DIFFERENTIAL EXPRESSION OF REGENERATION RELEVANT MOLECULES IN NEURONS OF ADULT RAT BRAIN AFTER INJURY AND THE IMPLANTATION OF PERIPHERAL NERVE GRAFTS

BY

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~ To mother, who is everything in my life ~
ABSTRACT

This study was concerned with the pattern of expression of several regeneration relevant molecules in the striatum, thalamus and cerebellum of adult rats and correlating this with differences in the regenerative potential of the neurons. The molecules investigated were neurotrophin receptors (Trk A and p75), the growth associated protein GAP-43, the immediate early gene c-jun and, in particular, the cell recognition molecules L1 and close homologue of L1 (CHL1). Segments of peripheral nerve were autografted into the striatum, thalamus or cerebellum and expression of these molecules was investigated by non-radioactive in situ hybridization with cRNA probes to detect mRNAs, and by immunocytochemistry to detect the proteins. In some cases these techniques were combined with retrograde labelling from the distal end of the graft to identify regenerating neurons. In all 3 sites CHL1 mRNA was upregulated in neurons which regenerated axons into the grafts. In the striatum, the neurons which displayed CHL1 mRNA upregulation included aspiny cholinergic interneurons and these neurons also upregulated c-jun mRNA, trk A as well as p75 expression. After graft implantation into the thalamus, CHL1 mRNA was upregulated almost exclusively in neurons of the thalamic reticular nucleus which were also identified as regenerating neurons by retrograde labelling. In the cerebellum, CHL1 mRNA was upregulated by regenerating neurons of the deep cerebellar nuclei, some of which also expressed L1, c-jun, and GAP-43 mRNAs, but not by non-regenerating neurons of the cerebellar cortex. However, some of Purkinje cells close to the graft expressed c-jun mRNA. These findings suggest that regenerating CNS neurons increase their expression of a group of molecules, including CHL1, which may play an important role in the regrowth of their axons.
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LIST OF ABBREVIATIONS

ABC – avidin biotin complex
AChE – acetyl cholinesterase
AP – alkaline phosphatase
BCIP – 5-bromo-4-chloro-3-indolyl-phosphate
BDNF – brain-derived neurotrophic factor
bFGF – basic fibroblast growth factor
BSA – bovine serum albumin
CAM – cell adhesion molecule
ChAT – choline acetyltransferase
CHL1 – Close Homologue to L1
CNS – central nervous system
CNTF – ciliary neurotrophic factor
DAB – 3,3’-diaminobenzidine tetrahydrochloride
DEPC – diethyl-pyrocarbonate
DIG – digoxigenin
DN – deep cerebellar nuclei
dpo – day(s) postoperative
DRG – dorsal root ganglia
DTT – dithiotreitol
EDTA – ethylenediaminetetra acetic acid
ELISA – enzyme linked immunoassay
EM – electron microscope/y
FGF – fibroblast growth factor
FITC – fluorescein isothiocyanate
GFAP – glial fibrillary acidic protein
HBSS – Hanks Balanced Salt Solution
HCl – hydrochloric acid
HRP – horseradish peroxidase
Ig – immunoglobulin
JZ – junctional zone
LM – light microscope/y
NADPH – nicotine adenyl dinucleotide phosphate (reduced)
NBT – nitroblue tetrazolium
N-CAM – neural cell adhesion molecule
NGBS – normal goat blocking serum
NGF – nerve growth factor
NHBS – normal horse blocking serum
NT-3 – neurotrophin-3
NT-4/5 – neurotrophin-4/5
NT-6 – neurotrophin-6
PB – phosphate buffer
PBS – phosphate buffered saline
PBZ – parenchymal border zone
PLP – paraformadehyde-lysine-sodium periodate
PN – peripheral nerve graft
PNS – peripheral nervous system
PRSS – post reaction and storage solution
RGC – retinal ganglion cell
RT – room temperature
SNpc – substantia nigra pars compacta
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SNpr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>TRN</td>
<td>thalamic reticular nucleus</td>
</tr>
<tr>
<td>wpo</td>
<td>week(s) postoperative</td>
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CHAPTER 1

Introduction

The poor regenerative capacity of the central nervous system (CNS) of adult mammals, in contrast to that of the peripheral nervous system (PNS), has long been recognized (Cajal, 1928), but the mechanisms underlying this phenomenon still remain highly speculative. For many years it was believed that neurons of the mammalian CNS were simply intrinsically unable to regenerate due to some unidentified genetic restriction (Barron, 1983). However, this view has been changed following the success of experiments in which CNS axons were shown to regenerate into peripheral nerve (PN) grafts (e.g., Aguayo, 1985, Berry et al., 1986; Bray et al., 1987a,b; Vidal-Sanz et al., 1987, 1988). These studies have suggested that CNS regeneration is not limited by an innate inability of CNS neurons to extend their processes, but rather reflects differences in the extraneuronal cellular and molecular environments of the CNS and PNS. Injured CNS neurons can exhibit morphological plasticity (e.g. sprouting), provided appropriate environment and trophic substances for axon growth are granted. For example, axotomized CNS neurons can regenerate axons into transplants which provide trophic factors, such as fetal brain tissues (e.g., Kromer et al., 1981; Itoh and Tessler, 1990), PN grafts (e.g., David and Aguayo, 1981; Aguayo, 1985; Bray et al., 1987b; Hagg et al., 1990b; Campbell et al., 1991; 1992; Morrow et al., 1993; Vaudano et al., 1995, 1998; for review, see Anderson et al., 1998) or a combination of embryonic/PN grafts (e.g., Houle and Ziegler, 1994; Houle and Ye, 1997; Cui et al., 1999; Lin et al., 1999). Nevertheless, not all CNS neurons are capable of regeneration in response to such permissive environments. For example, in the thalamus, only axotomized neurons of the thalamic reticular nucleus (TRN) vigorously regenerate axons into PN grafts, whereas such a response is not displayed by neurons of other thalamic nuclei (Campbell et al., 1991; Morrow et al., 1993; Vaudano et al., 1995). Purkinje cells in the cerebellum are another type of CNS neuron whose axons regenerate poorly even in the presence of growth promoting factors (Vaudano et al., 1993a; Bravin et al., 1997; Rossi et al., 1997). This suggests that the ability of CNS
neurons to regenerate axons may depend on the intrinsic regenerative ability of individual neuronal populations. Recent studies have shown that after injury, non-regenerating and regenerating CNS neurons express different patterns of growth-associated molecules, such as GAP-43, L1 and c-jun (Campbell et al., 1992; Vaudano et al., 1993a,b; 1995; 1998; Zhang et al., 1995; Woolhead et al., 1998; for review, see Anderson et al., 1998). This implies that the failure of particular types of CNS neuron to regenerate axons after injury results from an intrinsic inability to express growth-related molecules, which are essential (necessary) to promote axon growth in those neurons. It would, therefore, be very helpful in term of possible future clinical therapy for the CNS injuries, if it would be established which molecules need to be expressed to bring about axon regeneration in CNS neurons. The present study endeavours to correlate the expression of the growth-related molecules, L1, CHL1, c-jun and GAP-43 to the regenerative capacity of different populations of CNS neurons. The results are presented in the following Chapters. This Chapter briefly reviews some background on regeneration of mammalian CNS neurons and on the functions of the growth-related molecules examined in this study.

1. Regeneration in the mammalian CNS

The responses of neurons of the CNS and PNS to axotomy differ in a number of ways; these differences can be observed both in cell body responses to injury and in the extent of regeneration that occurs in each system. The responses of the cell body to injury of PNS axons involve the upregulation of genes that are not upregulated following comparable injuries to CNS neurons. The expression of particular genes following injury may be essential for regeneration to occur (reviewed by Fawcett and Keynes, 1990). In mammalian CNS neurons, axotomy close to their cell bodies leads to an earlier onset and greater extent of cell death than distal axotomy (Lieberman, 1974; Aguayo et al., 1991). Neurons axotomized proximally, however, are far more effective in regenerating their axons through a favourable growth environment, such as a grafted segment of a peripheral nerve, than those cut distally. On the other hand, CNS neurons lesioned far from their cell bodies rarely regenerate even with access to supportive environments, but simultaneously they are somehow protected from cell death.
Seemingly, it is a paradoxical matter; neurons have to be axotomized very close to their cell bodies if they are to regenerate their axons, but first of all, they must survive from such severe injury. Furthermore, unlike the PNS, the CNS lacks Schwann cells, non-neuronal cells actively involved in nerve repair (see Section 2.1B). After injury in the PNS, Schwann cells in the distal stump of a cut nerve rapidly proliferate, dedifferentiate and modify the synthesis of cell surface molecules and also become active to produce trophic factors essential for axon growth (e.g. Heumann et al., 1987a,b; Lindholm et al., 1987; Funokoshi et al., 1993; and see Fawcett and Keynes, 1990). Moreover, Schwann cells constitute a mechanical guidance channel (band of Bühner) for regenerating axons to follow towards their targets. In contrast, mature oligodendrocytes in the CNS do not produce growth factors or form band of Bühner after the axons they support are injured. Instead, they produce several inhibitory factors which impede axon regeneration (see below). In addition, the failure of CNS axons to regenerate has been attributed to the rapid formation of scar tissue and inhibitory molecules produced at the injury site, and a lack of trophic support (i.e., from neurotrophic or other growth-promoting factors) for the axotomized neurons. Nevertheless, the poor regenerative ability of CNS neurons is not explicable solely in terms of a non-permissive microenvironment, but also relates to the intrinsic differences in the growth potential of each type of neuron (Fawcett, 1992; Anderson and Lieberman, 1999).

Basically, the failure of axonal regeneration in the CNS involves the factors mentioned above. In the following sections, I will review some factors which stop or impede axon regeneration in the adult mammalian CNS, i.e., glial scars and inhibitory factors produced by CNS glia. In addition, the growth-promoting roles of neurotrophic factors, whose expression is markedly downregulated in adult CNS, will be considered.

1.1) Astrocytes and glial scars

Among the several factors which thought to lead to the failure of axon regeneration in the adult CNS is the non-permissive glial environment. Traumatic injury to the adult mammalian CNS results in a rapid gliosis involving reactive astrocytes, leading to glial scar formation that seals the wound site but blocks axonal regeneration in the process (reviewed by Reier and Houle, 1988). Astrocytes respond
to injury in the CNS in three principal ways; 1) by participating in the phagocytosis and removal of cellular debris, 2) by filling in large areas of extracellular space left by the degeneration of neuronal and glial elements and 3) by reconstituting a glial limiting membrane along aspects of the CNS that have become exposed to non-CNS environments (Berry, 1979; Reier et al., 1983; Reier and Houle, 1988). The glial scar is mainly composed of proliferated and hypertrophied astrocytes and also often contains other non-neuronal cells and elements such as oligodendrocytes, microglia, macrophages, meningeal cells and blood vessels (Reier et al., 1983, 1989).

Over the years, the glial scar has been proposed to be either a physical barrier blocking the advancement of the growth cone or a source of chemical factors actively inhibiting axon outgrowth (Cajal, 1928; Liuzzi and Lasek, 1987; Reier et al., 1988; Rudge and Silver, 1990). The most compelling evidence in favour of the glial barrier hypothesis has been obtained from studies of regeneration of injured dorsal root axons. It has been shown that regenerating dorsal root axons extend only as far as the PNS-CNS interface, the dorsal root entry zone (DREZ) where a dome of astrocytic processes defines the CNS-PNS interface (Berthold and Carlstedt, 1977). The majority of regenerating dorsal root axons terminate as large end bulbs at the glial fringe of the DREZ or deflect back towards the dorsal root ganglion (DRG) (Carlstedt, 1985; Stensaas et al., 1987). Surprisingly, the results of many studies show that axon regeneration is not seen even when glial reactivity is minimal (Gilson and Stensaas, 1974; Guth et al., 1981). This leads to the question of whether reactive astrocytes in the adult CNS are intrinsically incapable of supporting axon outgrowth or whether other factors serve to block the regeneration of CNS axons.

Over a period of a half of a century large number of researchers have made attempts to investigate the inhibitory effect of glial scars on axon growth. Nevertheless, analysis of glial scars and their function in vivo is complicated, by the large number of cell types present in the scars and around the lesion. Therefore, the studies of glial scars as a non-permissive substrate for regenerating CNS axons have commonly been done in vitro and in most cases attention has been focused on the role of reactive astrocytes. In general, the results from in vitro studies indicate that astrocytes act as a non-permissive
substrate for neurite outgrowth and that this effect is age-related. For example, cultured mature astrocytes, derived from lesioned CNS or from postnatal ages, provide only poor substrates for neurite extension, whereas immature astrocytes support axon growth (Fallon, 1985; Rudge and Silver, 1990; Smith et al., 1990, 1993; McKeon et al., 1991; Bähr et al., 1995). However, it is surprising to find that mature astrocytes can, on the other hand, provide a permissive substrate for neurite outgrowth in vitro (Hatten et al., 1984; Noble et al., 1984; Ard and Bunge, 1988; Ard et al., 1991; Neugebauer et al., 1988; Baehr and Bunge, 1990). Fawcett and colleagues (1989) investigated this contradictory data by studying the behaviour of growing axons from adult and neonatal neurons in three-dimensional astrocyte cultures, the behaviour of which is assumed to replicate the behaviour of axons in vivo. They reported that although monolayers of astrocytes support axon growth, axons from adult retina and postnatal DRG neurons are unable to penetrate purified three-dimensional astrocyte cultures.

In vivo, the evidence that reactive astrocytes act as a permissive substrate for injured cholinergic neurons has been shown when a source of NGF is provided (Gage et al., 1988; Kawaja and Gage, 1991; Chalmers et al. 1996). Eagle et al. (1995) found that regenerating axons of axotomized septal neurons, extending across the NGF-producing graft (placed between the cut ends of septo-hippocampal pathway) into the hippocampus, could occasionally be seen running parallel to the course of specific GFAP-immunoreactive processes of astrocytes. These data suggest that other factors such as NGF may play roles in allowing neurites to cross the astrocytic 'barrier'. The findings that reactive astrocytes can secrete NGF in vitro (Lindsay, 1979) and in vivo (Lee et al., 1995, 1998; Lorez et al., 1989; Shigeno et al., 1991; Hashimoto et al., 1992), and, importantly, can express the low-affinity NGF receptor p75 (Gage et al., 1989; Lee et al., 1995), possibly explaining the positive effect of astrocytes on regenerating axons, particularly those of NGF-responsive neurons. Moreover, reactive astrocytes can also secrete the neural cell adhesion molecule N-CAM (Chalmers et al., 1996). This molecule may be involved in regeneration-supporting cell-cell contacts between astrocyte processes and regenerating axons.
Additionally, some cytokines released following CNS injury may change the substrate properties of astrocytes. A recent *in vitro* investigation on the effects of many cytokines demonstrated that interferon gamma and transforming growth factor beta inhibit the ability of astrocytes to support migration of oligodendrocyte precursors and neuritic outgrowth, whereas, a combination of interleukin-1 alpha and basic fibroblast growth factor (bFGF) greatly increased axon outgrowth over these cells. These cytokine effects do not correlate with expression on astrocytes of laminin, fibronectin, tenascin, chondroitin sulphate proteoglycan, N-cadherin, polysialic acid NCAM (NCAM-PSA), tissue plasminogen activator or urokinase (Fok-Seang, 1998).

Seemingly, in addition to astrocytes, meningeal cells, another component of glial scars, may play an important, or even dominant role in obstructing growing axons. *In vitro*, oligodendrocyte precursors migrate poorly on astrocytes and meningeal cells compared to migration on laminin even though both astrocytes and meningeal cells express cell surface laminin, suggesting that both astrocytes and meningeal cells retard migration of oligodendrocyte precursors (Fok-Seang *et al*., 1995). Moreover, recent evidence from Hirsch and Bähr (1999), shows that meningeal cells, but not astrocytes, secrete high amount of keratan sulfate which probably determines the non-permissiveness of these cells and the glial scar as a whole. In co-culture experiments of embryonic retina explants on top of mixed layers of adult astrocytes and meningeal cells, retinal axons showed extensive growth on the astrocytes, but stopped growing or avoided meningeal cells by growing around them on the astrocytes (Hirsch and Bähr, 1999).

1.2) Growth inhibitory molecules

A) Inhibitory factors from glial scars

Neonatal astrocytes have been shown to exhibit permissive substrate properties for regenerating axons (Fallon, 1985; Rudge and Silver, 1990; Smith *et al*., 1990, 1993; McKeon *et al*., 1991; Bähr *et al*., 1995). It is well established that these immature astrocytes secrete a large number of growth-promoting molecules which may contribute to the suitable substrate properties of these cells. These molecules include N-CAM, NCAM-PSA, L1, laminin, fibronectin, vimentin, N-cadherin, and a group of
proteoglycans, including DSD-1-proteoglycan and heparan sulphate (Liesi, 1985; Liesi 
et al., 1986; Ard and Bunge, 1988; Tomaselli et al., 1988; Smith et al., 1990; Alonso 
and Privat, 1993; Frisen et al., 1995; Hirsch and Bähr, 1999). Expression of some of 
these molecules is apparently downregulated as a consequence of astrocyte maturation 
(Smith et al., 1990, 1993). Non-permissive or mature astrocytes produce potential 
inhibitors such as tenascin-C and proteoglycans of the chondroitin sulphate, dermatan 
sulphate, keratan sulphate and NG-2-proteoglycan varieties, all of which can be 
inhibitory for axon growth, particularly in vitro (Snow et al., 1990, 1991; McKeon et 
al., 1991; Smith-Thomas et al., 1995; Fok-Seang et al., 1995; Hirsch and Bähr, 1999). 
These inhibitory molecules have been reported to be concentrated where axons fail to 
grow in vivo (Snow et al., 1990; Cole and McCabe, 1991). For example, chondroitin 
sulphate has been found associated with scar tissue around lesion areas in the cerebral 
cortex (McKeon et al., 1991), and this proteoglycan can inhibits neurite outgrowth in 
vitro (Snow et al., 1990, 1991). Furthermore, the extracellular matrix (ECM) molecule, 
tenascin-C, has been found associated with glial scars (McKeon et al., 1991; Ajemian 
et al., 1994), suggesting a possible role of this molecule in non-permissive properties of 
mature astrocytes. Recently, a study in evolution of multiple sclerosis, in which 
astrogliosis is a prominent feature, shows that reactive astrocytes found in chronic 
multiple sclerosis plaque express tenascin-C, which may result in glial scar formation 
impeding remyelination and axonal repair in multiple sclerosis lesions (Gutowski et al., 
1999).

Surprisingly, recent data reveal that both neonatal and reactive adult astrocytes 
exist a very similar expression of growth promoting molecules (NCAM, L1, laminin, 
fibronectin, DSD-1 proteoglycan) and potential inhibitors (tenascin-C, chondroitin 
sulfate, and NG-2-proteoglycan) in vitro. These data parallel previous findings of Fok-
Seang et al. (1995) who failed to detect obvious differences in protein expression 
between astrocytic cells lines with different substrate properties, and reported that less 
permissive cell lines produced more abundant ECM. In addition, in the investigation of 
Hirsch and Bähr (1999), adult astrocytes did not produce the axon growth inhibitor, 
keratan sulfate proteoglycan, whereas meningeal cells, a less permissive substrate than 
astrocytes, highly expressed this proteoglycan, minimal amounts of N-CAM and
significantly high amounts of fibronectin (forming an extensive fibronectin-containing ECM network). In view of these results, an excess production of ECM, found in meningeal cells and mature astrocytes, but not immature ones, may be responsible for the non-permissiveness of both cell types, probably by containing unidentified inhibitors of axon growth or by reducing the accessibility of membrane associated growth promoters like N-CAM (Hirsch and Bähr, 1999). This observation is in line with previous studies which showed that proteoglycan-rich ECM produced by reactive astrocytes is directly associated with the failure of axon regrowth in vitro (Fawcett, 1994) and in vivo (Davies et al., 1997, 1999).

B) Inhibitory factors from oligodendrocytes

Some unidentified factors, produced as a by-product following CNS myelin breakdown, are supposed to suppress axonal elongation by affecting growth cone structure or function, altering perikaryal protein synthesis or interfering with binding sites for essential growth factors on the neuronal membrane (Berry, 1982). This hypothesis is supported by ample evidence. For example, following chemical injury in adult mammalian CNS, non-myelinated or thinly myelinated axons are apparently able to regenerate (Bjorklund and Stenevi, 1979). Similarly non-myelinated axons in the pituitary stalk appear to regenerate and reinnervate their targets after lesion (Adams et al., 1971). Savio and Schwab (1991) reported that when oligodendrocyte development and myelin formation were prevented by x-irradiation of newborn rats, myelin-free cortico-spinal tract transected at 2 weeks of age showed re-elongation of axons over many millimeters within 2-3 weeks after the lesion, whereas in normally myelinated controls, regenerative sprouts grew less than 1.7 mm caudal to the lesion. In vitro, regenerating axons from DRG neurons derived from newborn rats avoided growing on oligodendrocyte cultures; the axons grew all around these cells, but did not grow over them, and axons grew normally until their growth cone came into direct contact with an oligodendrocyte, when they suddenly collapsed (Fawcett et al., 1989). In light of these data, oligodendrocytes are therefore inhibitory to axonal growth, and this may partially explain the failure of axons to regenerate in the mammalian CNS.

How do oligodendrocytes inhibit axon growth? Myelin-associated inhibitors on the surface of oligodendrocytes, in particular, have been proposed to play an important
role in this phenomenon (Schwab et al., 1993). The first evidence for such inhibitors was provided by Schwab and colleagues (Caroni and Schwab, 1988a,b; Schnell and Schwab, 1990). Caroni and Schwab (1988a) isolated two components of oligodendrocyte membrane protein with molecular weights of 35 and 250 kDa, namely NI-35 and NI-250, both of which have been shown to be highly inhibitory to neurite outgrowth (e.g., Caroni and Schwab, 1988a,b; Bandtlow et al., 1990; Schnell and Schwab, 1990). The two inhibitors are selectively expressed on the surface of oligodendrocytes and their product—CNS myelin, and they are responsible for a dramatic and long-lasting paralysis of neurite growth as soon as growth cones come into contact with oligodendrocyte processes, a process often followed by a collapse of the growth cone structure (Bandtlow et al., 1990). Neutralization of NI-35 and NI-250 through the specific monoclonal antibody IN-1, raised against both inhibitory proteins (Caroni and Schwab, 1988b), allows neurites to grow over myelin or cultured oligodendrocytes (Caroni and Schwab, 1988b; Bandtlow et al., 1990). In vivo, application of these antibodies to spinal cord-lesioned rats results in a long-distance regeneration of lesioned corticospinal tract fibers (Schnell and Schwab, 1990). Due to the observation that the growth cones stop growing and collapse once they come into contact with differentiated oligodendrocytes (Fawcett et al., 1989; Bandtlow et al., 1990), it has been assumed that the inhibition of neurite growth exerted by oligodendrocytes is a contact-mediated phenomenon that can be triggered by the tip of growth cone filopodia (Bandtlow et al., 1990).

Ample evidence suggests an inhibitory action of the two proteins NI-35/250 on axon regeneration (Cadelli et al., 1992; Schnell and Schwab, 1990; Schnell et al., 1993; Weibel et al., 1994; Bregman et al., 1995). For example, in young rats with lesions of the corticospinal tract and implanted with a source of monoclonal antibody IN-1, axotomized neurons in IN-1-treated animals regenerated axons for over 10 mm in 2-5 weeks, while no regenerating fibers were detected further than 1 mm caudal to the lesion in controls (Schnell and Schwab, 1990; Cadelli et al., 1992). A similar, highly significant enhancement of regeneration was also found for the cholinergic septo-hippocampal pathway and for the optic nerve under the same condition (Cadelli et al., 1992). Application of IN-1 antibody accompanying the application of BDNF or FGF,
significantly enhances the growth of regenerating retinal axons after intracranial freeze-crush lesion of the optic nerve, and a similar result is obtained in the animals with myelin- and oligodendrocyte-free optic nerves produced by local x-irradiation (Weibel et al., 1994).

