here is most likely related to their integrative role in the adult basal ganglia, which requires some degree of synaptic plasticity. As a result of this it is possible that these molecules also endow such neurons with an increased regenerative ability as an advantageous bi-product of their presence.
Tenascin-C mRNA expression after graft implantation

Tenascin-C mRNA was only seen to be up-regulated in a discreet population of small cells closely lining the graft/brain interface, particularly at the graft tip. Unlike N-CAM or L-1, no mRNA for this extracellular matrix molecule was found within the striatal parenchyma far from the graft, although a small number of cells were seen medial to the graft. However, disruption of the striatal tissue lying between these tenascin-C mRNA positive cells and the graft indicates that they were probably not striatal in origin but may have migrated through the damaged tissue from the graft as some cells appear to have done along the lesion tract at the graft tip. More extensive observations in this laboratory have co-localized tenascin-C with GFAP in cells lining and extending from PN grafts implanted into the thalamus in a similar pattern to that seen in the present study (Zhang et al., 1995). These cells were interpreted as being reactive astrocytes and/or fibroblasts and indeed, both astrocyte and fibroblast processes were identified within the junctional zone between the graft and brain tissue in the ultrastructural study of the grafted striatum reported in Chapter 3.

Tenascin-C has been identified as having both growth inhibiting and growth promoting properties for extending PNS neurites grown in culture (Werhle and Chiquet, 1990; Faissner and Kruse, 1990) but appears to be involved in the growth-inhibiting process of astrocytic gliosis which is characteristic of injury in the CNS in vivo (Laywell et al., 1992; Mckeon et al., 1991; see Introduction, section 5c). The large numbers of CNS axons seen to invade striatal grafts (see Chapters 3, 6 and 7) and thalamic grafts (Morrow et al., 1991; Campbell et al., 1992; Vaudano et al., 1995), presumably regenerated through this layer of tenascin-C expressing glial cells and fibroblasts and entered the grafts where tenascin-C was even more strongly expressed. This does not appear to support the view that tenascin-C has an inhibitory effect on regenerating CNS neurons in vivo.

Ultrastructural observations of L-1 expression within the graft

Although the reaction product of the EM immunocytochemistry examined in the present study was very weak and the sample was taken from only two animals, initial observations of this tissue suggest that the pattern of L-1 protein expression in striatal PN grafts closely resembles that in thalamic PN grafts at similar survival times (Zhang et al., 1995a). In the present study faint L-1 reaction product was only seen where regenerating axons were in contact with Schwann cell or astrocyte processes or other axonal profiles within the graft or junctional zone. L-1 is a homophilic molecule and will bind strongly with the same molecule expressed by surrounding structures in its environment (Schachner et al., 1985; Rutishauser and Jessell, 1988). Since Schwann cells in PN grafts also express L-1, homophilic L1:L1 interactions between regenerating...
CNS axons and Schwann cells are possible. L-1 appears to be one of the most important molecules for the growth of neurites in culture on the surface of Schwann cells (Seilheimer and Schachner, 1988; Bixby et al., 1988; Zhang et al., 1995).

The role of the FGF receptor in neurite extension through PN grafts

Both L-1 and N-CAM have been shown to promote neurite extension from PC12 cells in vitro, independently of their homophilic binding properties (Williams et al., 1994). Surprisingly, the FGF receptor has been implicated in this pathway; FGF receptors contain a domain homologous to one found on L-1 and N-CAM, and peptides derived from this region inhibit neurite extension on N-CAM and L-1 positive surfaces (Williams et al., 1994). Similarly, cultured cerebellar neurons were also found to lose their ability to extend neurites in response to application of a soluble recombinant L-1Fc chimera when pretreated with antibodies made against the FGF receptor (Doherty et al., 1995). In view of these results, possession of FGF receptors may be an added advantage for regenerating axons which contact L-1 positive glial surfaces within the grafts (such as Schwann cell processes). Not only would these axons benefit from enriched cell-substrate adhesion but also receive a stimulus which would increase neurite outgrowth via the signal transduced through the FGF receptors. Significantly, neurons of the SNpc, which showed a strong regenerative response to striatal PN grafts (as demonstrated by retrograde labelling in Chapter 3) are known to express mRNA for the FGF-1 receptor (Wanaka et al., 1990) and this pre-adaptation may contribute to their ability to regenerate so well.

The importance of L-1 in CNS axonal regeneration

The expression of L-1 by regenerating some CNS neurons is therefore likely to be a considerable advantage for their growth through PN grafts, and may be one of the reasons for the differential axonal regeneration into PN grafts shown by CNS neurons in this and other studies (Campbell et al., 1992; Vaudano et al., 1993, abstract form only). In this way it would appear that both the population of large striatal neurons, presumptive cholinergic interneurons, and SNpc neurons, which were L-1 and N-CAM mRNA positive, are preadapted for axonal regeneration through PN grafts. However, no upregulation of L-1 mRNA expression was detected in these neurons in response to graft implantation. Zhang et al. (1995a) also correlated the regeneration of axonal profiles into PN grafts implanted into the thalamus with the up-regulation of L-1 mRNA in a discrete population of thalamic neurons, those of the thalamic reticular nucleus, which are known to show a strong regenerative response into such grafts (Campbell et al., 1991; Morrow et al., 1992; Vaudano et al., 1995). It is not clear how these results relate to those of the present study it may be that either the constitutive expression of L-1

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or the ability to upregulate the molecule following injury are requirements for successful regeneration.