However, in some circumstances, oligodendrocytes are not inhibitory to axon growth. An in vitro study of Ard et al. (1991) shows that neurites from embryonic rat retina grow on astrocytes in the presence of mature oligodendrocytes or among oligodendrocytes alone. Similarly, in a monolayer culture of large flat astrocytes with oligodendrocytes lineage cells on their surface, the extent of axon growth was unaffected by the presence or absence of oligodendrocytes (Fawcett et al., 1992). These results suggest that the presence of astrocytes probably alters the results of axonal contact with oligodendrocytes; in this case some factors secreted from astrocytes may suppress the inhibitory action of oligodendrocytes. Moreover, transplantation of embryonic tissue to the brain or spinal cord can induce axon elongation in some injured CNS neurons for a significantly long distance, and the axons also extend along the host white matter tracts (Wictorin et al., 1990; Li and Raisman, 1993). Seemingly, growth-promoting factors from embryonic tissue are able to overcome or repress inhibitory effects of oligodendrocytes and myelin sheath, leading to a greater extent of axon growth. Furthermore, there is evidence that the existence of NI-35 and NI-250 on myelin probably serves boundary and guidance functions for growing fibers in the postnatal period (Schwab and Schnell, 1991). Schwab and Schnell (1991) examined the development of the corticospinal tract (CST, which grows down the spinal cord during the first 10 postnatal days) in rats whose oligodendrocytes have been eliminated by x-irradiation of the littermates, or by application of antibody IN-1; significant anatomical aberration of the path of CST fibers was observed in these animals, such as the larger cross-section of the tract, and the aberrant CST fibers and fascicles intermixed with the neighbouring sensory ascending tracts.

Myelin-associated glycoprotein (MAG) on the oligodendrocyte membrane is another candidate inhibitor for neurite growth. In mature myelin internodes, MAG localizes primarily on the periaxonal membrane (Sternberger et al., 1979). MAG, a
member of the Ig superfamily, is a highly glycosylated protein constituting about 1% of all CNS myelin proteins (Trapp, 1990) and containing five immunoglobulin-like domains (Arquint et al., 1987, see also Walsh and Doherty, 1997). Because of the latter characteristic, it was proposed to play an adhesive role in myelination (Lai et al., 1987). However, many studies indicate that MAG is an inhibitor of neurite growth in vitro (Mukhopadhyay et al., 1994; McKerracher et al., 1994; David et al., 1995; Li et al., 1996; Shen et al., 1998; Shibata et al., 1998). It modulates growth cone behaviour and causes growth cone collapse (Li et al., 1996). Also, it seems likely that this glycoprotein is more inhibitory in regions of damage and myelin breakdown than in undamaged white matter; as evidence, there was no significant difference in neurite growth on myelin purified from MAG-/- and MAG+/+ mice, whereas a major inhibitory peak was seen when fractionated myelin from MAG+/+ mice was used (David et al., 1995; Li et al., 1996). Furthermore, a recent functional assay study shows that MAG is part of signalling pathway of tenascin-R for cell repulsion; when MAG is coated as a uniform substrate, it is inhibitory for neurite outgrowth of hippocampal and cerebellar neurons in vitro, whereas tenascin-R enhanced neurite outgrowth, and when tenascin-R is added to MAG, the neurite outgrowth-inhibitory effects of MAG is neutralized (Yang et al., 1999).

In contrast to a substantial number of in vitro studies, up to now, there have been only a few in vivo studies providing clear evidence of the role of MAG on axon growth and regeneration in adult CNS (for review, see Bartsch, 1996). Mostly, in vivo, the investigation of inhibitory action of MAG on axon elongation has been carried out in MAG null mice and in a greater extent, in vitro by using myelin extracted from MAG null and wild type mice. However, the results from these studies are rather ambiguous. For example, myelin proteins derived from MAG-deficient mice inhibit neurite outgrowth to a similar degree to the wild-type CNS myelin (Ng, et al., 1996). This is in line with the in vitro result reported by previous studies (David et al., 1995; Li et al., 1996). Moreover, a very small number of regenerating axons passing through the lesion areas were observed following thoracic lesions of the corticospinal tracts in MAG-/- mice (Li et al., 1996). The failure to detect differences in axon behaviour between MAG null and wild type mice may reflect a contribution to axon growth
inhibition by CNS inhibitory myelin molecules other than MAG, such as NI-35/250, which are likely to exert strong inhibitory effects on the growth of neurites in those animals. However, the evidence from a recent study demonstrated that the soluble form of dMAG (extracellular domain only) released in abundance from myelin as found in vivo, and chimeric MAG-Fc, can potently inhibit axonal regeneration, suggesting that soluble dMAG detected in vivo may lead to the lack of regeneration in the mammalian CNS after injury (Tang et al., 1997).

MAG is also present in peripheral nerves (Trapp, 1990). This is surprising due to the robust regenerative capacity of peripheral nerves. However, there is evidence that myelin purified from peripheral nerve also has neurite growth inhibitory activity and such activity can be masked by laminin, which is a constituent of the Schwann cell basal lamina. When laminin, which is largely absent from the normal adult mammalian CNS, is added to purified CNS myelin, it overrides the neurite growth inhibitory activity in CNS myelin (David et al., 1995). In agreement, a recent in vitro study reported that MAG presenting on PNS myelin is inhibitory for axonal regeneration and also for neurite branching (Shen et al., 1998). Torigoe and Lundborg (1998) reported that MAG-containing cells appear at 6 to 12 days after axotomy in the PNS. The presence of MAG on the surface of a segment of cut nerve can eliminate abundant sprouts and immature regenerating axons and this inhibitory effect is completely blocked by local application of antibodies to MAG (Torigoe and Lundborg, 1998).

Interestingly, MAG acts as a bifunctional molecule: in addition to inhibiting axon growth in adult, it is also able to promote axon elongation of neonatal neurons. For example, when myelin from MAG+/+ mice is used as a substrate, it promotes axon elongation for neonatal DRG neurons, but inhibits axon growth in older DRG and all other postnatal neurons (Shen et al., 1998). The evidence from MAG knockout mice reveals that MAG is not essential for the initiation of myelination; however, it plays an important role in maintaining a stable interaction between axons and myelin (Filbin, 1995; for review, see Bartsch, 1996). Mukhopadhyay et al. (1994) showed that there is a developmental switch from MAG-mediated adhesion to MAG-mediated inhibition with neuronal maturation.
1.3) Lack of permissive neurotrophic factors

Neurotrophic factors are endogenous soluble proteins regulating survival, growth, morphological plasticity, or synthesis of proteins for differentiated functions of neurons (according to the definition proposed by Hefti et al., 1993). It is now generally believed that neurotrophic factors regulate nervous system development, adult nervous system plasticity, and maintenance of structural integrity. There are a large number of proteins or factors which are considered as neurotrophic factors, including the neurotrophin family (NGF, BDNF, NT-3, NT-4/5 and NT-6), the epidermal growth factor family (e.g., EGF, TGFα, SDGF), the fibroblast growth factor family (e.g., aFGF, bFGF, FGF-5, FGF-6, KGF, INT-2, HST/KGF), the insulin-like family (e.g., IGF-1, IGF-2, Relaxin), Ciliary neurotrophic factor (CNTF), Platelet derived growth factor (PDGF), Glial derived neurotrophic factor (GDNF), transforming growth factor beta (TGFβ), Cholinergic neuronal differentiation factor (CDF).

This review will concentrate on the neurotrophins, and to a lesser extent, CNTF, which have particular influence on injured CNS mammalian neurons and regeneration of their axons. I will focus attention, in particular, on the roles of NGF and its receptors which have biological effects on cholinergic interneurons of the striatum, one of the CNS neuronal types studied in this research (see Chapters 3-5).

A) The neurotrophin family

The term ‘neurotrophin’ refers to a family of proteins that has common structural features. Although the first family member, nerve growth factor (NGF), was identified four decades ago, only in the past ten years has evidence been provided for the existence of additional neurotrophins. In addition to NGF, brain-derived neurotrophic factor (BDNF) of which 50% of amino acid sequence is identical to NGF, neurotrophin-3 (NT-3), neurotrophin 4/5 (NT-4/5), and neurotrophin-6 (NT-6) were subsequently isolated (Barde et al., 1982; Leibrock et al., 1989; Maisonpierre et al., 1990; Hallböök et al., 1991; Ip et al., 1992; Götz et al., 1994). NGF, BDNF, NT-3 and NT-4 have a wide range of effects in the development and regeneration of neural circuits. For example, in the visual system of vertebrates, they can increase the survival of retinal ganglion cells (RGCs) after axotomy or ischemia, promote the regeneration of RGCs and also rescue photoreceptors from degeneration (reviewed by von Bartheld,
NT-6, is the most recently identified neurotrophin (Götz et al., 1994), but whether or not NT-6 exists in mammals remains unclear.

Compared to other growth factor families, neurotrophins are of special interest to neurobiologists because they exert their biological actions primarily on cells of the nervous system. Neurotrophins are retrogradely transported by CNS neurons (DiStefano et al., 1992), although there is evidence that BDNF and NT-3 are also transported anterogradely by central and peripheral neurons (von Bartheld et al., 1996; Altar and DiStefano, 1998; Fawcett et al., 1998). They initiate biological actions by binding to cell surface receptors, followed by receptor mediated internalisation, increased cellular tyrosine phosphorylation, retrograde transport and the activation of numerous effector molecules, such as protein kinase, phospholipases and transcriptional activators (reviewed by Lewin and Barde, 1996). Importantly, these proteins can promote the survival of CNS neurons both in vitro and in vivo (for reviews, see Korsching, 1993; Lewin and Barde, 1996).

a) NGF

Almost 50 years ago NGF was first identified in two sarcoma tissues by Rita Levi-Montalcini and Vikor Hamberger (1951). NGF has a molecular weight of about 13,000 and is composed of three subunits alpha, beta and gamma (Varon et al., 1967a,b). The beta subunit is biologically active and promotes neurite outgrowth and cell survival (Greene et al., 1971). This subunit consists of two identical monomers, each of which has 118 amino acids, with six cysteines and three disulphide bridges. The two monomers of NGF form a covalent bound dimer with considerable similarity to insulin-like growth factors (Angeletti and Bradshaw, 1971).

NGF was the first neurotrophic factor demonstrated to be required for normal development. It has been shown to be essential for the survival and maintenance of developing sensory and sympathetic peripheral neurons (Levi-Montalcini, 1987). Anti-NGF antibodies injected into newborn rodents specifically destroy the peripheral sympathetic nervous system (Cohen, 1960), while prenatal exposure to NGF antibodies also causes the death of sensory neurons of spinal ganglia (Johnson et al., 1983).
postnatal day 7 (P7) rats, whose carotid arteries were ligated, intraventricular injection of NGF could protect against damage to both the striatum and cortex caused by hypoxia-ishemia (Holzman et al., 1996). Some PNS neurons such as sympathetic neurons much reduce their responsiveness to NGF once ontogenic development is finished (Angeletti et al., 1971), but they regain their NGF sensitivity and dependence during regeneration after axotomy (Bjerre et al., 1974). In contrast, DRG neurons retain their sensitivity to NGF through adulthood (Johnson et al., 1986), although NGF is no longer essential for their survival (Lindsay, 1988). In adult CNS neurons NGF also plays critical role in promoting axon growth. Houle and Ziegler (1994) showed that NGF-treated nitrocellulose implants in combination with fetal spinal cord tissue has the capacity to promote the extensive regrowth of ascending sensory axons across a traumatic spinal cord injury site, by comparison with animals implanted with the untreated nitrocellulose.

Many pieces of evidence demonstrate that NGF is also a trophic factor for adult CNS neurons, and is specifically involved in the maintenance and regeneration of cholinergic neurons of the embryonic (Alderson et al., 1989; Barde, 1989; Knusel et al., 1990) and adult rat basal forebrain (e.g., Hefti et al., 1990; Varon et al., 1989; Hagg et al., 1991; Wilcox et al., 1995), the type of neurons known as NGF-responsive neurons. High levels of NGF protein and its mRNA are found in the hippocampus and neocortex, the innervation territories of these cholinergic neurons (Korshing et al., 1985; Whittemore and Seiger, 1987). A substantial number of studies demonstrate that chronic supply of NGF can protect not only the phenotype but also prevent cell death effectively in lesioned central cholinergic neurons (e.g., Hagg et al., 1988,1989,1990a; Gage et al., 1988; 1990; Wilcox et al., 1995); however, the effects are significantly reduced if the treatment is delayed (Hagg et al., 1988, 1989, Vahlsing et al., 1991). For example, NGF administration into the lateral ventricle, staring immediately after a fimbria-fornix transection, can prevent the disappearance of axotomized medial septum cholinergic neurons, which are detected by their markers, acetylcholinesterase (AChE), choline acetyltranferase (ChAT), and low affinity NGF receptor (p75) (Hefti et al., 1986; Williams et al., 1986; Kromer, 1987; Gage et al., 1988, 1989; Hagg et al., 1988, 1989). Moreover, NGF can stimulate the production of both ChAT and p75 in
cholinergic neurons of adult rats, not subjected to experimental lesions; NGF treatments also increase the level of ChAT activity (Fusco et al., 1989) and expression of p75 mRNA (Higgins et al., 1989; Holtzman et al., 1992; Gibbs and Pfaff, 1994; Lee et al., 1995) in the hippocampus and septum respectively. After being damaged, cholinergic interneurons in the striatum which had lost their receptor, p75, during postnatal development resumed their NGF responsiveness by reexpressing this receptor (Gage et al., 1989).

NGF can be shown to induce axon growth or sprouting of axotomized adult rat cholinergic neurons and to promote their regeneration into nerve grafts (Hagg et al., 1990a,b, 1991, 1993; Eagle et al., 1995). Hagg et al. (1991) demonstrated that NGF pre-soaked acellular PN grafts, implanted between the cut ends of the transected cholinergic pathway between the septum and hippocampal formation, could promote vigorous regeneration of cholinergic neurons. Additionally, NGF at high concentration has a neurotropic effect, attracting axon sprouts to grow towards it (Hagg et al., 1992, 1993; Eagle et al., 1995; Förander et al., 1996). A good example has been shown in animals implanted with sciatic nerve grafts between the cut ends of the severed septo-hippocampal pathway, and concurrently infused with NGF into the fornix, proximal to the lesion and graft; cholinergic sprouting was oriented towards the infused fornix, whereas no fibers entered the nerve bridge (Hagg et al., 1993).

Normally, NGF is not expressed to a significant extent by non-neuronal cells of adult peripheral nerve. However, this situation changes dramatically after nerve lesion. A rapid, transient, and massive increase in NGF mRNA and protein occurs in the nerve region immediately proximal to the lesion and along the distal nerve segment (Varon et al., 1981; Lindholm et al., 1987; Heumann et al., 1987a,b). It has been suggested that denervated Schwann cells, in the distal segment of the cut nerve, synthesize this neurotrophin (Taniuchi et al., 1987; Liu et al., 1995, Carter et al., 1996). Concurrently, those Schwann cells also upregulate p75 (Heumann et al., 1987a,b; Taniuchi et al., 1987; Liu et al., 1995, Carter et al., 1996). Schwann cells in the distal segment shows prolonged period of NGF and p75 expression, suggesting that NGF produced by non-neuronal cells may be bound and stored by the p75 in Schwann cells in the distal nerve
segment prior to arrival of the regenerating axons (Liu et al., 1995). Interestingly, NGF seems to participate in Schwann cell migration; pre-treatment of denervated nerve sections by NGF enhances the rate of Schwann cell migration and this NGF response depends on p75, since antibodies to NGF or to p75 abolish this response (Anton et al., 1994).

Upregulation of NGF can be induced by macrophages in response to inflammation; interleukin-1 released by these cells is supposed to be the principal mediator of this inductive effect (Heumann et al., 1987a,b; Lindholm et al., 1987; Gulati, 1998). NGF in turn has been shown to enhance macrophage function and inflammatory response, suggesting that NGF levels and inflammation in injured tissue play an important role in determining the success of transplants and in tissue repair (Gulati, 1998). Moreover, after ischemia or nervous system inflammation, there are several pieces of evidence that reactive astrocytes can secrete NGF in vitro (Lindsay et al., 1979) and in vivo (Lorez et al., 1989; Shigeno et al., 1991; Hashimoto et al., 1992; Lee et al., 1995, 1998).

Generally, NGF is produced by targets of NGF-dependent neurons in limited quantities and promotes neuron survival through retrograde transport and signalling to the cell body (Thoenen et al., 1988; Altar and DiStefano, 1998). This paradigm is confirmed by the evidence that the interruption of retrograde axonal transport has the same effects as the neutralization of endogenous NGF by anti-NGF antibodies (Thoenen et al., 1988) and by the retrograde flow of immunoreactive endogenous NGF accumulating at a site distal, but not proximal, to a ligature or lesion of PNS and CNS neurons (Curtis and DiStefano, 1994). NGF acts on responsive neurons by binding to specific NGF receptors, p75 and trkA (see below), on axonal terminals, after which a critical biochemical signal is retrogradely transported, to the cell body (for review, see Chao and Hempsted, 1995). Carter et al. (1996) reported that in the absence of trkA, NGF binding to p75 activated the transcription factor nuclear factor kappa B (NF-kappa B) in Schwann cells isolated from rat sciatic nerve. Previous studies on Schwann cells and PC12 cells have demonstrated that NGF can upregulate the adhesion glycoprotein L1 in the absence of trkA activation (Seilheimer and Schachner, 1987; Itoh et al.,
These molecules may be involved in the cascade of NGF induced events in neurons.

**b) Other neurotrophins**

While the expression of NGF in the adult CNS is mostly confined in the target regions of basal forebrain cholinergic neurons (Korsching et al., 1985, 1986; Whittemore et al., 1986; Ernfors et al., 1990), BDNF seems to be broadly distributed throughout the CNS; it is apparently a major neurotrophin expressed in several areas of the adult brain (Ernfors et al., 1990; Phillips et al., 1990; Wetmore et al., 1990). Expression of BDNF is also found during development (Maisonpierre et al., 1990) and in the postnatal brain (Friedman et al., 1991). In contrast, NT-3 is the most narrowly distributed, with high levels of expression restricted to hippocampus CA2 stratum pyramidale, the dentate gyrus granule cells, and cerebellar granule cells (Ernfors et al., 1990; Maisonpierre et al., 1990), and dopaminergic neurons of the midbrain such as substantia nigra pars compacta (SNpc), ventral tegmental area and mesencephalic regions (Seroogy and Gall, 1993; Seroogy et al., 1994). The expression of NT-4/5 in the CNS seems to be very low (Timmusk et al., 1993). NT-6, a new member of the neurotrophin family, recently cloned from teleost fish *Xiphophorus*, is restricted to non-mammalian species, and its distribution remains largely unknown (Götz et al., 1994).

In the PNS, all neurotrophins are upregulated by Schwann cells in the distal part of the nerve after injury. Transection of the sciatic nerve leads to a very much larger increase in BDNF mRNA, than NGF mRNA. However, the time-course and spatial pattern of BDNF mRNA expression are distinctly different; while NGF mRNA is rapidly increased in non-neuronal cells of the damaged nerve, the slow increase of BDNF mRNA starts after 3 days post-lesion and reaches a maximum 3-4 weeks later (Meyer et al., 1992). NT-3, on the other hand, decreases shortly after the transection but returns to control levels 2 weeks later, while at this time, a marked increase in NT-4 is seen in the distal segment of the sciatic nerve (Funakoshi et al., 1993).

As reviewed earlier, a number of studies have shown that NGF maintains the survival of only some neuronal types, in particular central cholinergic neurons,
suggested that other population of neurons which do not respond to NGF can be supported by other neurotrophins and the response of distinct neuronal populations to each neurotrophin is also different. For example, survival and neurite outgrowth of placodally derived sensory neurons of the nodose ganglion are supported by BDNF and NT-3, but not NGF (Lindsay et al., 1985; Maisonpierre et al., 1990). In particular, BDNF, NT-3 and NT4/5, but not NGF, have been shown to exert trophic and protective effects on dopaminergic neurons both in vivo and in vitro (Knüsel et al., 1991; Hyman et al., 1991; Hagg, 1998). Interestingly, BDNF, NT-3 and NT4/5 show markedly different potencies to prevent the death of axotomized nigrostriatal dopaminergic neurons of adult rats, i.e., NT-4 > BDNF > NT-3 and while BDNF and NT-4/5 are predominantly involved in facilitating cell survival, NT-3 is more involved in regulating neurotransmitter function of these neurons (Hagg, 1998). Moreover, infusion of recombinant human BDNF or NT-4/5 into the vicinity of the axotomized rubrospinal neurons fully prevents atrophy of the neurons and stimulates the expression of GAP-43 and T-alpha1-tubulin mRNA and maintains the level of trkB expression, whereas, NGF, or NT-3 treatment has no such effects on these neurons (Kobayashi et al., 1997).

BDNF supports the survival of a much wider spectrum of neuronal types, including nodose ganglion sensory neurons in the PNS, spinal motoneurons, basal forebrain cholinergic neurons, RGCs, and mesencephalic dopaminergic neurons in the CNS in culture (Furukawa, 1993). The application of BDNF to chick embryos in ovo prevents death of RCGs at early developmental stages, whereas exogenously applied NGF and NT-3 have no such effect (Frade et al., 1997). There is evidence that BDNF supports the survival of developing and injured RGCs and enhances neurite outgrowth of retinal neurons in vitro (Johnson et al., 1986; Rodriguez-Tébar et al., 1989; Frade et al., 1997). However, treatment of BDNF alone, following intraorbital axotomy is unable to induce the program of changes in growth-associated tubulins that accompany regeneration of RGC axons of adult rats when a PN graft is implanted (Fournier and McKerracher, 1997), suggesting that in addition to BDNF, cooperative signalling with other substrate molecules may be required to allow RGCs to regenerate.
The evidence indicating the role of BDNF and NT-3 in promoting axon regeneration in CNS neurons is also substantial. Xu et al. (1995a) demonstrated that BDNF and NT-3 infusion significantly promoted axonal regeneration of specific distant populations of brain stem neurons into grafts (Schwann cells in semipermeable guidance channels) implanted at the mid-thoracic level in adult rat spinal cord. Infusion of BDNF promotes long-term survival of the lesioned motor neurons and induced abundant motor axon regeneration from the ventral avulsion zone along the spinal cord surface towards the BDNF source, whereas neither a PN graft nor a combination of PN graft with embryonic spinal cord transplant could prevent the retrograde degeneration of such neurons (Novikova et al., 1997). NGF, NT-3 and BDNF can promote intraspinal regeneration of axotomized ascending primary sensory fibers in the adult rat to grow across a predegenerated peripheral nerve graft and back into the thoracic spinal cord, although with BDNF, fewer fibers reach farther distances into the cord (Oudega and Hagg, 1999). Moreover, a recent study showed that transplants of fibroblasts genetically engineered to produce BDNF promote rubrospinal tract regeneration in adult rats and behavioural tests demonstrate that recipients of BDNF-producing fibroblasts show significant recovery of forelimb usage (Liu et al., 1999).

Although the evidence from in vitro and in vivo studies is clear, the role of BDNF, NT-3 and NT-4/5 on cell survival and even on axon regeneration in mature neurons, and the survival promoting effect of these neurotrophins on developing CNS neurons remain controversial. In particular, the results from the 'knockout' paradigm present many interpretative difficulties (Johnson and Oppenheim, 1994). The CNS of BDNF and NT-3 null (-/-) animals appear largely normal (for review, see Snider, 1994). While much evidence demonstrates the effect of BDNF on the survival of RGCs of wild type animals (Johnson et al., 1986; Rodriguez-Tébar et al., 1989; Thanos et al., 1989; Frade et al., 1997), there is no gross defects in the neural retina has been noted in BDNF -/- animals (Jones et al., 1994), and so far, no changes have been reported in the retina or in the optic nerve of NT-3 -/- mice. However, the result of a recent study found abnormal development of the cerebellum in BDNF -/-mice (Schwartz et al., 1997).
B) CNTF

CNTF is very abundant in the PNS, but much less so in the CNS (Henderson et al., 1994; Richardson, 1994). Like the neurotrophins, CNTF displays neurotrophic activity towards a wide range of central (Ip et al., 1991) and peripheral (Ye and Houle, 1997) neurons. In addition, CNTF has also been shown to have effects on cells other than neurons such as oligodendrocyte/astrocyte progenitor cells (Hughes et al., 1988), oligodendrocytes (Barres et al., 1993). There is strong evidence showing that reactive astrocytes can produce CNTF both in vitro (Rudge et al., 1995) and in vivo (Asada et al., 1995).

The role of CNTF as an effective promoter of neuronal survival and axon regeneration is supported by a large number of studies. For example, CNTF infused into the lateral ventricle can prevent degeneration and atrophy of almost all injured neurons of the medial septum (whereas NGF protects only the cholinergic ones) (Hagg et al., 1992). In agreement, a CNTF-like component in gelfoam extracts collected from injury sites in the striatum, has neuroprotective and neurite-promoting activity for different types of neurons in vitro (Asada et al., 1996). The effect of CNTF on axon regeneration seems to be very powerful compared to other neurotrophic factors. For example, following injury of the cervical region of the spinal cord, CNTF given 4 or 8 weeks after lesion can promote axon regeneration of the brainstem neurons into PN grafts in a comparable degree, whereas under the same condition, bFGF can promote axon regeneration in these neurons, but the number of regenerating neurons is much decreased if exposure to bFGF is later than 4 weeks after lesion (Houle and Ye, 1997).

CNTF, like BDNF and NT-3, can promote axon regeneration in injured CNS neurons after cervical spinal cord hemisection lesions, although each neurotrophic factor induces axon regeneration in different neuronal groups (Ye and Houle, 1997). CNTF, but not bFGF and neurotrophins such as NGF, BDNF, NT-3 and NT-4/5, substantially enhanced the regeneration of damaged axons of RGCs of hamster into sciatic nerve grafts (Cui et al., 1999). A recent in vitro study showed that CNTF has specific effects on axon outgrowth, and this effect is dissociable from its effects on cell
survival, whereas BDNF stimulated RGC survival nearly as well as CNTF, but had only minor effects on outgrowth (Jo et al., 1999).

C) Neurotrophin receptors

Neurotrophins mediate signal transduction through the Trk family of tyrosine protein kinases, whose members serve as high affinity neurotrophin receptors. NGF, BDNF, NT-3 and NT-4/5 selectively bind to distinct members of the Trk family of tyrosine kinase receptors; TrkA, TrkB and TrkC. While NGF signals through TrkA, BDNF and NT-4/5 interact with TrkB. However, a recent study has shown that BDNF may play a role in axon regeneration independent of TrkB; axons of DRG neurons which regenerate into PN graft, express BDNF, but not trkB (Cameron, 1997). NT-3 appears to be more promiscuous and binds to each of the Trk receptors, but its primary biological responses are mediated by TrkC (Ip et al., 1993; for reviews, see Barbacid, 1994; 1995). In addition, each of these neurotrophins binds to p75 with similar low affinity dissociation, a receptor structurally unrelated to the Trks; however, kinetic studies of neurotrophin-p75 binding indicates that in the rates of ligand dissociation; NGF > NT-3 >> BDNF (Chao, 1994). The biological significance of neurotrophin binding to p75 in cells that express Trk receptors has been difficult to ascertain (Rodriguez-Tebar et al., 1990; Hallböök et al., 1991; Barbacid, 1994, 1995; Chao, 1994). At the present time, whether NT-6 binds to any of these receptors is not known. In this review, I will focus on TrkA and p75 whose interactions with NGF have a critical impact both on cell survival and cell death, particularly of central cholinergic neurons.

a) p75 and TrkA

The neurotrophin receptor p75, a 75 kDa size molecule, is a member of a family of cell surface molecules which serve as receptors for tumor necrosis factor and related ligands (reviewed by Chao, 1994). NGF (including BDNF and NT-3) interacts with p75 with low affinity (Radeke and Feinstein, 1991; Welcher et al., 1991; for review, see Chao, 1994; Chao and Hempstead, 1995). However, the specific receptor of NGF is TrkA (also known as Trk, p140Trk or gp140Trk), the cell surface receptor which provides a high affinity binding site for NGF. NGF binds to TrkA in a manner of 'slow-on/slow-off' kinetics \(K_d \sim 10^{-11} \text{M}\), compared to 'fast-on/fast-off' kinetics \(K_d \sim 10^{-9} \text{M}\) with
the p75 receptor (Meakin and Shooter, 1991; Weskamp and Reichardt, 1991; Bothwell, 1995, 1996; Chao and Hempstead, 1995). Currrent views of the two neurotrophin receptors have therefore designated p75 as the low-affinity neurotrophin receptor and TrkA as the high-affinity NGF receptor (for reviews, see Barbacid, 1993, 1995; Chao, 1994). Chao (1994) proposed that TrkA and p75 should be considered together as subunits in neurotrophin function, since the majority of NGF responsive cells express both p75 and TrkA (Verge et al., 1992; Schechterson and Bothwell, 1992), in contrast to cells expressing TrkB or TrkC which may not necessarily express p75.

A widespread distribution of TrkA immunoreactivity is found throughout the adult rat CNS, predominantly in cholinergic neurons of the basal forebrain and the striatum, neuronal populations known to be NGF-sensitive (Holtzman et al., 1992; Merlio et al., 1992; Steininger et al., 1993; Sobreviela et al., 1994). In addition, trkA mRNA has been detected in two densely packed populations of non-cholinergic thalamic neurons (Venero and Hefti, 1993) as well as in magnocellular neurons of several brainstem nuclei (Merlio et al., 1992). Different from TrkA, the p75 receptors are much more widely expressed in the CNS and PNS; they have been identified in a wide variety of cells including neurons and non-neuronal cells, some of which do not respond to NGF nor to any of the other known members of the neurotrophin family (Emfors et al., 1988; Thomson et al., 1988; Yan and Johnson, 1989; Holtzman et al., 1992). The function of p75 in non-dependent neurotrophic cells is yet known. TrkA has mostly been found to coexpress with p75 in cholinergic neurons of basal forebrain, but not in cholinergic interneurons of the striatum (Gage et al., 1989; Springer et al., 1990; Sobreviela et al., 1994), whose p75 expression is lost during postnatal development (Martinez et al., 1985; Mobley et al., 1985; Johnson and Taniuchi, 1987).

Clearly, expression of p75, but not TrkA, in NGF-sensitive can be rapidly upregulated by NGF (Cavicchioli et al., 1989; Gage et al., 1989; Higgins et al., 1989; Figueiredo et al., 1995). For example, cholinergic interneurons of the striatum of adult rats, which normally do not express detectable level of p75, can reexpress this receptor when receiving chronic administration of NGF (Gage et al., 1989). Similar effects of NGF on p75 expression also occurs in the PNS (Miller et al., 1991). Apparently,
upregulation of p75 expression in non-neuronal cells is also induced in some circumstance such as ischemia or tissue damage. Following brain injury, expression of p75 is upregulated by astrocytes (Lee et al., 1995), which normally contain no detectable level of this neurotrophin receptor (DiStefano and Johnson, 1988; Kumar et al., 1990). In the PNS, p75 is not present on intact Schwann cells; however, the level of p75 is remarkably increased in these cells in the distal segment of the injured nerve (Taniuchi et al., 1986, 1988; Heumann et al., 1987a,b; Thoenen et al., 1987; Funakoshi et al., 1993) simultaneous to the increase of NGF in such cells (Heumann et al., 1987a,b; Lindholm et al., 1987; Taniuchi et al., 1988; Muir et al., 1989; Varon et al., 1981).

It has been suggested that p75 may facilitate NGF signalling through TrkA receptors (Barbacid, 1993; Chao, 1994), although little evidence presently supports an interaction between the two receptors. However, it is possible that the two receptors may lead to a greater sensitivity to neurotrophin factors (Hempstead et al., 1991). It has been shown that when p75 and TrkA are coexpressed in proper ratio, a high affinity site for NGF is observed (Mahadeo et al., 1994; Verdi et al., 1994; Twiss et al., 1998; for review, see Chao and Hempstead, 1995). Deletion mutations in p75 have been found to eliminate high-affinity binding (Hempstead et al., 1990). Furthermore, when both TrkA and p75 receptors are coexpressed, the rate of association of NGF is increased 25-fold to produce a higher affinity binding site. An increase in the rate of internalization was also observed, suggesting that the biological effects by NGF are derived from a novel kinetic binding site that requires the expression of both receptors. (Mahadeo et al., 1994). For example, PC-12 cells lacking TrkA do not respond to NGF in spite of containing abundant p75 receptors (Barbacid, 1995). A considerable amount of evidence showed that p75 collaborates with TrkA resulting in enhancement of TrkA phosphorylation and cellular responsiveness to low NGF concentrations (Barker and Shooter, 1994; Mahadeo et al., 1994; Verdi et al., 1994; Twiss et al., 1998).

In the absence of TrkA, p75 may generate intracellular signals which mediate apoptosis through its ligand, NGF. Such effects of p75 is obviously seen in non-neuronal cells which do not express TrkA, such as glioma cells, fibroblast,
oligodendrocytes and Schwann cells (Carter et al., 1996; Casaccia-Bonnefil et al., 1996; Frade et al., 1996). In these cells, binding of NGF to p75 activates the NF-kappaB transcription factor, c-jun amino-terminal kinase (JNK), spingomyelinase and ceramide (Dobrowsky et al., 1994, 1995; Carter et al., 1996; Casaccia-Bonnefil et al., 1996); the latter two factors have been shown to mediate death signal (Dobrowsky et al., 1998). During development, 25% of cholinergic basal forebrain neurons in normal mice, which express p75 but do not express trk A, die, between postnatal days 6 and 15. This loss does not occur in p75 -/- mice or in normal mice injected with p75 inhibiting peptide (Van der Zee et al., 1996). However, a controversial result from Fagan et al. (1997) showed that in trk A knock out mice, basal forebrain cholinergic neurons which express p75, although smaller than normal, remained alive. In addition to NGF, BDNF may be another ligand candidate for p75 to mediated cell death. A recent study showed that BDNF mediates activation of p75 signalling leading to naturally occurring neuronal death of sympathetic neurons, whereas in p75-/- mice or BDNF-/- mice, the normal period of sympathetic neuron death does not occur (Bamji et al., 1998).

In contrast, there are many reports indicating that expression of p75 can mediate signal tranduction in apoptotic pathways in both PNS and CNS neurons if this receptor is not bound by NGF (Rabizadeh et. al. 1993; Barrett and Barrett, 1994; Rabizadeh et al., 1994; Cheema et al., 1996; Bunone et al., 1997) and NGF binding appears to block this apoptotic activity, perhaps contributing to the well known effect of NGF on neuronal survival. For example, reducing the levels of p75 with antisense p75 oligonucleotides can prevent the death of sensory neurons in vitro (Cheema et al., 1996). Rabizadeh et al. (1994) demonstrated that the expression of p75 by wild type and mutant PC12 cells potentiated cell death induced by β-amyloid peptide. NGF binding to p75 inhibited the toxicity of β-amyloid peptide, whereas NGF binding to trkA enhanced it (Rabizadeh et al., 1994). NGF binding to p75 is both necessary and sufficient for the abrogation of apoptosis in neuroblastoma cells treated with antimitotic agents (Cortazzo et al., 1996). Moreover, in the absence of NGF, p75 can induce neuronal death by inhibiting ligand-independent Trk A autophosphorylation, that is, prevent the intrinsic survival-promoting signalling of Trk A (Greene and Kaplan, 1995, Barrett and Georgiou, 1996).
2. Peripheral nerve grafts and regeneration in the CNS

It is now widely accepted that although regeneration of adult CNS axons hardly takes place following injury, they are able to regrow over long distances when a permissive PNS environment is offered (e.g., David and Aguayo, 1981; Benfey and Aguayo, 1982). This indicates that the failure of axon regeneration in CNS neurons is not the result of an intrinsic inability to mount a successful regeneration of CNS neurons, as it was assumed for most of this century. Instead, it is chiefly as a consequence of non-permissive environment and inhibitory factors present in the CNS per se (see above). Thus, sustained regenerative elongation of CNS axons is possible in adult mammals if the non-neuronal environment of damaged axons is changed or modified. Based on this knowledge, various methods have been developed in animals to encourage CNS axons to regenerate by providing essential factors to create suitable environment for regeneration. One experimental strategy that has been used to change the CNS from a non-permissive to permissive one consists of grafting a segment of peripheral nerve into the CNS tissue.

2.1) PN grafts: the possibility to enhance regeneration in the CNS

It was in the early of this century that Ramón y Cajal (1928) and Tello (cited in Cajal’s paper, 1928) first introduced the idea of using a PN graft to bridge the lesion and to stimulate axonal growth from injured CNS neurons. Tello, Leoz and Arcuate were the first to demonstrate the regeneration of optic axons into sciatic nerve segments grafted to the cut end of an optic nerve (cited by Cajal, 1928). Their intriguing result has stimulated the idea that axotomized CNS neurons can regenerate axons provided auxiliary factors are supplied to the CNS environment. However, it was never possible to determine the source of the axons within the grafts and only fifty years later were such experiments repeated and extended with methods that could reveal the cell bodies of neurons with axons in the graft. The pioneering experiments were conducted in the laboratories of Aguayo in Montreal and Berry in London in the early 1980’s (e.g., Aguayo et al., 1981; Berry et al., 1986). They showed that injured CNS axons could regenerate into segments of peripheral nerve implanted into the brain, spinal cord or severed optic nerves. Also, by using a retrograde labelling technique with tracers, i.e.,
horseradish peroxidase (HRP) and/or fluorescent dyes, they could confirm that the regenerating axons found inside PN grafts unequivocally originated from axotomized CNS neurons (e.g., Richardson et al., 1980; David and Aguayo, 1981, 1985; Benfey and Aguayo, 1982; Berry et al., 1986; Vidal-Sanz et al., 1987). Since then a substantial number of experiments have taken advantage of these techniques to study the nature of CNS regeneration. The examples of the most successful experiments, showing that axotomized CNS neurons are capable of promoting axons through PN grafts and in particular re-innervating their CNS targets, are best documented in the visual system. For example, PN grafts bridged between the retinal stump of the optic nerve and the superior colliculus, can promote axotomized RGCs to regenerate axons for approximately 350μm (Vidal-Sanz et al. 1987, 1991; Villegas-Pérez et al. 1988; Carter et al., 1989) and these regenerating axons become ensheathed by CNS myelin in the superior colliculus where their terminals form asymmetric synapses with dendrites of neurons in the layers, in which retinal axons normally terminate (Vidal-Sanz et al., 1991; Carter et al., 1994). Ultrastrutural examination of the terminal fields of these regenerated RGC axons indicates that they form well-differentiated synapses in the superior collicullus and the structure of the regenerated RGC terminals, the type of synaptic contacts formed, the ratios of contacts to terminal perimeter, and the domains of the postsynaptic neurons contacted were all normal (Carter et al., 1989; also see Thanos, 1997). Thanos et al. (1992) reported that adult rats regain the pupillary light reflex if severed axons of the optic nerve are guided by PN grafts into the pretectal area. Moreover, if the distal end of the graft is implanted near the lateral geniculate nucleus, the restoration of pattern of vision is observed (Thanos et al., 1997).

In other area of the CNS, examples of complete regeneration in CNS neurons are also available. Salame and Dum (1985) showed that when an autologous nerve graft is bridged between the medulla and the ipsilateral cervical spinal cord in adult rats, CNS axons elongating within PN grafts are able to conduct action potentials and maintain functional synapses on CNS neurons. A PN graft, linked between the interrupted cholinergic pathway of septo-hippocampus, could induce AChE-positive fibers from the septal area entering the hippocampal formation and normal innervation
pattern and fiber density in the most rostral 1.5 mm of the dorsal hippocampal formation were observed by 6 months postlesion (Hagg et al., 1990b).

2.2) **How can PN grafts support regeneration in CNS neurons?**

As mentioned above, the transplantation of PN graft into the CNS tissue is an effective way to promote axon elongation in injured central neurons and in some cases, functional repair of the injured CNS is also achieved. When a fresh PN graft is inserted into the host brain or spinal cord, several changes occur as followed: a) axons and myelin within the PNS graft degenerate rapidly; b) Schwann cells divide and phagocytose axon and myelin debris; 3) Schwann cells remain aligned in columns of Büngner which extend along the length of the graft; 4) each column is surrounded by a continuous tube of basal lamina; and finally 5) there is growth of new axons along the graft (Campbell et al., 1992; Lu and Richardson, 1993; for review, see Anderson et al., 1998).

A) Interaction between PN grafts and CNS tissue: the responses of astrocytes

Until now, there have been relatively few detailed morphological studies of the responses of injured CNS axons to the presence of a PN graft. However, those available studies have provided rather clear explanation of the interaction between PN grafts and CNS host tissue. As with any injury to the CNS, implantation of a PN graft makes a large wound in the CNS parenchyma and as a consequence, stimulates responses from non-neuronal cells, particularly reactive astrocytes (see section 1.1). As early as 5 days post-grafting, accumulation of reactive astrocytes around the brain/graft interface and a layer of glia limitans are formed (Campbell et al., 1992). Ultrastructural examination shows that this layer consists of many astocytic processes and an incomplete basal lamina surrounding the graft. The formation of the glia limitans is progressive and continues until an almost continuous layer is formed, which separates the parenchyma border zone and the junctional zone of the graft (see definitions in Chapter 2, section 8.1), by 14 days and finally a complex layer is formed by 2 month (Campbell et al., 1992). The layer of glia limitans encloses the junctional zone, which contains fibroblasts, macrophages, oligodendrocyte processes, mixed bundles of astrocyte and Schwann cell processes, and non-myelinated axons associated with.
Schwann cell processes (Campbell et al., 1992). A recent EM examination of the CNS host-PN nerve interface in the rat spinal cord shows that a distinct scar formed by multiple layers of astrocyte processes is observed by 20 days after PN graft implantation and it completely envelops the transplant and confined Schwann cells and fibroblasts to the area enclosed by the scar (Sims et al., 1999). However, when glial cells are depleted by x-irradiation, there is no a scar formed; as a consequence, some Schwann cells can apparently migrate away from the transplant (Sims et al., 1999).

The view that astrocytic processes guide axons into the grafts, is still a matter of controversy (for review, see Anderson et al., 1998). Campbell et al. (1992) reported that at the earliest stages, the axon sprouts, whether single or in a small cluster, are often devoid of contact with glia cells. However, at later stages (14 days –2 months) astrocytic processes are present in the graft, at the periphery area and in some cases these processes appear to contain some axons (Campbell et al., 1992). Weinberg and Raine (1980), in their ultrastructural study of regeneration into PN graft implanted into the midbrain, described the presence of astrocytes as ‘bridges’ to support axons growing into PN grafts. However, subsequent studies show that the astrocytes processes follow rather than lead the axons into the grafts, probably acting to stabilize than establish the connections between the brain and the graft (Berry et al., 1988; Hall and Berry, 1989; Campbell et al., 1992). The finding that reactive astrocytes express N-CAM (Chalmers et al., 1996), may contribute to cell-cell interaction between astrocytic processes and the shaft of axons. Nevertheless, a strong argument emerges from a recent study of Sims et al. (1999). Their ultrastructural investigation of glia scar formation following the implantation of a segment of a sciatic nerve into the spinal cord, showed that the terminals from axons that appeared to have traversed the transplant during the period of astrocytic scar formation ended blindly in the scar (Sims et al., 1999).

Although structural and molecular changes, particularly in the graft, following implantation of PN grafts into the CNS seem to be similar to those changes in the distal stump of peripheral nerves in situ (see above), there is evidence to show that some differences between the responses of the distal stumps and PN grafts in the CNS still
exist. For example, preliminary observations from this laboratory indicate that the removal of axonal and myelin debris takes place more slowly than the same processes occur in the PNS after axotomy. Vaudano et al. (1996) found that Schwann cells in the proximal part of the grafts inserted into the thalamus express very low or undetectable levels of c-jun, whereas the expression of this molecule is massively upregulated by Schwann cells in the distal PN stump after axotomy. These differences are probably influenced by the CNS environment, which may provide some factors modifying the nature of non-neuronal cells in responding injury, especially of Schwann cells, within the PN graft.

B) Schwann cells: tool for promoting CNS regeneration

Schwann cells and their proliferation after injury seem to be the most important factor promoting axon regeneration in the PNS. Generally, regenerating axons of peripheral nerves are nearly always found in contact with Schwann cells or other peripheral glia (Anderson and Turmaine, 1986, 1987; Hall, 1986). There is evidence that axons from severed peripheral nerves have difficulties in emerging from the proximal stump if the proliferation of Schwann cells is inhibited (Hall, 1986). Severed axons can regenerate only for a short distance into freeze-thawed (acellular) grafts, in which contain non-viable Schwann cells (Nadim et al., 1990). These data undoubtedly indicate that Schwann cells are a key element in promoting axonal regeneration in the PNS.

As is the case in the PNS, Schwann cells can be considered as the principal factor to promote regeneration of damaged CNS axons into PN grafts. The role of Schwann cells in PN grafts is not only to form Schwann cell columns (bands of Büngner) for supporting axon elongation, but more importantly, to induce axon sprouting from injured CNS neurons and to support the growth of the sprouts over long distances (Anderson et al., 1998). For example, in a study comparing the effects of living PN grafts and acellular grafts (in which Schwann cells were previously frozen-killed) by implantation of one such graft into either side of the diencephalon of adult hamsters, numerous regenerating axons were found in the living grafts, but not in the freeze-killed grafts, although both types of graft contained basal lamina tubes (Smith
and Stevenson, 1988). This result supports the crucial role of Schwann cells in promoting axon regeneration and indicates that Schwann cell-derived basal lamina tubes, when isolated from their parent cells, are insufficient to initiate or sustain CNS axonal regeneration.

Many studies have demonstrated the growth-promoting role of Schwann cells by using other experiment models. For example, Schwann cell-seeded guidance channels transplanted between the proximal and distal stumps of the transected adult rat spinal cord promote a number of axons to regenerate along the channels (Paino et al., 1994; Xu et al., 1995b). Kromer and Combrooks (1985) demonstrated that septal axons traversing the fimbria-fornix bundle that normally show little regenerative capacity, regenerated very well and reached the target hippocampus within 14 days, if a Schwann cell graft supported on a collagen substratum was implanted between the cut ends of the fimbria-fornix bundle. These axons did not regrow, however, through acellular grafts containing only collagen (Kromer and Combrooks, 1985). Solid human Schwann cell grafts (formed by PAN/PVC guidance channels), placed between T9-T10 spinal cord of nude rats, significantly induced regenerating axons to enter the graft, although approximately 1% of the fibers that entered grafts, re-entered the host spinal cord (Guest et al., 1997). In keeping with these results, Xu et al. (1999) showed that a Schwann cell-containing mini-channel bridged between the two stumps of the hemisected rat spinal cord, stimulated many axons to grow into the graft, some of which became remyelinated, and these regenerating axons penetrated through the distal graft-host interface to re-enter the host environment. Moreover, terminal bouton-like structures were also observed in the grey matter, the destination of these regenerating axons (Xu et al., 1999).