**CONCLUSIONS**

The experiments reported in this thesis have confirmed that while many different populations of adult CNS neurons are able to regenerate axons long distances through PN grafts after injury, some types of neuron regenerate their axons more readily than others including neurons of the SNpc and certain populations of striatal neurons. This thesis has also given some clues as to the possible roles of trophic factors in the regeneration of CNS axons into these grafts and has established that transected segments of peripheral nerve continue to produce NGF, even after implantation into the brain. However, although neurons of one population which appeared to regenerate axons into the grafts, striatal cholinergic interneurons, are known to be sensitive to NGF, other neurotrophins, trophic factors and cytokines may additionally be involved in mediating the regenerative axonal growth of injured CNS neurons into PN grafts. In this regard it would be desirable to confirm which trophic molecules other than NGF are expressed by PN grafts and to perform *in situ* hybridisation for their receptors (eg the trk family of receptors in the case of neurotrophins) to determine how well regenerative ability is correlated with the capacity to respond to individual trophic molecules. Furthermore, experiments using blocking antibodies against these factors would be necessary to demonstrate how important such responses were. Neurons in the SNpc are known to be responsive to BDNF, which is only slowly upregulated by injured segments of PN. Our preliminary results suggest that the use of predegenerate PN grafts reduces the delay between graft implantation and the time when SNpc neurons can be retrogradely labelled. This needs further evaluation but suggests that BDNF may indeed play a role in the regeneration of these neurons.

In the present study the ability to regenerate axons through PN grafts has once again correlated with GAP-43 and L-1 expression. However, in contrast, to previous studies of the regeneration of thalamic axons (Vaudano et al., 1995; Zhang et al., 1995a) the neurons of the SNpc, which regenerated their axons most readily, exhibited high constitutive levels of expression of mRNA for these molecules and further upregulation in either L-1 or GAP-43 mRNA expression during regeneration could not be detected. Further L-1 in situ hybridisation experiments and EM immunocytochemical investigations of the expression of both GAP-43 and L-1 by regenerating axons would strengthen this correlation, as would a combined retrograde label/in situ hybridisation study to investigate the levels of expression in individual regenerating neurons. The role that these molecules play in regeneration would however, require a different type of
study, perhaps involving the infusion of antibodies to L-1 into the grafts, or the use of transgenic animals in which the expression of these molecules was altered.

Finally, this work provides further evidence that some CNS neurons are preadapted to regenerate their axons through PN grafts, by their constitutive expression, or ability to up-regulate the expression, of regeneration-related molecules such as trophic factor receptor molecules, GAP-43 or L-1. If PN grafts are to be used successfully in therapeutic attempts to repair damaged pathways in the CNS it seems likely that ways will have to be found to increase the expression of growth related molecules by wider populations of neurons, the technology for which is beginning to be developed.
REFERENCES


Andreason, T. J., Luetje, C. W., Heidman, W., Storm, D. R. 1983. Purification


Bjorklund, A. and Lindvall, O. 1975. Dopamine in dendrites of substantia


Davies, A.M., Thoenen, H. and Barde, Y.A. 1986. The response of chick


Donoghue, J.P. and Herkenham, M. 1986. Neostriatal projections from individual cortical fields conform to histochemically distinct striatal compartments in the
rat. Brain Res. 365: 397-403.


Faissner, A., J. Kruse, C. Goridis, E. Bock and M. Schachner. 1983. The neural cell adhesion molecule L1 is distinct from the N-CAM related group of surface
antigens BSP-2 and D2. EMBO J. 3:733-737.


Gerfen


Hall, S. and Berry, M. 1989. Electron microscopic study of the interaction of
axons and glia at the site of anastomosis between the optic nerve and cellular or acellular sciatic nerve grafts. J. Neurocytol. Apr; 18(2): 171-84.


Kawaja, M. D. and Gage, F. H. 1991 Reactive astrocytes are substrates for the growth of adult CNS axons in the presence of elevated levels of nerve growth factor. Neuron 7, 1030.


Kruger, L., Bendotti, C., Rivolta, R., Samanin, R. 1993. Distribution of GAP-


Martini, R. 1994. Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of


protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. Proc. Natl. Acad. Sci. USA. May; 83(10): 3537-41.


Nozaki, K., Moskowitz, M.A., Maynard, K.I., Koketsu, N., Dawson, T.M.,


Tello, F. 1911. La influencia del neurotropismo en la regeneracion de los centros nerviosos. Trabajos del Laboratorio de Investigaciones Biologicas de la Universidad de Madrid, 9, 123 - 159.


Thanos, S. 1992. Adult retinofugal axons regenerating through peripheral nerve


Neuron. Sep; 13(3): 583-94.