Viable Schwann cells are suggested to substantially influence regeneration in both the PNS and CNS because they contribute both trophic and tropic factors to the injured neurons (Kromer and Combrooks, 1987; Bunge, 1993, 1994; Anderson et al., 1998). Studies in the past decade have shown that Schwann cells produce a remarkable number of neurotrophic factors as well as expressing a variety of cell adhesion molecules, known to influence neurite growth, on their surface. Among these are the
neurotrophins: NGF (e.g., Lindholm et al., 1987; Heumann et al., 1987a,b; Muir et al., 1989; Liu et al., 1995), BDNF (Acheson et al., 1991; Meyer et al., 1992), CNTF (Friedman et al., 1992; Rande et al., 1992), the cell adhesion molecules: L1 and N-CAM, and ECM components such as tenascin and laminin (Kromer and Cornbrooks, 1985; Seilheimer and Schachner, 1988; for reviews, see Bunge, 1993 and Martini, 1994). A good example demonstrating that trophic factors secreted from Schwann cells play a crucial role in promoting neurite growth, derives from the study of Hagg and colleagues (1991). They showed that debris-free acellular peripheral nerve segments treated before implantation with purified beta-NGF, placed between the disconnected septum and the hippocampal formation, promoted nearly as many regenerating cholinergic axons as did fresh cellular nerve grafts. Furthermore, the tropic effect of Schwann cells has been demonstrated by experiments in which cografts of neonatal rat Schwann cells and dissociated fetal tectal cells were injected into the midbrain of newborn rats. Schwann cells in such cografts altered the characteristic pattern of host retinal growth into a tectal graft, by inducing axons to grow away from appropriate target areas (in the midbrain) (Harvey and Plant, 1995; Harvey et al., 1995). It has been suggested that Schwann cells are able to stimulate axons to grow into inappropriate (non-target) regions in the CNS, presumably by producing growth-promoting factors which mask or compete with signals released from the target neurons (Harvey and Plant, 1995; Anderson et al., 1998).

2.3) Differential abilities of CNS neurons in regenerating axons into a PN graft

The implantation of PN grafts into the CNS is a good strategy for inducing adult CNS axons to regenerate and this model also well illustrates the differences in regenerative ability among different CNS neuronal populations. For example, following implantation of PN grafts into the thalamus, the major population of axons growing along the graft originate from neurons of the TRN (Benfey et al., 1985; Morrow et al., 1993; Vaudano et al., 1995) and occasionally from neurons of the zona incerta and the posterior nucleus (Vaudano et al., 1995; Dr. G. Campbell—personal communication). Neurons in the cerebellum also exhibit differential axon regeneration into implanted PN grafts: neuron of the DN and the brainstem (which project axons to the cerebellum) are able to regenerate axons into the grafts (Dooley and Aguayo, 1982; Vaudano et al,
1993a,b), whereas Purkinje cells and other neurons of the cerebellar cortex fail to do so (Vaudano et al., 1993a,b, 1998). Woolhead et al. (1998) demonstrated that cholinergic interneurons of the striatum and neurons of the substantia nigra pars compacta are the only two types of CNS neuron which regenerate axons vigorously into PN grafts placed in the striatum. Interestingly, a recent result from Guest et al. (1997) shows that following the implantation of human Schwann cell grafts between T9-T10 of the nude rat spinal cord, several neuronal populations including propriospinal, sensory, motoneuronal, and brainstem neurons regenerate axons into the grafts, but only propriospinal and sensory neurons re-enter the host spinal cord.

It is still equivocal why some injured neurons fail to respond to the permissive environment created by PN grafts. It is possible to demonstrate that most neurons that regenerate their axons into the grafts are axotomized close to their cell bodies (Salame and Dum, 1985; Bray et al., 1987; Clatterbuck et al., 1998; see also Anderson and Lieberman, 1999). However, proximity of cell bodies to the grafts appears to be a prerequisite for a good regenerative response to the graft in only some CNS neurons, e.g., neurons of the olfactory bulb (Friedman and Aguayo, 1985), certain neurons of the spinal cord (Salame and Dum, 1985) neurons of the anterior thalamic nuclei (Clatterbuck et al., 1998), cholinergic interneurons of the striatum (Woolhead et al., 1998). In contrast, some CNS neurons, albeit situated far from the implantation site of the PN graft, apparently regenerate axons vigorously into grafts such as TRN neurons following grafts in the thalamus (Campbell et al., 1991; Morrow et al., 1993) and SNpc neurons after implantation of grafts in the striatum (Woolhead et al., 1998). It would therefore appear that differing degree competence of CNS neurons to regenerate axons into PN grafts may result from variable intrinsic regenerative abilities of these neurons rather than being determined exclusively by external factors such as the site of axotomy.

Recently, the ability of CNS neurons to regenerate axons into PN grafts has been correlated with the expression of some growth-related molecules by the injured neurons. For example, neurons of the TRN and DN, which regenerate axons very well along PN grafts upregulate high levels of expression of c-jun, GAP-43 (Vaudano et al., 48
1993a,b; 1995, 1998) and L1 (Zhang et al., 1995) following axotomy and such expression is maintained if a PN graft is implanted. Conversely, neurons which regenerate axons poorly such as Purkinje cells do not express these molecules under the same conditions (i.e., axotomy and implantation of PN grafts) (Vaudano et al., 1993a,b; 1998). Neurons of SNpc neurons express GAP-43, L1 constitutively and this neuronal population regenerates axons robustly into PN grafts, even though the site of axotomy is far from their cell bodies (Woolhead et al., 1998). In the light of these results, the intrinsic regenerative potential of CNS neurons may, in part, be associated with their ability to express the growth-promoting molecules in response to injury.

3. Growth-related molecules

3.1) Cell adhesion molecules

Diverse glycoproteins of cell surfaces operationally termed "cell adhesion (or recognition) molecules (CAMs)" are important in the specification of cell interactions during development, maintenance and regeneration of the nervous system. These adhesion molecules have distinct functions involving different cells at different developmental stages, but may cooperate when expressed together. These molecules are also involved in the molecular mechanisms of contact between cells and surrounding extracellular matrix components, and are important in plasticity of the nervous system. CAMs have been grouped into three classes: the cadherins, integrins, and immunoglobulin (Ig) superfamily (Schachner et al., 1990; Takeichi, 1990; Stappert and Kemler 1993) or into four classes which includes a another group of molecules; selectins (reviewed by Hynes and Lander, 1992). The Ig superfamily is the largest family of related molecules, with well over 100 members (Williams and Barclay, 1988). The members of this superfamily can be subdivided into at least nine groups of related molecules based on the number of Ig-like domains, fibronectin type III (FNIII)-like repeats and the mode of membrane attachment (for review, see Walsh and Doherty, 1997), for example, N-CAM, L1, TAG-1, MAG, DCC. These molecules play key roles in neuronal migration, neurite outgrowth promotion, neurite fasciculation, pathfinding, target recognition, synaptogenesis and myelination (for review, see Brummendorf and Rathjen, 1995). Mutations in these proteins may result in neurological disease
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(Uyemura et al., 1996). In this brief review, concentrate on L1 and CHL1 (a recently identified member of the L1 family) which were a major focus of the work reported in this thesis.

A) L1

Among the members of the Ig superfamily, the L1 family shows striking consistency in function and structure. Members of the L1 family are characterized by six Ig-like domains at the N-terminal end and at least four FNIII homologous repeats. These molecules can be either transmembrane glycoproteins or linked to the cell surface by a glycosylphosphatidyl inositol (GPI) anchor. The members of the L1 family are either strongly homophilic, i.e., self-binding partners, or predominantly heterophilic, i.e., they bind to non-self-binding partners, which often comprise other members of the Ig superfamily, integrins or extracellular matrix molecules (Holm et al., 1996; Hortsch, 1996).

L1 glycoprotein was purified from postnatal mouse brain by Rathjen and Schachner (1984). The molecular structure of L1 is homologous with NGF-induced large external glycoprotein NILE (Stallcup and Beasley, 1985); and very similar to glycoproteins isolated from chickens; neuro-glia cell adhesion molecule NgCAM (Grumet and Edelman, 1984, Grumet et al., 1984), NgCAM related molecules NrCAM/Brovo (Grumet et al., 1991), 8D9 (Lemmon and McLoon, 1986), and G4 (Rathjen et al., 1987). L1 has molecular weight range between 200-230 kDa. Its extracellular section consists of six immunoglobulin-like domains and five FNIII repeats (Rathjen and Schachner, 1984, Moo et al., 1988). Similar to other members in the family, L1 can bind other CAMs either homophilically or heterophilically (Hortsch, 1996; Hillenbrand et al., 1999).

L1 is involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites, cell migration, and neurite outgrowth on Schwann cells (Seilheimer and Schachner, 1988; Moo et al., 1988). Its expression also mediates axonal guidance and synaptogenesis during neural development, and mutations in its gene result in severe neurological defects (Gopinath et al., 1996; for review, see Hortsch, 1996). During the development of the PNS, L1, which shows a pattern of expression similar to N-CAM,
has been identified on the surface of axons, their growth cones and filopodia, and on Schwann cells (Martini and Schachner, 1991). For example, in sciatic nerves of newborn and early postnatal mice, L1 is expressed on fasciculating axons as well as on the Schwann cells that ensheath them (Mirsky et al., 1986; Martini and Schachner, 1986). Also, this molecule is expressed at both Schwann cell-Schwann cell and Schwann cell-axon contacts when axons and Schwann cells are already separated from more immature axonal bundles. L1 (and also N-CAM) is however, drastically downregulated when myelination starts, but remains present on non-myelinating Schwann cells and their associated axons, through to adulthood (reviewed by Martini, 1994). Similarly, CNS axons have been shown to downregulate L1 to undetectable levels when oligodendrocytes (which do not express L1) start to myelinate them (Bartsch et al., 1989).

L1 is abundant in the nervous system and is concentrated on axons. In vitro, L1 is a potent promoter of neurite outgrowth and elicits specific growth cone behaviour (Bixby et al., 1988; Potoltorak et al., 1997, for review, see Burden-Gulley et al., 1997). This molecule, like N-CAM, is also able to modulate the activity of protein tyrosine kinase pp60^{src} or phosphatases in axonal membranes; the presence of this molecule can induce downregulation of pp60^{src}-dependent tyrosine phosphorylation of α and β tubulin in growth cone membrane, which could in turn enhance tubulin polymerization and help to stabilize the cytoskeleton of elongating axons (Atashi et al., 1992). Moreover, in recent years it has become clear that nerve growth stimulated by L1 and other CAMs (e.g., N-CAM, N-cadherin) does not necessarily rely on the adhesion function of these molecules, but instead also involves the activation of second messenger cascades in neurons. A considerable amount of evidence has shown that L1 in neurons is capable of directing neurite outgrowth and neuronal FGF receptor function (FGFR) is required for this response; antibodies which bind to this receptor specifically inhibit neurite outgrowth stimulated by this adhesion molecule (Williams et al., 1994a,b; Doherty et al., 1995; Doherty and Walsh et al., 1996; Walsh et al., 1997; also reviewed by Walsh and Doherty, 1997). Harper et al. (1994) reported that calcium influx through L- and N-type calcium channels is also required for L1 to promote cell contact-dependent axonal growth.
In addition, expression of L1 is apparently regulated by NGF; antibodies against NGF abolish the effects of L1 expression (Seilheimer and Schachner, 1987; Schachner et al., 1990). Further investigation of such effects has been carried out recently by Itoh et al. (1995) who showed that when PC12 cells are exposed to NGF, L1 mRNA levels are increased fourfold. The levels of L1 mRNA are also increased in NGF-treated mutant PC12 cells (PC12nnr5) that lack the high-affinity NGF receptor. The effect of NGF on L1 mRNA is greatest in cells cultured at high density, but its effect on cells cultured at low density is augmented by antibody to L1 (to mimic L1 homophilic binding). Various extracellular matrix components had no differential effects on L1 mRNA levels in either the presence or absence of NGF. These findings suggest that NGF regulates L1 expression by a mechanism that is independent of the high-affinity NGF receptor and that this regulation is modulated by cell-cell contact but not by cell-extracellular matrix interactions.

Expression of L1 can be induced or enhanced in neurons following axotomy (e.g., Poltorak et al., 1993, 1997, Harper et al., 1994; Aubert et al., 1998). In addition, it is strongly upregulated by denervated Schwann cells (reviewed by Martini, 1994; Zhang et al., 1995). The upregulation of L1 expression in axotomized neurons has been correlated with axonal sprouting during self-repair and axon regeneration in the CNS (Kobayashi et al., 1995; Styren et al., 1995; Zhang et al., 1995; Dezawa and Nagano, 1996; Jung et al., 1997; Aubert et al., 1998; Schmidt and Schachner, 1998). For example, fibroblast L cells (genetically modified to express the L1 molecule) grafted to a lesion of rat spinal cord immediately after hemisection, strongly promote regeneration of the axons in the injured spinal cord 2 weeks after grafting (Kobayashi et al., 1995). Moreover, experiments in which PN grafts were implanted to promote axon regeneration in RGCs have clearly shown that L1 is expressed by regenerating neurons and its immunoreactivity is confined to the interface between axons and astrocytes and between axons and Schwann cells (Dezawa and Nagano, 1996; Jung et al., 1997). Interestingly, Jung et al. (1997) found that in addition to L1, regenerating RGCs also expresses GAP-43. Following the implantation of a PN graft into the thalamus, neurons of the TRN, which regenerate axons very well into PN grafts (Morrow et al., 1993), express high levels of L1, whereas other thalamic neurons, which show a lesser
regenerative propensity, fail to upregulate expression of this molecule (Zhang et al., 1995). These results suggest that the intrinsic ability of CNS neurons to express L1 after injury may be related to, and may even be necessary for successful regeneration in such neurons.

B) CHL1

CHL1, or close homologue of L1, has recently identified in mouse by Holm and his colleagues (1996). To date, very little is known about CHL1, in particular its function in neurons; only a few publications have appeared concerning the characteristics and possible roles of this molecule in vitro (Holm et al., 1996; Hillenbrand et al., 1999). The similarity in structural features between CHL1 and other members of the L1 family, however, suggests an important role for CHL1 as a cell recognition/adhesion molecule in developing and regenerating neurons.

The molecule, CHL1 structurally comprises an N-terminal signal sequence, six immunoglobulin (Ig)-like domains, 4.5 fibronectin type III (FN)-like repeats of which four are highly homologous to the FN-like repeated region of other L1 family members, a transmembrane domain and a C-terminal, most likely intracellular domain of about 100 amino acids. Other structural characteristics of CHL1 shared between members of the L1 family are high level of N-glycosidically linked carbohydrate, which include the HNK-1 carbohydrate structure, and a pattern of protein fragments comprising a major 185 kDa band and smaller fragment of 165 and 125 kDa. The partial FN-like repeats of CHL1 localize to the region of the molecule situated adjacent to the cell membrane, and is the most variable region in L1 family members (Holm et al., 1996).

CHL1, like other members of the L1 family, is predominantly expressed in the developing nervous system at later developmental stages. It is not detectable in the brain before embryonic day 15 (E15). CHL1 is expressed by the major cell types in the CNS and PNS (Holm et al., 1996; Hillenbrand et al., 1999; Zhang et al., submitted for publication). In the CNS, CHL1 and L1 show overlapping but distinct patterns of expression (Holm et al., 1996). For example, CHL1 and L1 mRNAs are expressed by ganglion cells of the retina of P7 mice. Additionally, L1 transcripts are detectable in amacrine and horizontal cells located in the inner nuclear layer whereas CHL1 mRNA
is occasionally present in only a few such cells. Glial cells in the optic nerve do not contain significant levels of L1 mRNA, but, in contrast, they express strong levels of CHL1 mRNA. Detectable levels of CHL1 expression are found in neurons of the thalamus of P7 mice but not after P21. In contrast, L1 is expressed both prenatally and throughout postnatal life in the thalamus of the mouse (Hillenbrand et al., 1999). In the cerebellar cortex of P14 mice, L1 mRNA is detected in the stellate and basket cells located in the molecular layer and in Golgi and granule cells located in the internal granular layer. The same cell types in the granule cell layer express CHL1 mRNA but not those in the molecular layer (Holm et al., 1996). Detectable levels of CHL1 and L1 mRNAs are not found in Purkinje cells (Holm et al., 1996). In other regions of the brain, the expression patterns of CHL1 and L1 are more similar. For example, in the cerebral cortex, pyramidal neurons in layer V express both CHL1 and L1 (Hillenbrand et al., 1999). Generally, more neurons express L1 than CHL1, although in some instances, e.g., the hippocampus, some neurons express CHL1 but not L1. Expression of CHL1 signal in many regions of the brain, such as the telencephalon and thalamus is downregulated during postnatal development (Hillenbrand et al., 1999).

The most striking differences between CHL1 expression and L1 expression, is that CHL1 is also expressed by certain CNS glial cells both in vitro and in vivo, whereas L1 is not expressed by CNS glia under normal condition. In vitro, most GFAP-positive astrocytes express CHL1. In vivo, the expression of CHL1 mRNA by astrocytes appears to be restricted to certain subpopulations, e.g., astrocytes in the lamina cribrosa of the optic nerve head (Holm et al., 1996); however after injury in the brain, CHL1 gene expression by GFAP-positive reactive astrocytes is common at the site of injury (see Chapters 4, 6, 7 and 8). Oligodendrocyte progenitor cells express CHL1 in vitro and downregulate CHL1 expression as they differentiate (Hillenbrand et al., 1999). In agreement, mature oligodendrocytes do not express CHL1 mRNA in vivo (Holm et al., 1996). In contrast to CHL1, the L1 family members are expressed by mature oligodendrocytes and not by progenitors (Moscoso and Sanes, 1995). In the PNS, immature and non-myelinating Schwann cells express both CHL1 (Hillenbrand et al., 1999) and L1 (Seilheimer and Schachner, 1987) in a similar pattern.
Although roles of CHL1 in the nervous system are not yet clear, the structural relatedness to L1 and members of the L1 family suggests that CHL1 is involved in cellular interactions and neurite outgrowth promotion. Hillenbrand et al.(1999) showed that CHL1 acts as an adhesion molecule (but not by homophilic binding as in the case of other members of the L1 family), in \textit{in vitro} experiments using CHL1-tranfected fibroblast cell line (L cells). The ligand for CHL1 interaction remains to be identified. However, the existence of a ligand for this molecule that evokes a cellular response is suggested by the ability of CHL1-tranfected cells to promote neurite outgrowth \textit{in vitro}. When hippocampal neurons of embryonic rats and small cerebellar neurons of early postnatal mice were maintained on a monolayer of CHL1-tranfected L cells, neurite outgrowth was induced and interestingly, this promotion effect was more pronounced on CHL1-tranfected cells more than L1-tranfected cells. The finding that CHL1 promotes neurite outgrowth in cultured small cerebellar neurons, which do not express detectable levels of CHL1, suggest that this process is mediated by heterophilic interactions of CHL1 (Hillenbrand et al., 1999).

Additionally, the recent results from our laboratory show upregulation of CHL1 mRNA expression by DRG neurons and motor neurons of the spinal cord following sciatic nerve crush in adult rats (Zhang et al., submitted for publication). The latter study also showed that upregulation of CHL1 mRNA expression in these injured neurons occurred in the same period when regenerating axons would be expected to grow towards their targets, and was eventually downregulated at the time at which contacts with peripheral targets would be expected to be re-established. The possibility that CHL1 expression is regulated by signal from peripheral targets is supported by evidence for prolonged upregulation of CHL1 in such neurons when the sciatic nerve was cut and ligated to prevent regenerating axons reconnecting with their targets. This evidence suggests a potential role of CHL1 in promoting axonal regeneration in PNS and CNS neurons \textit{in vivo}. However, the roles and functional mechanism of CHL1 interaction \textit{in vivo}, especially in CNS neurons of the brain where the expression of this molecule is rather widespread, remain to be elucidated.
3.3) Growth Associated Protein GAP-43

The growth-associated protein, which is now widely known as GAP-43, was discovered independently in several laboratories, which implicated it in a range of phenomena that include neuronal regeneration and development, growth cone motility, signal transduction, phospholipid metabolism, calmodulin-binding, and synaptic potentiation. Accordingly, in addition to GAP-43, a variety of names were termed including 48K4.8 (or GAP-48), B-50, F1, pp46, protein 4, and p57 (neuromodulin) (for review, see Benowitz and Routtenberg, 1997).

Within cells, GAP-43 is bound to the inner surface of the axonal membrane and concentrated in neuronal growth cones (Skene et al., 1986). Its promoter contains an activator protein 1 (AP-1) site (Eggen et al., 1994) that contributes to regulation of its expression (Waber and Skene, 1998). GAP-43 is a protein kinase C substrate and phosphorylation by protein kinase C is particularly significant, linked with both nerve-terminal sprouting and long-term potentiation (Jacobson et al., 1986; Routtenberg, 1986; Lin and Bazan, 1995). Interestingly, a recent study has shown that addition of soluble cell adhesion molecules (CAMs) to isolated growth cone preparations from mouse or rat brain leads to enhanced phosphorylation of the GAP-43 protein providing a link between the cell surface and the cytoskeleton (Walsh et al., 1997). GAP-43 appears to regulate Go, the major non-cytoskeletal protein, a member of G protein family that links receptors and second messengers, in the growth cone membrane (Strittmatter et al., 1990, 1991). In addition, this protein has been shown to preferentially bind calmodulin in the absence of calcium (Andreason et al., 1983).

GAP-43 is a neuron-specific phosphoprotein expressed by most neuron populations during processes of axon development and synaptogenesis. Developmental and regenerative axon growth is frequently accompanied by elevated synthesis of this protein and once it is synthesized in the cell body, it is rapidly transported to its active sites in the growth cone or nerve terminals (Benowitz et al., 1981; Jacobson et al., 1986; Skene et al., 1986). There is no evidence of the appearance of GAP-43 in growing dendrites or dendritic growth cones (Goslin et al., 1988). GAP-43 has now been found to be a major component of growth-cone membranes in developing rat...
brains. Relative to total protein at the growth cone, GAP-43 is approximately 12 times as abundant in growth-cone membranes as in synaptic membranes from adult brains (Skene et al., 1986). Although GAP-43 was originally thought to be a neuron-specific protein (Basi et al., 1987; Benowitz et al., 1988), there is evidence suggesting that GAP-43 is also synthesized by non-neuronal cells of the developing and adult nervous system (Vitkovic et al., 1988; Vitkovic and Mersel, 1989; Vitkovic, 1992; Woolf et al., 1992).

Many studies have provided evidence that GAP-43 plays an important role in axon growth and synaptogenesis, particularly during development. Overexpression of GAP-43 results in striking axonal sprouts and unusual axonal labyrinths composed of tightly packed whorls of neuronal membrane (Aigner et al., 1995; Holtmaat et al., 1997). Even in non-neuronal cells, overexpression of the GAP-43 gene has been found to induce extensive process outgrowth: such cells, which under normal conditions extend very few processes, give rise to many fine, long filopodial processes under these condition (Zuber et al., 1989). Conversely, suppression or blockage of GAP-43 expression has adverse effects on axon outgrowth (Aigner and Caroni, 1993; Shea et al., 1991; 1995).

It is possible that GAP-43 alone may not be essential for the development of the nervous system. A strain of mutant mice, lacking for the GAP-43 gene, is apparently able to develop a normal nervous system (Strittmatter et al., 1995). However, recent studies showed very clearly that GAP-43 expression is required to initiate correct pathfinding from the optic chiasma to form a normal optic tract (Kruger et al., 1998; Sretavan and Kruger, 1998). GAP-43 null mouse embryos in which the enlarged optic chiasma are developed subsequent to failure of the earliest RGCs axons to progress laterally through the chiasm-tract transition zone to form optic tract and are delayed temporarily in the midline region (Kruger et al., 1998; Sretavan and Kruger, 1998).

Most neurons cease to express high levels of GAP-43 after the completion of synaptogenesis (Jacobson et al., 1986); however, certain brain regions contain considerable amounts of the protein throughout life (Oestreich and Gispen, 1986;
Oestreicher et al., 1986; Yeo et al., 1993; Augood et al., 1995). In adult rat, GAP-43 mRNA is more abundant in the forebrain than in the lower brainstem. Weak to moderate hybridization signals are also widely expressed in thalamus, hypothalamus, and midbrain (Yeo et al., 1993). Most cholinergic neurons of the striatum, basal forebrain and lateral tegmental nucleus (most of which express choline acetyl transferase (ChAT)) are also enriched in GAP-43 (Augood et al., 1995). The expression of GAP-43 in restricted brain regions in the adult, is thought to be related to functional plasticity and structural remodelling (for reviews, see Skene, 1989; Benowitz et al., 1990; Benowitz and Routtemberg, 1997). In the hippocampal formation, in which GAP-43 continues to be abundant in the adult (Neve et al., 1988; Yeo et al., 1993), reactive synaptogenesis occurs following lesions of the perforant pathway (Cotman and Nieto-Sampedro, 1984).

A considerable number of studies indicate that many adult neuron populations upregulate GAP-43 after axotomy (e.g., Skene, 1989; 1992; Doster et al., 1991; Tetzlaff et al., 1991; 1994; Verhaagen et al., 1993; Schaden et al., 1994; Vaudano et al., 1995). Many pieces of evidence indicate that upregulation or reexpression of GAP-43 in mammalian neurons after injury is induced by deprivation of trophic factors derived from target cells. Examples in this regard have been obtained from both PNS and CNS neurons (Woolf et al., 1990; Schreyer and Skene, 1993; Chong et al., 1994a; Zagrebelsky et al., 1998). For example, after cutting or crushing the sciatic nerve in adult rats, GAP-43 immunoreactivity can be detected in axotomized DRG neurons and in central terminals of primary afferent neurons in the superficial laminae of the dorsal horn of the lumbar enlargement; blockade of axon transport in the sciatic nerve with vinblastine or capsaicin was found to produce a pattern of GAP-43 immunoreactivity in the dorsal horn identical to that found after a crush injury (Woolf et al., 1990). Similar evidence has been shown for Purkinje cells of the cerebellum; this cell type is highly resistant to axotomy and fails to express GAP-43 after axonal interruption, although strong upregulation of this protein can be induced by the injection of colchicine into the uninjured adult cerebellum (Zagrebelsky et al., 1998).
There is some controversy as to whether or not the expression of GAP-43 in adult injured neurons is dependent on the site of injury. Doster et al. (1991) made the observation that proximal axotomy to RGCs seems to encourage a stronger increase in GAP-43 expression in those neurons than distal axotomy. However, Jones and Aguayo (1991), could not detect the differences in the GAP-43 expression in RCGs following either proximal or distal optic nerve transection. In the PNS, sites of injury seem not to affect the expression of GAP-43 in axotomized neurons; the pattern of upregulation of GAP-43 immunoreactivity in DRG neurons following lesions at different distances, proximal, distal and far-distal injuries, along the sciatic nerve was similar (Liabotis and Schreyer, 1995).

In the brain, there is evidence that certain populations of adult neurons are able to upregulate their level of GAP-43 synthesis when regenerating axons into PN grafts. Campbell et al. (1991) demonstrated that 11-14 days following implantation of a segment of peroneal or tibial nerve into the thalamus, GAP-43 immunoreactivity was found in perikarya of neurons around the graft and also in the sprouts within the graft. Further investigations were carried out by Vaudano et al. (1995) who showed that, although mechanical injury was a sufficient stimulus to induce transient expression of GAP-43 mRNA in thalamic neurons, prolonged expression of this mRNA was only seen in the presence of the PN graft and mostly in neurons of the thalamic reticular nucleus (TRN) which regenerate axons vigorously into the graft. In addition, a direct correlation between TRN neurons expressing GAP-43 mRNA and TRN neurons could be established (Vaudano et al., 1995). A recent study from Elliott et al. (1997) showed that cortical neurons, a neuronal population with poor regenerative responses, did not upregulate GAP-43 mRNA above background levels following axotomy caused by a stab wound, even though the lesions constituted a very close axotomy.

Correlation between expression of GAP-43 and axon regeneration in RGCs in the presence of a PN graft has been shown by a considerable number of studies (e.g., Schaden et al., 1994; Ng, et al., 1995; Jung et al., 1997; Wouters et al., 1998). Wouter et al. (1998) demonstrated that all RGCs with axons growing into the grafts were positive for GAP-43; however, not all RGCs showing GAP-43 immunoreactivity were
extending axons into the grafts. Surprisingly, Chong et al. (1994b) found that high level of GAP-43 protein were still present in peripheral axons which had regenerated into freeze-killed PN grafts although they ceased to regenerate any further. In the view of these results, it can be deduced that expression of GAP-43 by itself is not sufficient to ensure regeneration of injured neurons and suggest that not only GAP-43, other factors may also incorporate with this protein to determine success in such regeneration in individual neurons. Jung et al. (1997) reported that regenerating axons of RGCs expressed both L1 and GAP-43 in grafted rats. Further investigation is needed to elucidate the combinations of GAP-43 and other factors which are essential to promote axon regeneration.

3.4) Immediate early gene: c-jun

Jun and other inducible transcription factors, including Fos, Krox, Myb, Myc, NGFI-C, and SRF belong to class of the immediate early genes, which have been so termed because they display rapid and transient induction following stimulation of cells with various physiologically relevant stimuli and in the absence of de novo protein synthesis (reviewed by Sheng and Greenberg, 1990; Morgan and Curran, 1990; 1991; Herdegen and Leah, 1998). Induction of immediate early genes is likely to be very important following brain injury and during subsequent regeneration because these genes regulate the expression of a variety of specific target genes (Morgan and Curran, 1991) which may include regeneration relevant genes.

Jun was first described as the oncogene of the avian sarcoma virus 17 (ASV 17), a retrovirus capable of causing fibrosarcoma in chicken (Cavalieri et al., 1985). ASV 17 also provided the name for the ‘jun’ oncogene (‘ju-nana’ is the Japanese expression for ‘number 17’). Shortly afterwards, the cellular counterpart, c-jun, was isolated from human cells (Bohmann et al., 1987). Other members of Jun family include JunB and JunD, which show different expression patterns primarily because of differences in their promoter regions (Herdegen and Leah, 1998). In this review, I focus only on c-jun.
The proto-oncogene c-jun is rapidly induced in cells stimulated with growth factors, phorbol ester, hormones, cytokines or neurotransmitters (Angel & Karin, 1991). It belongs to the leucine zipper family of transcription factors and was shown to be the major constituents of activator protein 1 (AP-1), the binding site for a transcription factor (Bohmann et al., 1987; Rauscher et al., 1988b). Its protein product (c-Jun) dimerizes both with itself and with another leucine-zipper-containing molecule or other members of the Fos and Jun families to form the transcriptionally active AP-1 complex, which binds to specific sequences on DNA to modulate gene expression (Bohmann et al., 1987; Halazonetis et al., 1988; Sheng and Greenberg, 1990; Morgan and Curran, 1991). Apparently, Fos/Jun dimers, the most transcriptionally active complex, bind to the AP-1 sequence with 50-fold greater efficiency than Jun/Jun dimers in vitro (Halazonetis et al., 1988). It is likely that the particular sets of hetero- or homodimers formed within any cell depend on the intracellular concentration of each protein (Morgan and Curran, 1991).

During development, the level of c-jun expression is higher than present in adult brain (Pennypacker et al., 1993). Johnson and colleagues (1993) provided clear evidence that expression of c-jun during development is not required for cellular proliferation and differentiation up to mid-gestation; instead, it may required for survival past that stage as well as for the mitogenic response of the embryonic cells. The role of c-jun in the developing nervous system has also been reported to include the promotion of apoptotic cell death (Ferrer, 1996a,b, 1997). Overexpression of c-jun results in neuronal death, and suppression or antagonization of c-Jun prevents neuronal death in neonatal hippocampal neurons (Schlingenseipen et al., 1993. 1994). Nevertheless, c-jun is not essential to promote cell death; a study on c-jun null embryos showed that programmed cell death could take place in embryos lacking Jun (Roffler et al., 1996).

In adult CNS, expression of c-jun is widely distributed in many areas, but expression in the spinal cord and brainstem seems to be more extensive than in the forebrain (Herdegen et al., 1992,1993c; Harlan and Garcia, 1995; Herdegen et al., 1995). Surprisingly, the level of c-jun mRNA expression found in each brain area is
apparently less than the level of its protein (Harlan and Garcia, 1995). Potential
differences between these results based on immunocytochemistry and in situ
hybridization may involve differences in sensitivity of the two techniques or differences
in the stability of the protein vs the mRNA.

Upregulation of c-jun in adult neurons can be induced by various stimuli such as
seizures, cellular stress, or UV irradiation (Herdegen et al., 1993c, 1997a; Grass et al.,
1992, 1993, 1997; MacGibbon et al., 1995; William and Jope, 1994). It is also well
established that expression of c-jun in neurons of the PNS and CNS can be induced by
axotomy (e.g., Herdegen et al., 1991; 1992; 1993a,b; Jenkins et al., 1993a,b,c; Leah et
al., 1993; Brecht et al., 1994; De Felipe and Hunt, 1994; Broude et al., 1999; De Felipe
and Belmonte, 1999). Ostensibly, when axotomy provokes very little functional
response in the injured neurons, it also fails to induced c-jun. For example, after
transection of the thoracic spinal cord, distally axotomized rubrospinal neurons neither
die nor regenerate axons through the supportive environment of a peripheral nerve
graft, and they do not express c-Jun (Jenkins et al., 1993; Tetzlaff et al., 1994). By
contrast, more proximal lesions in the cervical spinal cord induce both cell death and
regenerative competence as well as a robust expression of c-Jun in the rubrospinal
neurons (Jenkins et al., 1993).

On the basis of a substantial amount of evidence, it has been postulated that the
induction of c-jun after axotomy may arise from the interruption of a target-tissue
derived and retrogradely transported, trophic substances which normally arrives at the
nucleus from the neurons' targets (Leah et al., 1991, 1993; Herdegen et al., 1991,
1997b). For example, peripheral nerve transection (or crush) induced strong c-Jun
immunoreactivity in motor neurons and DRG neurons, which normally showed a faint
basal immunoreactivity for Jun proteins; this result can be imitated by microinjection
with colchicine- or vinblastine-induced block of axonal transport (Leah et al., 1991).
While c-jun was upregulated in cholinergic neurons in rat medial septum/diagonal band
after lesion of the fimbria-fornix, treatment with either NGF or NT-4/5 could suppress
this c-jun expression (Hughes et al., 1997).
Activation of c-jun expression following axotomy suggest its role in mediating functional responses of injured neurons, but it does not show precisely whether c-jun is linked directly to cell death, survival or regeneration. A large amount of evidence indicates that c-jun is involved in the process of cell degeneration, or even that it acts as a killer protein both \textit{in vivo} and \textit{in vitro} (e.g. Ham \textit{et al.}, 1995; Anderson \textit{et al.}, 1996; Schlossberg, \textit{et al.}, 1996; Isenmann and Bähr, 1997; Miglieli \textit{et al.}, 1997; Araki \textit{et al.}, 1998; Watson \textit{et al.}, 1998; Herdegen \textit{et al.}, 1998). The clear evidence that links c-jun to apoptosis, is the association of DNA strand breaks, a morphological signs of apoptosis, in the same group of cells which express c-jun (Anderson \textit{et al.}, 1996; Isenmann and Bähr, 1997). A recent study suggested that the tumor suppressor gene p53, a critical regulator of the cellular response to DNA damage, requires the expression of c-Jun to induce apoptosis in cerebellar granule cells \textit{in vitro} (Araki \textit{et al.}, 1998).

A straightforward correlation of c-jun with the neurodegenerative state, is complicated by the fact that phosphorylation on serine 63 by Jun N-terminal kinase (JNK) and AP-1 activity is necessary for apoptosis (Ham \textit{et al.}, 1995; Mosieniak \textit{et al.}, 1997; Herdegen \textit{et al.}, 1998; Watson \textit{et al.}, 1998). For example, \textit{in vitro}, when sympathetic neurons are deprived of NGF (the condition in which cell death occurs), the level of c-Jun protein significantly increases and becomes more phosphorylated, probably on its amino terminal transactivation domain (Ham \textit{et al.}, 1995). Further study from the same group showed that c-Jun phosphorylation on serine 63 is necessary for apoptosis and clearly, AP-1 activity is necessary for cell death in cerebellar granule cells in which c-jun is the only member of the AP-1 family that is expressed (Watson \textit{et al.}, 1998). However, the result from a recent \textit{in vivo} study has correlated an increase of c-Jun phosphorylation and AP-1 activity in injured neurons to axon regeneration (Kenney and Kocsis, 1998); in axotomized DRG neurons, chronic activation of JNK concomitant with the elevation of c-Jun and AP-1 activity persists while they are making regenerative attempts.

As with cell death, expression of c-jun is implicated in the control of growth and differentiation of a variety of cell types, and importantly in axon regeneration of injured neurons (e.g., De Felipe \textit{et al.}, 1993; De Felipe and Hunt, 1994; Pfarr \textit{et al.}, 1994;
A link between expression of c-jun and competence for axonal regeneration has been demonstrated by a considerable number of studies over the past decade. Many groups of researchers who have concentrated on the study of the pattern of expression of c-jun/c-Jun in axotomized neurons both in PNS and CNS have reported that the increased levels of c-jun protein and mRNA are maintained if the damaged nerve is ligated or blocked, but return to basal level if the peripheral nerve is allowed to regenerate (Leah et al., 1991, 1993; Herdegen et al., 1991; Jenkins and Hunt, 1991; Jenkins et al., 1993a,b,c).

The role of c-jun in regulating the process of axon regeneration in neurons is shown by experiments in which PN grafts are used to encourage axon regeneration by axotomized CNS neurons (e.g., Robinson et al., 1994; Wu, 1996; Vaudano et al., 1993a). For example, after axotomy and replacement of the optic nerve with an autologous peripheral nerve graft to allow axonal regrowth, identified RGCs with regrown axons were shown to contain nuclear c-Jun immunoreactivity (Robinson et al., 1994). The evidence relating expression of c-jun to axon regeneration derives from the recent comparative studies showing that c-jun is expressed in axotomized CNS neurons which regenerate axons into PN grafts, such as TRN neurons (Vaudano et al., 1998), DN neurons (preliminary study of Vaudano et al., 1993a), brainstem neurons (Broude et al., 1999) and neurons of the inferior olivary nucleus (Bravin et al., 1997; Buffo et al., 1998), but not expressed by neurons which regenerates axon poorly into the graft such as Purkinje cells (Buffo et al., 1998; Vaudano et al., 1998).

Ample evidence shows that the induction of c-jun transcription is regulated by some factors such as JNK (see above) and the activation transcription factor-2 (ATF-2). ATF-2 rapidly decreases in axotomized neurons during the period when c-Jun is rapidly expressed (Herdegen et al., 1997a; reviewd by Herdegen et al., 1997b). Buschman et al. (1998) showed that explantation of DRG evoked a dramatic and rapid induction of c-Jun in DRG neurons in which prominent apoptosis (visualized by transferase dUTP nick end-labelling (TUNEL) and immunoreactivity of ATF-2 declined or disappeared in those DRG neurons. These results suggest that the presence or absence of ATF-2
might determine the action of c-jun and that ATF-2 is involved in regulating regeneration or degeneration. Moreover, some neurotrophic factors such as NGF, BDNF, NT-4/5 and CNTF have been shown to mediate expression of c-jun in axotomized neurons and suggested that these factors may evoke a pathway promoting cell survival by elevating the levels of c-Jun (Courtney et al., 1997; Hughes et al., 1997; Houle et al., 1998; Hsieh et al., 1998). In agreement, following the implantation of a PN graft, a source of neurotrophic factors, particularly of NGF (Woolhead, 1995), into the thalamus could prolong the expression of c-jun in TRN neurons, the group of neurons which regenerated axons vigorously into the graft (Vaudano et al., 1998).

As reviewed, expression of c-jun can mediate either the increased vulnerability of axotomized neurons to undergo programmed cell death or neuronal survival and a regeneration response. However, which pathway is followed must depend on additional signalling events that modulate the transcriptional activation of c-jun.

4. Aims of the study

Although previous work, as reviewed above, has established a correlation between the expression of the growth-associated molecules GAP-43, c-jun and L1, and axon regeneration in the CNS, up to now there has been no evidence to demonstrate the relationships among these molecules in term of their ability to promote axon regeneration. In other words, it is still not clear if axotomized CNS neurons express all these molecules during axon regeneration, or whether the expression of all of them is essential to this process. According to previous studies, it is apparent that these individual molecules may require coexpression of other molecules or factors to promote successful axon growth from injured CNS neurons. Particularly, in the case of c-jun which appears to be able to function as a bi-potential mediator for neuronal death on the one hand and survival and regeneration on the other hand, the existence of other intrinsic factors in injured neurons must be necessary to selectively determine the cascade of events initiated by this transcription factor. Additionally, CHL1, the close homologue of L1, is a very interesting molecule; it is included in the group of growth-related molecules, because it appears to be a more powerful neurite growth promoter,
than L1, *in vitro* at least. Thus, the aim of this study was to compare the pattern of expression of four regeneration relevant molecules L1, CHL1, c-jun and GAP-43 in axotomized CNS neurons and to correlate these patterns with differences in the regenerative potential of those neurons. The study is particularly concentrated on CNS neuronal populations in three different brain regions: the striatum, thalamus and cerebellum, regions which previous studies have shown that different populations of neurons have greatly differing ability to regenerate axons into PN grafts (Morrow *et al.*, 1993; Vaudano *et al.*, 1993a,b; 1995; 1998; Zhang *et al.*, 1995; Woolhead *et al.*, 1998; also reviewed by Anderson *et al.*, 1998), and axon regeneration in these CNS neurons is induced by PN graft implantation. Since the expression of some molecules has been previously examined in this laboratory i.e., the expression of GAP-43 and L1 in the thalamus and striatum and c-jun in the thalamus (Vaudano *et al.*, 1995; Zhang *et al.*, 1995; Woolhead *et al.*, 1998), the present study focuses chiefly on the expression of CHL1, L1, c-jun and GAP-43 in regions in which their expression has not been fully investigated. Finally, since cholinergic interneurons of the striatum, a CNS neuronal population showing high regenerative capacity (Woolhead *et al.*, 1998), are NGF sensitive, possible correlation between the expression of the high- and low-affinity NGF receptors, trkA and p75, respectively, and axon regeneration of this cell type, is also examined.
CHAPTER 2
Materials and methods

1. Animals and surgical procedures

1.1) Animals

All experiments were done using adult female Sprague-Dawley rats weighing 200-300g supplied by UCL Biological Services. The animals were housed in standard polypropylene cages and kept under a 12 hour light and 12 hour dark illumination cycle and received food pellets and water ad libitum.

1.2) Surgical procedures

All surgical procedures were carried out in the UCL Biological Services operating theatre, which is fitted with high specification equipment for surgical procedures. The environment of the surgery area was always maintained hygienically.

A) Sterilization of the equipment

Before surgery, all instruments, drapes and swabs were autoclaved for 20 minutes at 121°C.

B) Anaesthesia

Anaesthesia in all animals was induced by a mixture of 1.5% oxygen, 3% nitrous oxide and 4% Halothane (Fluothane, ICL) delivered through a scavenging nose cone from an IME portable anaesthesia station or central control station supplied by UCL Biological Services. Once deep anaesthesia was established, i.e., no corneal blink reflex and no reflex after toe pinching, the Halothane level was reduced to 1.5-2% at which level it was maintained throughout the surgical procedure.
C) Animal preparation

The operation area i.e., left hind leg and scalp were shaved using veterinary hair clippers and the exposed skin was antiseptically cleaned with 70% alcohol. Animals were then placed on a heated pad covered with a sterile drape and the anaesthesia nose cone was secured in place. Except for the cutaneous incision, all surgical procedures were performed under an operating microscope (Zeiss, Germany).

D) Tibial nerve graft preparation

An incision was made in the skin of the left thigh following the direction of the femur and the underlying quadriceps muscles were exposed and separated. Then, the sciatic nerve was located and the tibial branch was carefully separated from the common peroneal and sural branches. A segment of the tibial nerve about 15 mm in length was excised to make a graft. A point 6 mm from the proximal end of the graft was marked using a 10/0 monofilament suture (Ethicon Ltd). The graft was left between the quadriceps muscles bathed with Hanks’ Balanced Salt Solution (HBSS, Gibco, Life Technologies Ltd.) before autografting.

E) Graft implantation

The scalp was opened along the midline of the skull and the periosteum was removed to expose the sagittal and coronal cranial sutures. The position of the striatum, thalamus or cerebellum was identified using stereotaxic co-ordinates from Paxinos and Watson (1986). A craniotomy was made at the marked point (for the striatum: on the left coronal suture 3 mm away from the bregma; for the thalamus: 3.5 mm caudal to the bregma and 3.5-4.0 mm left to the midline; for the cerebellum: 11.6mm caudal to the bregma and 2.5 mm left to the midline) on the skull using a dentists’drill (burr size RdP.C. 7, round) until only a thin layer of bone was left. The remaining bone was carefully removed using microvessel forceps to make the hole wide enough for insertion of the graft. The dura mater was opened with a fine syringe needle to allow access to the brain. Subsequently, the graft was taken from the leg and its proximal end was gently pushed vertically into the brain to a depth of 6 mm by using a glass micropipette. The graft and its distal end were glued to the dura and skull respectively by Histoacryl adhesive (B.Braun, Melsungen AG, Germany) to prevent
graft displacement once the scalp was closed. The scalp was sutured using Mersilk 4/0 braided suture thread (Ethicon Ltd.) and the leg wound was closed with 10 mm Michel clips (International Market Supply). Finally, the animals were given 15 mg/kg Clamoxilina (Beecham), a long term antibiotic, subcutaneously and 2.5 mg/kg Finedyne (Flunixin) a long term analgesic (Schering-Plough Animal Health) into the right quadriceps muscles.

Grafts to be used as freeze-killed controls underwent a 6 cycles of freezing and thawing in liquid nitrogen before being implanted into the brain. This procedure kills all living cells in the graft but leaves basal laminae and extracellular connective tissue matrix intact.

F) Postoperative care

After surgery, the animals were left in a warm incubator to recover full consciousness before being returned to their cages.

G) Application of retrograde tracers to the graft

Animals assigned for retrograde tracer labelling experiments were reoperated two days before they were killed. They were anaesthetised and prepared as described above. The scalp was reopened with care to avoid disturbing the graft lying underneath. Connective tissue and blood vessels surrounding the graft were carefully removed using fine forceps and fine spring bow scissors. An 8/0 suture was inserted immediately beneath the graft approximately 2 mm from its distal end and knotted loosely over the graft. Meanwhile, the retrograde tracer was prepared by mixing 3 μl of cholera toxin subunit-B conjugated HRP solution (Quadrature) with 7 μl of sterile water for injection (Antigen Pharmaceuticals). The tracer was drawn into a clean 5 μl Hamilton syringe to which a fine cannular had been attached. A small nick was made in the perineurium of the distal end of the graft through which the fine cannula was inserted and pushed along the inside of the graft so that its tip of the cannula was at least 2 mm beyond the suture. The knot was then tightened around the barrel of the needle and the solution was slowly injected to avoid rupturing the graft and flooding the surrounding tissue with the solution. The suture was tightly knotted to form a
ligation around the graft after withdrawing the needle to prevent the tracer leaking out of the graft. The graft was gently crushed 2-3 times to facilitate tracer uptake and transport. The scalp wound was closed and animals received the same postoperative care as described above.

2. Fixation

With the exception of animals for in situ hybridization (see below) all animal were killed as follows. The animals were lightly anaesthetized by exposure to ether vapour and were then injected intraperitoneally with an overdose of Sagatal (sodium pentobarbitone, Rhône Mérieux). When the corneal blink reflex was lost but before respiration failed, the animal was transferred to the dissection tray in a fume cupboard. The abdominal cavity was rapidly opened, the diaphragm and both sides of the rip cage were cut using a large scissors. The rib cage was pulled back and secured with clamps to reveal the heart. The pericardial sac and fatty tissue around the heart were carefully removed. A blunt cannula attached to tubing passing through a Watson-Marlow peristaltic pump was immediately inserted into the left ventricle and up into the aorta and a small cut was made in the right atrium to release venous blood. The animal was first exsanguinated with 200-250ml of 0.1 M phosphate buffered saline (PBS), pH 7.4 at a flow rate 20-25ml/minute and then perfused with 500ml of freshly prepared fixative; the first 200ml of the solution was perfused at a flow rate of 20-25ml/minute and the remaining solution was perfused at 15ml/minute. The types of the fixative used were varied depending on experimental design as follows.

2.1) For LM immunocytochemistry for trk A and p75

Animals were perfused with chilled paraformdehyde-lysine-sodium periodate (PLP) fixative prepared as follows:

For 1 litre of the fixative

1) 20g paraformaldehyde (BDH) was dissolved in 250ml distilled water by heating to 60°C, cooled and then filtered.

2) 750ml of L-lysine solution was prepared by dissolving 6.8g of L-lysine, 1.42g of Na2HPO4 (anhydrous) and 0.39g of NaH2PO4 in 750ml distilled water
3) The solutions in 1 and 2 were chilled on ice and mixed before use and before adding 2.13g of sodium periodate.

The pH of the final solution was adjusted to 7.2-7.4 by 1M hydrochloric acid (HCl) or 1M sodium hydroxide (NaOH).

2.2) For the tetramethylbenzidine (TMB) method

After being exsanguinated with 0.1 M PBS, animals were perfused with 1% paraformaldehyde containing 1.25% glutaraldehyde in 0.1 M PBS, pH7.4. The fixative was made as followed:

For 1 litre of fixative

1) 10g paraformaldehyde (BDH) was dissolved in 200ml distilled water on a hot plate while heating the solution to 60°C.

2) The solution was cleared by adding a few drops of 1 M NaOH, left to cool and then filtered.

3) 50ml 25% glutaraldehyde and 500ml 4M standard PBS, pH7.4 was added to the solution.

4) The final volume of the fixative was made up to 1 liter with distilled water.

5) The fixative was filtered and adjusted to pH7.2-7.4 by 1M HCl or 1M NaOH.

2.3) For in situ hybridization

Animals used for in situ hybridization experiments were not perfused, but were overdosed with Sagatal and quickly decapitated once all reflexes had disappeared. The brain was then immediately removed (see below).

3. Dissection

When the following technique was applied to the tissue prepared for in situ hybridization, all instruments and equipment were washed by strong detergent such as D90 and treated with absolute ethanol prior to use.
To isolate the brain tissue for sectioning, animals were decapitated using large scissors. The scalp was then opened and the connective tissue carefully cleared away to expose the external portion of the graft. The temporalis muscle was trimmed away from the bone, using a scalpel. To remove the cerebellum, the right side of the occipital bone covering this part of the brain was cautiously trimmed off using bone forceps so that the graft in the left cerebellum could be seen through the subcranial space. The graft was cut from the overlying bone just above the surface of the cerebral cortex by using fine scissors. The left occipital bone was then removed and the exposed cerebellum excised. To remove the striatum and thalamus, the occipital bone covering the cerebellum was chipped away with bone forceps, and subsequently the right temporal, parietal and frontal bones were removed. Fine scissors were inserted beneath the left parietal bone to separate the graft from the underlying bone and then the remaining bone was removed. The cerebellum, olfactory bulbs and cranial nerves were trimmed away as the cerebrum was lifted out of the cranial base. The portion of brain containing the graft was trimmed into a block ready for cryoprotection.

4. Tissue preparation and sectioning

4.1) For immunocytochemistry and the TMB method

After dissection the block of brain tissue was postfixed in the fresh fixative (the same as used for perfusion) for 4 hours at 4°C or overnight in case the perfusion was not perfect. Then the tissue block was transferred to 30% sucrose (BDH) in 0.1M standard phosphate buffer (PB) at 4°C until it was fully infiltrated (indicated by the sinking of the tissue) before being sectioned.

From 30% sucrose, tissue blocks were briefly washed in 0.1M PB and were positioned on a freezing microtome to be rapidly frozen with solid carbon dioxide using OCT mounting medium (Miles Inc.). Serial coronal sections were cut at 40μm and collected in 0.1M PBS.

4.2) For in situ hybridization

Immediately after being removed from the skull, blocks of non-perfused brain tissue were placed caudal side downward, onto an appropriate sized plastic mould using
OCT mounting medium, and slowly frozen in iso-pentane precooled in liquid nitrogen. The frozen blocks were wrapped in parafilm and kept at -70°C until sectioned.

For in situ hybridisation, the tissue blocks were cut at 12 μm by using a cryostat (Jung-Reichert Cryocut 1000) set at -18°C. Serial sections of the area of interest were by thaw-mounted directly onto slides coated with 3-aminopropyl-triethoxy-silane and quickly immersed in RNase-free 4%paraformaldehyde in 0.1 PBS.

5. Preparation of slides

5.1) Slides for LM immunocytochemistry and histochemistry

Gelatine-chrome alum coated (subbed) slides were used to mount cut sections processed for immunocytochemistry and histochemistry. The subbing solution was made by dissolving 14.5g gelatine (Sigma) in 240ml distilled water and was heated to 50°C to allow the gelatine to dissolve fully. Chrome alum (BDH) was added to the cooled gelatine solution (3g in 60ml distilled water). The subbing solution was well filtered before being used. Cleaned slides were loaded into staining racks and washed briefly in distilled water. Then they were dipped into the subbing solution for a few minutes on a shaker to ensure that all slides were well coated. The coated slides were dried in an oven at 60°C.

5.2) Slides for in situ hybridization

Silane-coated slides were use to mount cut sections processed for in situ hybridization. Clean slides were washed in 10% D90 for 2 hours, rinsed in tap water for 2 hours, followed by dipping in distilled water for a few minutes. The slides were immersed in a solution consisting of 15ml 37% HCl and 985ml 95% ethanol for 15 minutes and rinsed in distilled water for 2 minutes. They were then dried overnight at 160°C. The next morning the slides were removed from the oven and allowed to cool before being dipped in 6% Silane in 100% acetone for 5 minutes. The slides were then washed twice in acetone for a few minutes and then twice in distilled water for a few minutes. The silanized slides were left to air dry at room temperature before being used.
6. Histochemical procedures

6.1) Tetramethylbenzidine (TMB) method for visualization of HRP

A) TMB method for free floating sections

Free floating sections cut at 40 μm by a freezing microtome were collected in 0.1M PB and rinsed briefly 3 times in the buffer. The sections were stored at 4°C while the following solutions were prepared.

0.2M Acetate buffer: for 1 litre of the solution
1) 13.61g sodium acetate was dissolved in 200ml distilled water
2) 1M HCl was added to adjust pH to 3.3
3) distilled water was added to make the final volume 1 litre

Solution A: 0.3g sodium nitroferricyanide (Sigma) dissolved in 280ml distilled water and 15ml acetate buffer.

Solution B: 0.015g 3,3’, 5,5’ TMB (Sigma) dissolved in 7.5ml absolute ethanol.

Solution C: 0.3% hydrogen peroxide in distilled water (300μl H₂O₂ in 30ml distilled water).

PRSS (post reaction and storage solution) 200ml acetate buffer diluted in 800ml distilled water.

Solution A, B, C, and PRSS were chilled on ice. Solutions A and B were combined seconds before the introduction of the tissue sections.

To start the reaction process for HRP visualization, the sections were rinsed 6 times in distilled water for 10-15 seconds each time, before starting the reaction. The following procedures were carried out on an ice tray (to reduce the level of background and slow down the TMB reaction) that was placed on a rocker table. The sections were incubated in a mixture of 49ml solution A and 1.25ml solution B for 20 minutes. Then, the sections were changed to a fresh mixture of solutions A and B with an addition of 2ml solution C (5x5 minutes incubation). The reaction was stopped by rinsing the sections in PRSS solution (6x5 minutes). Finally, the sections were mounted onto gelatinised slides and left to air dry for a couple of hours at room temperature for and then overnight at 4°C. The dried sections were dehydrated rapidly in 95% and absolute
alcohol before being immersed in Histoclear clearing reagent for 5-10 minutes. The examination of TMB reaction product was done under both bright and dark-field illumination.

B) TMB method for *in situ* hybridization sections

For brain specimens processed both for *in situ* hybridization and the TMB method to visualise HRP, alternate sets of consecutive sections were cut at 12 μm in a cryostat and collected on silanized slides. All sets of sections were quickly fixed in RNAase free 4% paraformaldehyde and sections prepared for *in situ* hybridization were further processed as described in 6.3B. Sections prepared for TMB processing were left in the fixative for 30-60 minutes before being rinsed in 0.1M PBS three times for 5 minutes each. The following procedures were performed as described in 6.1A. The sections were washed briefly 6 times in distilled water and incubated in a mixture of pre-cooled solutions A and B for 20 minutes. The incubation solution was then changed to the combination of solutions A, B and C. Since the sections were cut thinly, the TMB reaction occurred very rapidly, i.e., within a few minutes of the first incubation of combined solution A, B, and C. The reaction was stopped by washing the sections in PRSS solution 6 times for 5 minutes each wash before being left to air dry at room temperature for 1-2 hours or in some cases, overnight at 4ºC. Lastly, the sections were cleared in Histoclear for 5 minutes and coverslipped with DPX mounting medium.

6.2) Immunocytochemistry and histochemistry

The followings are details of antibodies, normal sera, and buffer used in immunocytochemistical study, both ABC and immunofluorescence methods, for trk A and p75

**Buffer:** 0.1M Tris-HCl buffer saline (TBS), pH 7.4

For 1 litre of buffer

1) 13.22g of Tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl, Sigma) and 1.94g of Tris[hydroxymethyl]aminomethane (Tris-base, Sigma) was dissolved in 1 litre of distilled water and adjusted pH to 7.4 by adding 1M HCl or 1M NaOH.

2) 9g of sodium chloride was added to the buffer.
Primary antibodies:
1) Rabbit polyclonal antibody for trk A, a gift from Professor L.F. Reichardt’s laboratory, San Francisco, U.S.A., was used at dilutions of 1:4000 for the ABC method and of 1:400 for immunofluorescence.
2) Mouse monoclonal antibody for p75 (clone 192, Boehringer Mannheim) was used at dilutions of 1:3000 for the ABC method and of 1:150 for immunofluorescence.

Primary antibodies could be recycled by keeping in clean Eppendorfs left at 4°C and used within a week.

Secondary antibody:
1) Biotinylated goat anti-rabbit IgG (Vector) and Biotinylated horse anti-mouse IgG (Vector) were used with the ABC method at a dilution of 1:200.
2) Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antibodies to rabbit IgG (Sigma) and fluorescein isothiocyanate (FITC)-conjugated goat antibodies to mouse IgG (Sigma) were used for the immunofluorescence method at a dilution of 1:60.
3) Streptavidin Texas Red (Amersham Life Science) was used for immunofluorescence at dilution of 1:400.

Normal blocking serum (NBS): NBS was used to incubate tissue sections in order to reduce the occurrence of non-specific antibody binding and was also used as a diluent for primary antibodies.
1) Normal goat blocking serum (NGBS) comprised 10% normal goat serum (Sigma), 1% bovine serum albumin (BSA; Sigma) and 0.05% triton-X 100 (Sigma) diluted in 0.1M TBS.
2) Normal horse blocking serum (NHBS) comprised 2% normal horse serum (Sigma), 0.5% BSA and 0.05% triton-X 100 (Sigma) diluted in 0.1M TBS.
Table 2.1 Summary of solutions, dilutions and incubation times for trkA and p75 immunocytochemistry using the ABC and immunofluorescence methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>1° Antibodies (dilution)</th>
<th>Normal sera</th>
<th>Incubation time</th>
<th>2° Antibodies (dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>TrkA (1:4000)</td>
<td>NGBS</td>
<td>60 hrs at 4°C</td>
<td>Biotinylated goat anti-rabbit IgG (1:200)</td>
</tr>
<tr>
<td></td>
<td>p75 (1:3000)</td>
<td>NHBS</td>
<td>48 hrs at 4°C</td>
<td>Biotinylated horse anti-mouse IgG (1:200)</td>
</tr>
<tr>
<td>Immuno-fluorescence</td>
<td>TrkA (1:400)</td>
<td>NGBS</td>
<td>60 hrs at 4°C</td>
<td>FITC-goat anti-rabbit IgG (1:100)</td>
</tr>
<tr>
<td></td>
<td>p75 (1:150)</td>
<td>NHBS</td>
<td>48 hrs at 4°C</td>
<td>Biotinylated horse anti-mouse IgG (1:200) and Strepavidin Texas Red (1:400)</td>
</tr>
</tbody>
</table>

A) Avidin-biotin complex method (ABC method) for trk A and p75

Free floating sections of PLP-fixed brains were cut at 40μm by a freezing microtome and collected in 0.1M PBS; alternate sections were collected separately and immunostained for trkA or p75. Sections were rinsed twice for 10 minutes in 0.1M TBS before incubation for 30 minutes in 0.3% hydrogen peroxide in 0.1m TBS to reduce endogenous peroxidase inside cells and tissues. These sections were subsequently treated to diminish non-specific antibody binding by incubation in normal blocking serum: NGBS for trk A sections and NHBS for p75 sections for 1 hour at room temperature. Then, the sections were incubated with either antibody against trkA diluted in NGBS or antibody against p75 diluted in NHBS. The time and temperatures for incubation are shown in Table 2.1. After incubation, sections were rinsed in TBS (3x10 minutes), and followed by incubation in a secondary antibody (biotinylated goat anti-rabbit IgG for trkA and biotinylated horse anti-mouse IgG for p75) diluted 1:200 in TBS for 90 minutes at room temperature. Meanwhile, the avidin-biotin signal amplification complex (Vectastain Elite ABC kit) was prepared 30 minutes before use at a dilution of 1:200 in TBS. Sections were washed in TBS three times for 10 minutes apiece before incubation in this ABC mixture for 90 minutes at room temperature.
Again, sections were washed in TBS 3x10 minutes, and were then incubated in 0.05%
3.3’-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.03% hydrogen peroxide
until dark brown reaction product was seen. This step was carried out under a fume
hood to prevent hazardous effects and the DAB solution was neutralised with sodium
hypochloride (Sechelle) before disposal. The DAB reaction was stopped in 0.1M TBS
for 3x10 minutes before the sections were mounted onto gelatinised slides. After
leaving the slides to air dry overnight at room temperature, sections were dehydrated in
two changes of 70% and 95% ethanol for 5 minutes each and 100% ethanol for 10
minutes and cleared in Histoclear for 10 minutes as shown in Table 2.2. Finally,
sections were coverslipped with DPX (BDH).

Table 2.2 Dehydration steps and times for histochemical and immunocytochemical
procedures

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 minutes</td>
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<tr>
<td>95% ethanol</td>
<td>5 minutes</td>
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<tr>
<td>100% ethanol</td>
<td>10 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Histoclear</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

B) Double Immunofluorescence for trk A and p75

Sections for double immunofluorescence for trk A and p75 were processed and
cut as those for ABC immunocytochemistry. After cutting, sections were rinsed in
0.1M TBS and incubated in 0.1% sodium borohydride for 20 minutes at room
temperature to reduce auto-fluorescence in cells. This was followed by incubation in
antibodies against trkA diluted at 1:400 in NGBS for 60 hours at 4°C. Sections were
washed in 0.1M TBS for 3x10 minutes at room temperature before application of
antibody against p75 diluted at 1:150 in NHBS. Sections were left to incubate in the
second primary antibody for 36 hours at 4°C. After washing in 2% non-fat dried milk in 0.1M TBS for 2x10 minutes, sections were incubated for 2 hours in FITC-conjugated goat antibodies to rabbit IgG (1:100) and biotinylated horse anti-mouse IgG (1:200) diluted in 0.1M TBS. Sections were rinsed in 2% non-fat dried milk for 10 minutes twice before incubation in Strepaavidin Texas Red diluted in 0.1M TBS at 1:400 for 3 hours. Finally, sections were rinsed in TBS 2x10 minutes before being mounted in a mixture of glycerol and PBS (9:1) containing 2.5% DABCO and using nail polish to seal around the edge of coverslip to prevent vaporisation of the mounting medium. Sections were examined under a Leica laser confocal microscope.

Control sections for ABC and immunofluorescence methods were processed at the same time and under identical conditions except the primary antibody was omitted from the incubation solution.

C) Combined ABC and TMB methods for trkA/p75 and HRP localization

The protocol for the combination of ABC and TMB methods used in this study was modified from the protocols described in by Bolam (1992), Rye et al. (1984) and Lue and Dessem (1996). Animals used for this experiment were perfused with PLP (see 2.1) and in some cases by 4% paraformadehyde. Free floating section were cut at 40 μm and collected in 0.1M PBS. Alternate sections were processed for TMB/trk A and TMB/p75 staining. Sections were washed briefly 6 times in distilled water and were then processed for the TMB reaction as described in 6.1A. After washing several times in PRSS solution to stop the TMB reaction, sections were rinsed and left in 0.01M acetate buffer (diluted from 0.2M acetate buffer). Meanwhile, the following solutions were prepared.

Stabilising solution: for 50ml of solution

1) 0.025g DAB was dissolved in 0.1M PB.

2) 1ml of 1% cobalt chloride (CoCl₂, Sigma) was slowly added to DAB solution (drop by drop).

3) 15μl of 30% H₂O₂ was added.
Since TMB reaction product was not stable at pH 7.2-7.4, which was the working condition for immunocytochemistry, sections were incubated in stabilising solution for 3-4 minutes on a rocker table to stabilise this reaction product. The stabilising reaction was stopped by washing the sections for 3x10 minutes in 0.1M PB. Sections were then transferred to 0.1M TBS to await immunostaining for trk A and p75 by the ABC method as described in section 6.2A.

6.3) *In situ* hybridization

A) Probes

All of the probes were cRNA probes labelled with digoxigenin. Apart from the CHL1 cRNA probe which was a gift from Professor M. Schachner's laboratory (Zentrum für Molekulare Neurobiologie, D-20246 Hamburg), other probes, both sense and anti-sense were prepared in this laboratory by Dr. Y. Zhang.

**CHL1**: Sense and anti-sense probe were obtained from pBlue CHL1-extra, which comprised 3.4 Kb of the mouse CHL1 cDNA (Holm *et al*., 1996) coding for the extracellular part of CHL1.

**L1**: Sense and anti-sense probe were obtained from pBluescript L1, which comprised 3.3 Kb of the mouse L1 cDNA (Moos *et al*., 1988) coding. L1 was inserted into EcoRI site of pBluescript, which contains HindIII in the upstream and XbaI in the downstream. To produce antisense cRNA probe the plasmid was digested with HindIII and T7 RNA polymerase was used for RNA transcription. To obtain sense cRNA probe the plasmid was digested with XbaI and T3 RNA polymerase was used for RNA transcription.

**c-jun**: The open-reading frame of mouse c-jun DNA comprising 1127 base pairs was subcloned into pBluescript SKII- vector (Stratagene) to produce c-jun-OPRF plasmid. To achieve this the OPRF of c-jun was digested with NcoI and ScaI from pGEMI/c-jun. This resulted in removal of about 350bp fragment containing untranslated sequence. The OPRF of c-jun was subcloned into NcoI and PvuII site in a modified plasmid pRSET7c, which contained HindIII in the upstream of c-jun OPRF and BamHI in the downstream of c-jun OPRF. The OPRF of c-jun was then digested with HindIII.
and BamHI and subcloned between HindIII and BamHI sites of pBluescript SKII-. To produce antisense c-jun cRNA probe pBluescript SKII-/c-jun OPRF was digested with HindIII to linearize the plasmid. T3 RNA polymerase was used for cRNA transcription. To obtain c-jun sense probe the plasmid was digested with BamHI and T7 RNA polymerase was used cRNA transcription.

**GAP-43**: The open-reading frame of rat GAP-43 DNA comprising about 700 base pairs was subcloned between HindIII and EcoRI site of pCDNA (Stratagene) to produce the pCDNA GAP-43 plasmid. This plasmid was a gift from Dr. Joost Verhaagen’s lab. HindIII is in the upstream of GAP-43 OPRF and EcoRI is in the downstream of GAP-43 OPRF. To produce GAP-43 antisense probe the plasmid was digested with HindIII and cRNA was transcripted using SP6 RNA polymerase. To obtain GAP-43 sense probe the plasmid was digested with EcoRI and cRNA was transcripted by T7 RNA polymerase.

Anti-sense and sense cRNA probes labelled with digoxigenin were all generated according to the manufacturer's recommendations using an RNA labelling kit (Boehringer Mannheim, Germany). After transcribing, CHL1 and L1 probes were digested under alkaline conditions to obtain an average length of approximately 250 or 300 nucleotides.

**B) In situ hybridization procedures**

*In situ* hybridisation was carried out as described by Zhang et al.(1995). All glassware and instruments used during the procedures were kept RNAase free either by being autoclaved in an oven at 160°C or by washing with a strong detergent and treatment with autoclaved 0.2% DEPC treated water (DECP-H$_2$O). All solutions used before the post-hybridization washing step were prepared or made with molecular grade chemicals and dissolved or diluted in autoclaved 0.2% DEPC-treated millipore water. Gloves were worn at all times while performing the experiment to prevent DNA contamination from hands.
**a) Fixation and pre-treatment of tissue**

After cutting and mounting cryostat sections on the slides coated with 6% silane (see 5.2), the slides were immediately immersed into pre-cooled 4% paraformaldehyde in 0.1M PBS, pH 7.2-7.4 made up with DEPC-H₂O, for at least 2 hours to overnight at 4°C. All the following steps were done at room temperature. Sections were washed for 3x5 minutes in 0.1M PBS before being dehydrated in 70% ethanol. Sections can be stored in 70% ethanol at 4°C for several days before being further processed. Then the sections were washed in DEPC-H₂O 2x5 minutes and subsequently treated in 0.1M HCl for 10 minutes. To reduce the ability of charged probes to bind 'electrostatically' to the sections, the sections were permeabilized in a solution of 0.1M triethanolamine (BDH), pH 8.0 consisting 0.25% acetic acid anhydride (BDH) for 20 minutes followed by rinsing 2x 5 minutes in 0.1M PBS. The sections were then dehydrated through an ascending ethanol series i.e., 70%, 80% and 100% ethanol respectively for 5 minutes each. The slides were taken out from 100% ethanol to air dry for a few of minutes.

**b) Pre-hybridization**

From this step, all solutions used were applied directly onto the sections mounted on slides. To prevent leakage of the solution from the slides and minimise the amount of the solution used, the sections on every slide were encircled by a PAP-pen (Dako). The slides were placed in an incubation chamber humidified with strips of filter paper soaked with a 1:1 mixture of deionized formamide and 0.1M PBS. A 1:1 mixture of deionized formamide and prehybridization mix (consisting of 25mM ethylenediaminetetraacetic acid (EDTA, Sigma), 50mM Tris-HCl (Sigma), pH 7.6, 2.5x Denhardt’s solution (Sigma), 0.25mg/ml tRNA (Yeast tRNA, Boehringer Mannheim), and 20 mM NaCl (BDH)) was applied to the sections and gently spread over them to ensure that all were completely covered. The incubation chamber was sealed with tape and placed in an oven at 37°C for at least 3 hours to overnight.

**c) Hybridization**

Before beginning this step, the hybridization solution was prepared by the 1:1 combination of deionized formamide and the hybridization buffer consisting of 20mM Tris-HCl (pH 7.5), 1mM EDTA, 1x Denhardt’s solution. 0.5mg/ml tRNA, 0.1mg/ml
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poly A RNA (Sigma), 0.1M dithiotreitol (DTT, Sigma), 10% dextran sulphate (Sigma) and 0.3M NaCl. Anti-sense and sense probes were then added into the hybridization solution at concentrations of: 5μl/ml for GAP-43 and c-jun; 6μl/ml for CHL1; and 3μl/ml for L1 probes, and mixed well. The hybridization mix (containing probes) was left warm in a 55°C waterbath until use. The following procedures were carried out as quickly as possible to prevent low stringency hybridization. The incubation chamber was removed from the 37 °C oven and one at a time, the slides were removed from the chamber and the pre-hybridization mix was blotted. The slides were returned to the chamber and prewarmed hybridization mix was applied onto the sections of each slide. Once all done, the chamber was re-sealed with tape and left incubated overnight in an oven set at the appropriate temperature for hybridizing each probe, i.e., 57 °C for CHL1 and L1; 62°C for c-jun and GAP-43.

d) Post-hybridization washing

The washing solutions including 0.2x standard saline citrate, pH 7.0 (SSC, diluted from 20X SSC consisting of 30mM NaCl, and 3mM tri-sodium citrate) and 0.1x SSC/50% formamide (made up from equal volumes of 0.2xSSC and formamide) were prepared and left standing in a 57 °C waterbath. The slides were removed from the incubation chamber and transferred into a coplin jar before being rinsed briefly with prewarmed 0.2 SSC to removed the hybridization mix. The slides were then washed twice in 0.2 SSC for 30 minutes each wash and 3 times in 0.1xSSC/50% formamide for 1 hour each wash; through this step the coplin jar containing slides and the washing solutions were left in the waterbath to maintain the same temperature. Finally, the slides were removed from the waterbath and rinsed in 0.2x SSC for 10 minutes at room temperature.

e) Visualisation of the probe

Sections were equilibrated in buffer 1, pH 7.5 (diluted from 10x buffer1 consisting 100mM Tris-HCl, 150mM NaCl) for 10 minutes at room temperature before being immersed in modified buffer 2 (blocking medium, consisting of 1% Boehringer blocking reagent, 0.5% BSA fraction (Sigma) dissolved in buffer1) to block non-specific binding sites. The slides were then placed into a humid incubation chamber,
moistened with buffer 1. The solution of alkaline phosphatase (AP)-coupled antibodies to digoxigenin (Fab fragments, Boehringer Mannheim) prepared at a dilution of 1:700 in modified buffer 2 was applied onto the sections. The incubation chamber was left at 4°C overnight. The following steps were all done at room temperature. The sections were rinsed 2x15 minutes in buffer 1 and then equilibrated in buffer 3, pH 9.5 (consisting of 100mM Tris-HCl, 100mM NaCl and 50mM MgCl₂) for 2 minutes. The slides were returned to the incubation chamber and the sections were covered with AP-developer (consisting 0.34mg/ml 4-nitroblue tetrazolium chloride (Boeringer), 0.175mg/ml 5-bromo-4-chloro-3-indolyphosphate (Boeringer) and 0.25mg/ml levamisol (Sigma) diluted in buffer 3). The slides were then left to develop in the dark until the reaction product could be seen in the wet sections. It usually took overnight for the colour reaction in the sections to be visible. Checking the colour reaction of the wet sections by light microscopy before stopping the reaction was essential. To stop the colour reaction, the sections were washed in buffer 4, pH 8.0 (consisting of 10mM Tris-HCl, 1mM EDTA) for 10 minutes and 3x5 minutes each wash in distilled water. For permanent storage, the slides were air dried overnight and immersed in Histoclear before being coverslipped with DPX mountant (BDH).

Specificity of the hybridization signal was verified by using anti-sense and sense probes of comparable specific activity hybridized under identical conditions. No hybridization signal was observed in control sections in which the anti-sense probes were replaced by sense probes.

C) Thionin staining for in situ hybridization sections

To reveal and distinguish types of labelled cells in the in situ hybridization sections, a nuclear stain, thionin, was selected to double stain such sections. After stopping development of colour reaction in buffer 4 as described above, sections were rinsed 3 x 5 minutes in distilled water. Subsequently, without being dried, sections were stained with 0.05% thionin for 20-30 seconds before bathing in several changes of distilled water to wash out the excess stain from the sections. After being left to air dry, sections were immersed in Histoclear for 10 minutes and coverslipped with DPX.
D) *In situ* hybridization and immunofluorescence double staining for GFAP/trkA

Sections were processed for *in situ* hybridization as described above until reaching the step of stopping the colour reaction by buffer 4 and washing in distilled water (see 6.3B, section d). Then, the sections were rinsed briefly in 0.1M TBS before being incubated with monoclonal antibody against glial fibrillary acidic protein (GFAP; Sigma), a marker for astrocytes or polyclonal antibody against trk A diluted in normal goat blocking serum at a concentration of 1:200 for GFAP or 1:400 for trkA. The slides were placed in a humid incubation chamber moistened with TBS and left at 4°C for 60 hours (for trkA) or 24 hours (for GFAP). The following steps were all carried out at room temperature. The sections were rinsed briefly in 0.1M TBS and followed by washing in 2% non-fat dried milk in 0.1M TBS for 3x10 minutes. Section were then incubated in the dark in 0.1M TBS containing (TRITC)-conjugated goat antibodies to rabbit IgG (Sigma) for trk A or TRITC-conjugated goat antibodies to mouse IgG at a dilution of 1:100 for 2 hours. The sections were rinsed 2x10 minutes in 0.1M TBS and finally the slides were coverslipped using anti-fading mounting medium comprising 90% glycerol, 10% 0.1M PBS and 2.5% DABCO (Sigma). The sections were viewed and photographed soon after, using a Leica fluorescence microscope.

E) Fluorescence *in situ* hybridization (FISH) and GFAP immunofluorescence double staining

Sections were processed as for *in situ* hybridization until the step of immersion in modified buffer 2 (see 6.4B, section d). At this step, modified buffer 2 and AP-coupled antibody to digoxigenin conjugated FITC (Boehringer Mannheim) were mixed at a dilution of 1:100 to detect *in situ* hybridization signal. Additionally, antibody against GFAP was also added to the same solution at a concentration of 1:200. The solution containing both α-GFAP and α-digoxigenin conjugated FITC was applied to the sections which were placed in the incubation chamber humidified with buffer 1. Following incubation at 4°C for 24 hours or overnight in the dark, the sections were washed briefly in buffer 1 and then 0.1M TBS for 3x10 minutes. Subsequently, the sections were incubated in a solution containing TRITC-conjugated goat antibodies to mouse IgG diluted in 0.1 TBS at a dilution of 1:100 for 90 minutes. Finally, they were
washed in 0.1M TBS for 2x10 minutes. The slides were coverslipped using the anti-fading mounting medium DABCO and examined under a fluorescence microscope.

7. Storage of slides

For the most part, processed sections, in particular those prepared for immunofluorescence were examined and photographed in an appropriate microscope soon after being coverslipped and left to dry for a short time. This approach yielded the best results. However, labelling and staining could be preserved for a certain period of time if sections were stored in an appropriate environment.

7.1) Immunocytochemistry (ABC) and in situ hybridization slides

As the reaction products occurred from immunocytochemistry and in situ hybridization procedures were relatively stable, slides from both experiments were stored in a dark box and kept at room temperature. No evidences of fading immunostain or loss of in situ hybridization signal in the sections stored in this way.

7.2) TMB and immunofluorescence slides

Slides processed for HRP retrograde labelling and those for immunofluorescence were stores in a dark box and kept at 4°C to maintain stability of the reaction products. For immunofluorescence slides, however, under this condition, the intensity of labelling signal was still decreased within months of the storage.

8. Data analysis

8.1) Terminology

In this study, the distribution area of labelled cells found in tissue sections was described according to the distinct anatomical regions of the brain/graft interface as defined by Campbell et al. (1992). The interface regions between the brain and the graft are illustrated in Figure 2.1 and the following are the definitions of the terms used:
A) Graft

'The distal graft'— the portion of the graft which passes through the cerebral cortex and remains outside the brain.

'The proximal graft'— the portion of the graft embedded in brain tissue and close to the graft tip.

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

B) Brain/graft interface

'The graft proper'— the part of the graft inside the brain tissue which contains Schwann cell columns and myelin debris.

'The junctional zone'— the zone which lies between the glia limitans and the graft proper.

'The glia limitans'— the thin layer formed by astrocyte processes produced by graft implantation at the site of the damage.

'The parenchymal border zone'— The area of brain tissue containing degenerating axons and reactive axons, axon terminals and dendrites, reactive glial cells and axonal sprouts. This zone extends no more than about 100-150μm from the interface between the brain and the graft and gives way to normal brain parenchyma in which very few structural abnormalities are apparent.
Figure 2.1 Schematic drawing illustrating an implanted peripheral nerve graft within the corpus striatum; (A) in a coronal plane and (B) in a horizontal plane cut at the level of the dash line (B), showing features of the graft brain/interface. Ac, anterior commissure; Cc, Corpus callosum; CC, Cerebral cortex; G, graft; V, lateral ventricle;

8.2) Cell size measurement
A) Measurement of cell size in sections prepared for in situ hybridization

Where the size of a labelled cell on the in situ hybridization sections was focused, the measurement was made to the shortest and longest axes of its visible perikaryon. The average length of both axes was recorded as the 'diameter' of that cell. By using a Leica microscope with a camera lucida attachment, such perikarya were drawn at high magnification (x100) and the measurement was made from the drawing. To avoid an error in the estimation of cell size as a consequence of different planes of
sectioning, at least three consecutive sections were examined for the appearance of the same labelled perikaryon before the biggest part of it was selected to measure.

B) Measurement of cell size in sections prepared for immunocytochemistry

The estimation of the size of an immuno-labelled cell was made only if its labelled perikaryon appeared round or oval shaped with an unambiguous, complete cell outline and appearance of two or more distinct cell processes. Although very rare evidence of the appearance of the same cell in consecutive sections was recorded, careful examination through a set of three adjacent sections was done before measurement. The “diameter” of the selected perikaryon was measured in the same way as described above.

8.3) Matching corresponding cells

In Chapters 6, 7 and 8 probe-labelled neurons were examined in adjacent sections after processing to visualise different probes or retrograde HRP labelling. Cell matching was done twice over to confirm precision. First, by using a camera lucida attached to a Leica light microscope, the area of two consecutive sections containing promising matching cells were drawn and the positions of all labelled cells inside the areas were plotted on tracing paper. Importantly, at least two landmarks within the particular areas such as small arteries were also located to use as fidicial points for cell matching. The corresponding neurons were then compared and numbered. Second, the same areas were photographed under both phase-contrast (to clearly visualize fidicial markers) and bright field (to show all labelled neurons). The matching neurons were traced directly on the photomicrographs using tracing paper.
CHAPTER 3
Expression of TrkA and p75 neurotrophin receptor in the striatum following peripheral nerve graft implantation

Cholinergic interneurons of the striatum have been established as one of the CNS neuronal types, which can regenerate axons into a peripheral nerve (PN) graft (Woolhead et al., 1998). Similar to other CNS cholinergic neurons, cholinergic striatal interneuron expresses high level of choline acetyltransferase (ChAT) and TrkA, the phenotypic characteristics of NGF-responsive neurons. However, in contrast to the cholinergic neurons of basal forebrain, cholinergic neurons in the striatum possess very low, to undetectable, levels of p75 in the adult, and their responsiveness to NGF has been reported to decline dramatically during postnatal development (Martinez et al., 1985; Mobley et al., 1985). However, Gage et al. (1989) demonstrated that after injury, cholinergic interneurons can reexpress p75 in presence of NGF, suggesting the resumption of NGF responsiveness in this cell type. Also, injured cholinergic striatal interneurons can regenerate their axons very well in the presence of a PN graft and this ability is believed to be mainly a consequence of the action of NGF produced from living Schwann cells in the graft (Woolhead et al., 1998). Together, these findings suggest that cholinergic interneurons in adult striatum regain their regenerative response by become NGF responsive again and both TrkA and p75 may be required for NGF to promote axon regeneration in this neuronal type. In other words, cholinergic interneurons whose axons have regenerated into PN grafts would presumably express both TrkA and p75. To investigate this hypothesis, the work described in this chapter examined (i) the expression of TrkA and p75 proteins in striatal neurons in response to PN graft implantation, (ii) the colocalization of these markers; and (iii) the expression of TrkA and p75 in regenerating striatal neurons.
1. Summary of methods used
(see Chapter 2 for details)

Autologous tibial nerve grafts were implanted into the left striatum of 31 adult Sprague Dawley rats. At postoperative survival periods of 3 days (n=7), 1 week (n=7), 10 days (n=1), 2 weeks (n=4), 4 weeks (n=4), 7 weeks (n=2), 12 weeks (n=1) and 29 weeks (n=1), the animals were perfused with PLP. The brains were removed and cut at 40 μm by using a freezing microtome. The same procedures were carried out in 4 animals implanted with freeze-killed grafts and sacrificed at 3 days (n=2), 1 week (n=1) and 2 weeks (n=1). Sections of the striatum and substantia nigra were collected as 2 parallel series of alternate sections and were processed for immunocytochemistry (by the ABC method) to detect p75 in one series and TrkA in the other series. Eight animals from the survival times of 3 days to 7 weeks were selected to examine colocalization of p75 and TrkA proteins in striatal neurons by the double immunofluorescence histochemical staining method.

Three animals at 7, 12 and 29 weeks post-operation (wpo) were injected with the tracer, CT-HRP, 2 days before perfusion, to trace the cell bodies of neurons with axons which had regenerated into the graft. Then the brains were processed for dual visualisation of HRP (by the TMB method) and TrkA or p75 (by immunocytochemistry).

2. Results

2.1) Localization of TrkA and p75 neurotrophin receptor in the intact striatum
A) TrkA

TrkA immunoreactivity was generally present in cholinergic interneurons of the striatum of adult rats as described by Steininger et al. (1993) and Sobreviela et al. (1994) (Fig 3.1). Numerous strongly immunoreactive neurons were scattered throughout the corpus striatum. Most of them appeared to be medium to large neurons (perikaryal diameter approximately 15-25 μm) with a few short neurites extending from the cell soma. Commonly, the immunoreactive neurons showed bipolar, fusiform or polymorphic appearances (Figs. 3.1b, 3.4b). Neuropil of the striatum displayed weak to
moderate immunoreactivity for TrkA. There was no evidence for a correlation between immunoreactivity of this high affinity NGF receptor and the patch-matrix organization of the neostriatum.

In the substantia nigra, neurons of both SNpc and SNpr contained low levels of TrkA immunoreactivity.

B) p75

Very few p75 immunoreactive neurons were observed in the striatum of unoperated animals or in the striatum contralateral, to a PN graft, although a few moderately p75 immunoreactive neurons with large perikarya could be seen sporadically distributed in the lateral area of caudal striatum. The neuropil of the striatum also exhibited very low to insignificant levels of p75 immunoreactivity. Intense p75 immunoreactivity was, however, seen in neurons within the medial septum and vertical limb of the diagonal band nucleus. The immunoreactivity of these neurons served as a useful positive control for this method (Fig. 3.6).

Neurons of the SNpc and SNpr displayed negligible levels of p75 immunoreactivity.

2.2) Localization of TrkA and p75 neurotrophin receptors in the striatum following implantation of a living PN graft

A) TrkA

After insertion of a PN graft into the corpus striatum, changes in striatal TrkA immunoreactive were obvious between 3 days post-operation (dpo) and 2 wpo. At early survival times of 3-4 dpo, neuropil around the graft showed intense TrkA immunoreactivity. Numerous TrkA immunoreactive cells with cell bodies of variable size were present around the graft (Fig. 3.2). Among these immunopositive cells, which displayed very strong immunoreactivity for TrkA and were almost certainly cholinergic striatal interneurons on the basis of their size and shape. The labelled cells with smaller perikarya (diameter less than 15 μm) were less strongly immunoreactive and were only located close to the graft. It is difficult to identify precisely the phenotypes of these
small immunopositive cells at the light microscopic level and by this staining technique alone. However, these cells probably included small and medium sized striatal neurons and probably glial cells also. One week later, the numbers of such small TrkA immunoreactive cells was dramatically reduced.

Up to 2 wpo, TrkA-labelled perikarya located close to the graft (within 1mm of the brain/graft interface) and particularly lying close to the graft tip, were often large with round or oval shapes (Fig. 3.2-3.4) and in some cases, showed abnormally long dendritic and axonal extensions (Fig. 3.2c,f, 3.3b). Examples of axons from these TrkA immunoreactive neurons running along the lateral edges of the graft tip were occasionally detected (Fig 3.2c, 3.3b), but were not observed crossing from the host brain into the graft itself. Measurement of perikaryal size of such cells (see Chapter 2 for how this was done), gave a diameter range of 25-30 μm, larger than the average size of such cells in normal brain (Fig 3.4). Also, these large perikarya were more strongly TrkA immunoreactive than TrkA immunoreactive cells on the contralateral side of the brain in the same sections. This evidence showed that a particular group of TrkA immunoreactive striatal neurons appeared to respond to PN graft implantation by perikaryal hypertrophy, enhancement of TrkA synthesis and elongation of their axons. Nevertheless, it should be noted that the number of such TrkA immunoreactive neurons found in different animals of the same survival group was varied. This might be a consequence of differences in the graft position and the degree of axon damage. TrkA immunoreactive neurons showing such responses to the graft were reduced in number at survival times longer than 2 wpo, when a few cholinergic interneurons adjacent to the graft appeared to display much stronger TrkA immunoreactivity.

Occasionally, long, thin fibres with moderate TrkA immunostaining, presumably the products of axonal sprouting, could be seen near the graft (Fig 3.5c). In addition, axons which exhibited ‘bead and string’ characteristics were occasionally found (Fig. 3.16). However, the number of TrkA immunoreactive regenerating fibres or axonal sprouts appearing in brain parenchyma, was much smaller than seen in p75 sections (see below).
In the substantia nigra, there was no signal of upregulation of TrkA immunoreactivity in neurons of SNpc or SNpr in response to PN graft implantation.

B) p75

Schwann cells in PN grafts implanted into the striatum were strongly immunoreactive for p75 and a wide zone of neuropil surrounding the implantation site was also intensely p75 immunoreactive (Fig. 3.8). Short columns of presumptive Schwann cells with strong p75 immunoreactivity extending into the striatal parenchyma around the graft (Fig. 3.12) were often apparent particularly between 1 and 2 wpo. By 4 wpo, the appearance of such Schwann cells columns outside the graft as well as the intensity of p75 immunoreactivity in the surrounding neuropil had decreased and was insignificant by 6-7 wpo.

The presence of a PN graft appeared to promote upregulation of p75 neurotrophin receptor protein in striatal cells; p75 immunoreactive cells were detectable around the graft in the ipsilateral striatum from the earliest survival times examined, i.e., 3-4 dpo. Similar to the TrkA immunostaining described above, a striking level of p75 immunoreactivity was displayed in striatal cells of various sizes around the graft at this early survival time (Fig. 3.7). The large immunopositive cells were presumably cholinergic striatal interneurons (perikaryal diameter range 15-30μm) whereas the smaller cells (perikaryal diameter less than 15 μm), containing less immunoreactivity, were likely to be small and medium sized neurons, and possibly also glial cells. The smaller p75 immunoreactive cells were rarely found after 1 wpo whereas large p75 immunoreactive cells could be detected at all time intervals examined. The number of large p75 immunoreactive neurons present in the vicinity of the graft subsequently declined over the following weeks after graft implantation. Very few immunopositive cells were detected by 4 wpo. Nevertheless, it was noted that the position of the graft seemed to be a factor determining the number of immunopositive cells. When the graft was inserted into the caudal striatum and the graft tip ended laterally, large numbers of presumptive cholinergic striatal interneurons in the lateral caudal striatum showed upregulation of p75 immunoreactivity (Fig. 3.8a,b, 3.9). This was also the case for an animal at 7 wpo in which the graft embedded in lateral caudal region of the corpus
striatum; in this animal many p75 immunoreactive cells were apparent, most of them located lateral to the graft.

Similar to TrkA immunoreactive neurons responding to the presence of the graft, strong p75 immunoreactivity was mostly found in large fusiform or oval shaped multipolar neuronal perikarya with long process (Fig. 3.10). The perikarya of these immunopositive neurons had a diameter range of 20-30μm. Also, p75 immunoreactive neurons exhibiting hypertrophic perikarya were detected at early postoperative survival times (3 dpo-1 wpo). Slightly different from TrkA staining, the long p75 immunoreactive neurites were very distinctive because both dendritic and axonal processes immunostained very intensely (Fig. 3.10). The presence of long, p75 immunoreactive processes was peculiar to this cell type, which was presumably the cholinergic interneuron. In keeping with the TrkA results, examples of p75 immunoreactive neurons with axons extending toward the graft were infrequently seen (Fig. 3.7c). There was no evidence of p75 immunoreactive cells showing morphological features indicative of cell death or necrosis.

Additionally, exclusively between 1 and 2 weeks following graft implantation, large numbers of long fibres with strong p75 immunoreactivity were also found in the striatal tissue surrounding the grafts. These axon-like fibres were identified as long fine lines of reaction product and some had a beaded morphology (Figs 3.9d,e, 3.11) while others were slightly smoother similar to the processes emanating from labelled perikarya within the striatum (Figs 3.10e, 3.11b). Examples of these unidentified fibres, likely to be regenerating, running close to the brain/graft interface (Fig. 3.9d) or crossing from the host brain into the graft region (Fig 3.12) were also seen. Surprisingly, it was difficult to detect such immunoreactive fibres in the central core of the graft. The parent neurons of the presumptive regenerating axons could not be identified in the vicinity. Possibly, some of these fibres were axonal sprouts from neurons of other brain regions projecting their axons to the striatum, such as nigrostriatal neurons. The p75 immunoreactive fibres described above were rarely seen by 4 wpo.
Immunoreactivity for p75 was not detectable in neurons of either SNpc or SNpr.

2.3) Colocalization of TrkA and p75 immunoreactivity in striatal neurons following implantation of a living graft

By using the double immunofluorescence technique, TrkA and p75 receptor proteins could be colocalized in the same cells. Apparently, most of the p75 immunoreactive neurons found near the graft as described in section B were also TrkA immunopositive (Fig. 3.14, 3.15). Very few neurons displayed immunoreactivity for p75 alone.

2.4) Expression of TrkA and p75 proteins in identified regenerating neurons

The possible colocalization in individual cell bodies of HRP (indicating that the axon of the neuron had regenerated along the graft) and of p75 or TrkA (as markers of the cholinergic phenotype) was examined in 3 animals at survival periods of 7, 12 and 29 weeks after graft implantation into the striatum. In all of these animals CT-HRP had been injected into the distal end of the graft 2 days before the animals were killed. In the striatum, many cell bodies and proximal neurites containing HRP reaction product were also immunoreactive for TrkA (Fig. 3.17) or for p75 (3.18). In both cases, the reaction product for HRP appeared as blue-black granular deposits and the immunoreactive product as dust-like brown colouration. The number of cells labelled with HRP alone was greater than the number of double labelled cells (HRP+p75), but p75 immunoreactive cells almost always contained HRP reaction product. There were many TrkA immunopositive cells that were not HRP labelled and some that were clearly double labelled (as already described) but in the most heavily HRP-labelled cells it was difficult to determine if TrkA immunoreactivity was also present. The observation that many of the HRP-labelled cells displayed the presence of TrkA or p75 indicates that these cells are probably cholinergic interneurons.

In the SNpc, none of the numerous HRP labelled cells displayed p75 and TrkA immunoreactivity.
2.5) **Localization of TrkA and p75 immunoreactivity in the striatum following implantation of a freeze-killed graft**

Freeze-killed grafts displayed only weak immunoreactivity for TrkA and p75 by comparison with living grafts. This is as expected. The Schwann cells within the graft had been destroyed by the freeze-thaw protocol (see Chapter 2). In the living graft, denervated Schwann cells showed very strong p75 expression. Neuropil around the area injured by the graft did not showed stronger immunoreactivity for either antibody than the background level. At 4 dpo, in the sections stained for p75, only a few immunopositive cells and structures, some resembling cell debris, were detected close to the graft (Fig. 3.13). Similar groups of cells were also found in the sections stained for TrkA. In the ipsilateral striatum of animals implanted with freeze-killed grafts no significant change in TrkA immunopositivity responses could be seen at any survival time. No p75 immunopositive neurons were detected in the ipsilateral striatum of such animals at 1 and 2 wpo.

As for animals with living grafts, there were no TrkA- or p75-immunoreactive neurons in the SNpc or SNpr.

### 3. Discussion

The present study has clearly shown that in response to the implantation of a living PN graft into the corpus striatum, many striatal neurons around the graft strikingly re-expressed p75 neurotrophin receptor protein at high level. Such upregulation was not detected in the striatum implanted with a freeze-killed graft confirming that expression of p75 was particularly influenced only from the graft containing living Schwann cells. Most of the p75 immunoreactive striatal neurons, according to their characteristics, were similar to and assumed to correspond to large, aspiny cholinergic interneurons. This assumption was firmly supported by the evidence of the coexpression of this receptor in TrkA immunoreactive neurons. Since TrkA is expressed by large cholinergic striatal interneurons (Steininger *et al.*, 1993; Sobreviela *et al.*, 1994), it is reasonable to imply that upregulation of p75 immunoreactivity chiefly occurred in this subtype of striatal neurons. Although it was not always the case that
p75 immunoreactivity was present in TrkA immunoreactive neurons, only rarely were neurons immunoreactive for p75 alone found. When retrograde HRP labelling and immunocytochemical techniques were applied, the presence of TrkA/p75 immunoreactivity in some of the retrogradely labelled neurons whose axons had regenerated along the graft was demonstrated and all the double labelled neurons also exhibited the morphological features of cholinergic interneurons. In this study, no attempt was made to verify co-expression of p75 and TrkA in regenerating neurons; nevertheless, the co-existence of both neurothrophin receptors in the corresponding regenerating neurons could be postulated. In contrast, many neurons of the SNpc ipsilateral to the graft were strongly HRP labelled, testifying to the strong regenerative propensity of this neuronal population (Woolhead et al., 1998). However, none of the SNpc neurons were immunopositive either for TrkA or p75. The present data therefore provide strong evidence for the expression of TrkA and p75 neurotrophin receptors in cholinergic interneurons of the striatum during regeneration suggesting a crucial role of both receptors in mediating cell survival and promoting neurite outgrowth in this neuronal type but not in the SNpc neurons.

3.1) **TrkA immunoreactive neurons in response to living graft implantation**

Following the implantation of a PN graft into the striatum, some TrkA immunopositive neurons close to the graft underwent hypertrophy and the intensity of the immunostaining of these neurons appeared to be enhanced. In this study no attempt was made to determine the size of all TrkA immunoreactive neurons in both experimental (grafted) and control striatum, but the hypertrophy of these cells on the grafted side was very obvious and was confirmed by diameter measurements on samples of such cells from grafted and control striatum (see Chapter 2 for details). In addition, the comparison of the intensity of immunoreactivity between the affected and intact cells was made only within the same section to avoid errors due to uneven staining in different sections. The enlargement and increase in immunoreactivity of TrkA immunopositive cells was observed only when the striatum had been implanted with a graft containing living Schwann cells; it was not seen when a freeze-killed graft was inserted. This indicates that perikaryal hypertrophy of these neurons is induced by the presence of the living graft and is not simply a consequence of mechanical damage.
**Factor(s) inducing hypertrophy in TrkA immunoreactive neurons**

Denervated segments of peripheral nerve are well known to be a source of many trophic factors including neurotrophins which can enhance the regeneration of injured CNS neurons in the host brain (see Chapter 1). After axotomy, Schwann cells in the distal stump of transected peripheral nerve have been shown to upregulate many different growth-promoting molecules, including BDNF (Meyer *et al.*, 1992; Funakoshi *et al.*, 1993), NT-3 (Maisonpierre *et al.*, 1990), NT4/5 (Hallböök *et al.*, 1991; Ip *et al.*, 1992; Funakoshi *et al.*, 1993), CNTF (Sendtner *et al.*, 1992) and NGF (Heumann *et al.*, 1987a,b; Lindholm *et al.*, 1987; Taniuchi *et al.*, 1988; Liu *et al.*, 1995). The production and rapid accumulation of these neurotrophic factors in the distal stump of transected nerves has been attributed to retrograde transport of these proteins from the target tissue (Funakoshi *et al.*, 1993). If so, the same modulation of neurotrophin synthesis from the periphery, as occurs in the cut nerves following injury in the PNS, would not be expected to take place in the PN graft after implantation to the CNS tissue since the graft is removed from its target very shortly after being cut. This implies that the level of neurotrophic factors produced in a PN graft would probably be lower than within the distal stump of transected PN nerves in situ. Nevertheless, the amount of such factors, probably including other growth-associated factors secreted from the graft, seems to be adequate to induce axon regeneration in some axotomized CNS neurons. This assumption is supported by many experiments in which a PN graft was used successfully to encourage axon outgrowth from injured CNS neurons (see Chapter 1 for review and references).

Undoubtedly many neurotrophic factors synthesized in the graft can enhance neuronal survival and promote axonal regeneration in CNS neurons of the host brain. Nevertheless, expression of specific neurotrophin receptors on the surface of target cells is essential to mediate signal transduction and to initiate internalization of the neurotrophin ligands. In the striatum of adult rats, trkC, the specific receptor for NT-3 and trkB, the high affinity receptor for BDNF and NT-4, are mostly expressed by small and medium sized neurons; only some of the large neurons (presumptive cholinergic interneurons) display these receptors (Merlio *et al.*, 1992; Schmidt-Kastner *et al.*, 1996). In addition, preliminary study in this laboratory has shown that there is no
evidence of upregulation of gene expressions for trkC and trkB in striatal neurons after PN grafting at least up to 3 weeks following implantation of the observation. Thus, cholinergic striatal interneurons are unlikely to be the principal targets for BDNF, NT-3 or NT4/5 produced from the graft. Conversely, almost all (>99%) large, aspiny cholinergic striatal interneurons normally express both ChAT and TrkA, the high affinity receptor for NGF (Steininger et al., 1993; Venero and Hefti, 1993; Sobreviela et al., 1994). The existence of both phenotypic markers in cholinergic striatal interneurons, similar to other CNS cholinergic neurons such as basal forebrain neurons, indicates that they are NGF sensitive. Thus, the hypertrophic response as well as upregulation of immunoreactivity in TrkA immunoreactive neurons found in this study suggest the action of NGF upon this cell type. Corresponding results had been demonstrated by Forander et al. (1996) who reported that TrkA immunoreactive striatal neurons underwent hypertrophy following chronic infusion of recombinant human NGF (rhNGF) into the striatal parenchyma. In the same study, upregulation of ChAT-, TrkA- and p75 mRNAs were also detected. Supporting evidence showing that NGF induces biological responses in cholinergic neurons is extensive. Intraventricular infusion of NGF can induce gene expression of TrkA and p75 in basal forebrain cholinergic neurons (Cavicchioli et al., 1989; Gage et al., 1989; Higgins et al., 1989; Holzman et al., 1992; Gibbs and Pfaff, 1994; Förander et al., 1996). Hypertrophy in ChAT immunoreactive cholinergic neurons following NGF infusion into the lateral ventricle (Gage et al., 1988, 1989, Hagg et al., 1989; Vahlsing et al., 1991) and striatum (Förander et al., 1996) has also been demonstrated. Furthermore, NGF has also been shown to ameliorate the age-related atrophy of cholinergic neurons of the striatum, as well as the basal forebrain (Fischer et al., 1987). These results provide strong support for the hypothesis that CNS cholinergic neurons are responsive to NGF and firmly suggest that NGF is the main factor responsible for biological changes in TrkA immunoreactive neurons in the striatum after graft implantation. The source providing NGF in the grafted striatum is discussed below.

Much evidence shows that under some circumstances such as tissue damage and inflammation, NGF is produced and its synthesis modulated by non-neuronal cells at the site of injury. Reactive astrocytes can secrete NGF in vitro (Lindsay, 1979; Yoshida
and Gage, 1991) and in vivo after ischemia or neuronal inflammation (Lorez et al., 1989; Hashimoto et al., 1992; Lee et al., 1995, 1996, 1998). Heumann et al. (1987b,c) and Lindholm et al. (1987) reported that interleukin-1 released from macrophages accumulated at the site of injury is the principal mediator of the synthesis of mRNAs of NGF and its low affinity receptor, p75. However, the level of NGF produced by non-neuronal cells at the site of injury, (e.g., the tissue surrounding a PN graft), may be insufficient to induce morphological changes in axotomized cholinergic neurons. The failure to detect any change in TrkA immunoreactive neurons in the animals implanted with freeze-killed grafts in this study is good supporting evidence for this hypothesis. Another good example comes from Asada et al. (1996) who showed that gelfoam extract collected from traumatized rat striatum appeared to contain CNTF-like and BDNF-like components that had neuroprotective and neurite-promoting activity for DRG explants, dissociated ciliary ganglion and human dopaminergic neuroblastoma cells (SH-SY5Y); but they failed to detect the activity of NGF in the extract. CNTF can increase or induce p75 immunoreactivity and prevent degeneration in cholinergic neurons in medial septum and neostriatum, but, unlike NGF, it is unable to induce ChAT synthesis or cause hypertrophy of those neurons (Hagg et al., 1992). Moreover, the study of C.L. Woolhead (1995) showed that PN grafts continue to produce NGF after being implanted into the striatum and the level of NGF within the graft is much higher than that in surrounding host tissue. Taken together, these data suggest that a high dose of NGF is necessary to elicit a hypertrophic response from cholinergic neurons after injury.

Nevertheless, this cannot imply that low concentration of NGF fails to promote axonal sprouting in injured neurons. Hagg and his colleagues (1993) demonstrated that low dose administration of NGF infused to the lateral ventricle of animals with fimbria-fornix transection induces more p75 immunopositive fibres to become detectable than under normal conditions, although high doses of NGF induce and even more extensive p75 immunopositive fibre plexus. Interestingly, even though a substantial number of studies have demonstrated the requirement for a high level of NGF to upregulate the phenotype of axotomized cholinergic neurons and to promote axonal sprouting in such neurons, a variable susceptibility of different populations of CNS cholinergic neurons
to NGF has also been suggested. Vahlsing et al. (1991) found that the hypertrophic response of normal striatal neurons required less NGF than did that of medial septum neurons. This evidence is in line with the expectation that NGF production from PN grafts, although comparatively less than that secreted from Schwann cells in the distal stump of lesioned peripheral nerves and much less than the amount of NGF provided from chronic intraventricular infusion, is nevertheless sufficient for promoting axon regeneration in striatal neurons, especially in cholinergic interneurons (Woolhead, 1995). However, the possibility that not only NGF but also other factors may participate and/or co-participate in the instigation of regeneration of the axons of cholinergic striatal neurons cannot be excluded.

3.2) Re-expression of p75 neurotrophin receptor in aspiny cholinergic striatal interneurons following implantation of a living graft

The present study has established that PN grafts can induce p75 immunoreactivity in striatal neurons, most of which presumably are large, aspiny cholinergic interneurons. These immunopositive neurons shows the typical morphological characteristics corresponding to large, aspiny striatal intrinsic neurons, which are immunopositive for choline acetyltransferase (ChAT) described by Kubota and Kawaguchi (1993) and Kawakuchi (1997) and also correspond to the large, aspiny cholinergic interneurons (Type I neurons) described by Bolam et al. (1984). The finding that almost all p75 colocalized in TrkA immunopositive cells also firmly suggests the presence of this receptor in the cholinergic striatal interneuron subtype. Likewise, Gage et al. (1989) found that upregulation of p75 after chronic infusion of NGF into the striatum was seen in neurons immunopositive for ChAT, a specific phenotype marker for cholinergic neurons. p75 neurotrophin receptor is expressed by CNS cholinergic neurons in the rat both during development and in the adult (Richardson et al., 1986; Gage et al., 1989). In contrast, cholinergic neurons in adult striatum lose p75 expression and their responsiveness to NGF during postnatal development (Martinez et al., 1985; Mobley et al., 1985; Gage et al., 1989). Thus, this low affinity neurotrophin receptor is not normally expressed in the adult rat neostriatum, although a few p75 immunopositive neurons are found in the most ventrolateral part of caudate-putamen (Gage et al., 1989; Springer et al., 1990).
Re-expression of p75 in adult tissue can be directly induced by NGF. For example, several studies have shown that upregulation of p75 in NGF-sensitive cells is regulated by NGF both in vitro (Calissano and Shelanski, 1980; Doherty et al., 1988; Hartikka and Hefti, 1998) and in vivo (Caviccholi et al., 1989; Higgins et al., 1989; Holtzman et al., 1992; Gibbs and Pfaff, 1994; Figueiredo et al., 1995). In normal adult rats, intraventricular infusion of NGF can induce gene expression of both trk A and p75 in basal forebrain cholinergic neurons (Cavicchioli et al., 1989; Higgins et al., 1989; Holtzman et al., 1992; Gibbs and Pfaff, 1994). Similar results to those of the present study have been presented by Gage et al. (1989) and Förander et al. (1996) who showed that in the damaged adult rat neostriatum, the cholinergic neurons which lose their p75 neurotrophin receptor can reexpress this receptor after chronic infusion of NGF for 2 weeks. The results of both research groups are consistent with the present findings and strongly suggest that the upregulation of p75 immunoreactivity in cholinergic striatal interneurons following PN graft implantation is induced by NGF produced from the graft.

Taken together, these results suggest that a high level of NGF is necessary to induce expression of p75 and to promote axonal regeneration of striatal cholinergic neurons. This level would be higher than is produced locally at injury sites in the absence of a PN graft or exogenous infusion of NGF. The small number of p75 immunoreactive cells observed in the striatum of animals implanted with freeze-killed PN grafts, in which there are no living Schwann cells to produce NGF, strongly suggests this hypothesis.

However, inconsistent results have been available from Gage and his colleague (1989). According to their findings, upregulation of p75 in presumptive cholinergic interneurons appeared in animals both with and without NGF directly infused into the striatum. Moreover, the number of p75 immunoreactive cells found in the animals which received only vehicle, was comparable to number in those animals which received chronic infusion of high doses of NGF and some of them also showed perikaryal hypertrophy. One possible explanation for this discrepancy is differences in the details of the experimental procedures. In the Gage experiments, a cannula attached
to a small mini-osmotic pump was implanted into the corpus striatum and the pump was placed subcutaneously in the dorsal neck/back area. With this method, it is likely that the indwelling cannula which is a hard object would move from time to time when the animals moved. This could cause more serious damage in the host brain tissue than is caused by a PN graft. Furthermore, continuous infusion of fluid into the lesion site for 2 weeks could possibly induce: (1) cell swelling as a result of excess fluid in the tissue rather than hypertrophy due to cellular responses to injury alone, and (2) additional injury to the brain. In this case, larger numbers of astrocytes and macrophages aggregating around the area of damage may have been stimulated, by comparison with the number of such cells induced by PN graft implantation alone, thus resulting in higher levels of NGF production by these non-neuronal cells (see section 3.1). Furthermore, the level of CNTF, a cytokine secreted by reactive astrocytes (Asada et al., 1995) at the site of injury in this case was probably high enough to induce expression of p75 (Hagg et al., 1992).

Another discrepancy arises from the findings of Holtzman et al. (1992) who reported that following regular injection of NGF for 2 weeks through cannulae implanted bilaterally into lateral ventricles, expression of TrkA and ChAT (mRNAs and immunoreactivity) in neurons of the striatum and basal forebrain, and also p75 mRNA in basal forebrain neurons were significantly increased; they failed, however, to detect p75 mRNA in striatal neurons. In this case, the site of NGF administration is possibly the main variable. The amount of NGF diffusing from the ventricle is enough to upregulate p75 expression in basal forebrain neurons which normally possess high levels of this receptor, whereas it may be insufficient to induce reexpression of p75 in striatal neurons which have switched off expression of this receptor. Moreover, in their data analysis, only two brain sections of the striatum hybridized with a radioactive probe for full length p75 mRNA were chosen for quantitation of grains. Thus, undetectable expression of p75 mRNA above the background after NGF infusion in the experiment of Holtzman et al. (1992) may have resulted from studying such a small sample. In keeping with this possibility, the evidence from the present study is that the distribution of p75 immunoreactive cells is not even in all sections: numerous immunopositive cells might appear in some sections but comparatively few in others.
In conclusion, the reexpression of p75 in cholinergic neurons observed in this study is significant and most likely to be promoted by NGF derived from the graft, the evidence from previous literature is not uniformly consonant with this conclusion.

3.3) **Presumptive regenerating fibres and gradients in neurotrophin concentrations**

Abundant, strong p75 immunopositive fibres were found distributed in ipsilateral striatum and most of them were concentrated within the PBZ. These fibers showed a distinct beaded morphology which resembled that of regenerating axons. Although observations of fibres of this kind running across the brain/graft region into the central core of the graft were very rare, a significant number of the fibres running towards the graft and/or coursing adjacent to and parallel with the brain/graft interface, commonly very close to the junctional zone were found. In addition, extensive long, thin and smooth fibers sprouting from presumptive axons were apparent in numerous p75 immunoreactive neurons. TrkA immunoreactive fibers showing the beaded characteristics were also seen but the number was much smaller than that of p75 immunoreactive fibres. In addition, putative long axonal sprouts, such as were in association with p75 immunoreactive neurons, were not commonly observed to originate from TrkA immunoreactive neurons. As discussed earlier, most of the p75 immunoreactive neurons are believed to correspond to the population of neurons showing TrkA immunoreactivity. Therefore, it is not clear why only a few presumptive regenerating fibers displayed TrkA immunoreactivity. Possibly, the distribution of TrkA and p75 on the cell surface is different; p75 may distribute throughout the cell body and along all of the processes of neurons (p75 is a good maker for neurites) whereas TrkA accumulates mainly in the cell body and proximal portion of axon and dendritic tree. However, there is at the present no evidence to support this hypothesis.

It remains unclear which type(s) of cells gave rise to presumptive regenerating fibres displaying p75 immunoreactivity, as very few neurons giving rise to such fibres appeared in the same section. However, two possibilities may be suggested: (1) the parent cells are mostly cholinergic interneurons since they are the majority of neurons displaying p75 immunoreactivity, and (2) the parent cells may be extrinsic neurons
such as neurons of SNpc. The argument from the second possibility is very strong because the major afferent pathway projecting to the striatum comes from SNpc neurons and this neuronal group can regenerate axons vigorously into PN grafts (Woolhead et al., 1998). The weakness of this explanation is, however, that SNpc neurons display only low to insignificant levels of p75 immunoreactivity in control animals and at all survival times examined. Therefore, the speculation that the parent neurons of strong p75 immunoreactive axons resembling regenerating axons found in the brain parenchyma of grafted striatum are mostly cholinergic interneurons is more likely to be correct. Indeed, the number of cholinergic striatal interneurons which can regenerate their axon into the graft detected by retrograde HRP-labelling is not abundant (Woolhead et al., 1998; and observations in the present study). In this study and the study of Woolhead et al. (1998), most of the neuronal perikarya which can be retrogradely labelled from the graft in the striatum are found in the SNpc. This occurrence raises the question that if NGF produced from the graft can promote axonal regeneration in cholinergic striatal neurons and if the abundant p75 immunoreactive fibres are regenerating axons from these neurons, why does only a small number of these axons grow into the graft. Some possibilities are discussed below.

It is well documented that NGF can induce sprouting in axotomized cholinergic neurons in basal forebrain and that NGF plays critical role in cholinergic axons regeneration into the graft (Hefti, 1986; Kromer, 1987; Hagg et al., 1989, 1990a, 1991, 1992, 1993; Eagle et al., 1995; Förander et al., 1996). One piece of very strong evidence demonstrating this trophic function of NGF in promoting axonal outgrowths is to use NGF pre-soaked acellular PN graft implanted between disconnected cholinergic pathway of septum and hippocampal and formation; such a graft can promote vigorous regeneration of cholinergic neurons (Hagg et al., 1991). Interestingly, not only the presence of NGF but also the high gradient of this neurotrophin seems to be an important factor for attracting regenerating axons to grow towards. This fact was clearly demonstrated by Hagg et al. (1993) who investigated the potential neurotropic action of NGF in the adult rat cholinergic septohippocampal regeneration model. When NGF was infused into the lateral ventricle, the number of cholinergic axons entering the sciatic nerve graft implanted between the disconnected septum and hippocampal
formation was much reduced and instead, numerous axon sprouts from septal neurons were induced with a gradient towards the lateral ventricle (Hagg et al., 1993). Moreover, Eagle et al. (1995) found that the implantation of primary fibroblasts, genetically modified to produce NGF, between the cut ends of the lesioned septohippocampal pathway, could promote the outgrowth of axotomized septal neurons and regenerating axons stained for p75 and TrkA were extremely dense within the NGF-producing grafts rather than extending into the hippocampus. Additionally, continuous NGF infusion through a dialysis fibre implanted in the striatum could increase nerve fibre density around the implant (Förander et al., 1996). The lessons from these studies indicate that regenerating axons from NGF-sensitive neurons grow preferentially towards the available highest concentration of NGF. Therefore, the fact that there were more p75 immunopositive regenerating fibres around the graft than there were inside the graft suggests the possibility that the concentration of NGF inside the graft may not be much different from that in the PBZ and surrounding parenchyma.

There is, however, the following paradox to consider. After axotomy in the PNS, NGF and p75 are markedly upregulated in Schwann cells distal to the lesion (Heumann et al., 1987b,c; Lindholm et al., 1987; Taniuchi et al., 1988; Liu et al., 1995). A rising level of NGF and of its low affinity receptors is sustained in the distal stump of transected nerve for a prolonged period suggesting that NGF may be bound and restrained in the p75 receptors at the surface of Schwann cells at this site prior to arrival of the regenerating axons (Liu et al., 1995). Following implantation of a piece of peripheral nerve in CNS tissue, the neurotrophic factor concentrations produced by these non-neuronal cells is unlikely to be the same as occur in the PNS; however, the process of trophic factor production would be expected to be similar. Interleukin-1 released by macrophages at the site of PN nerve lesion is suggested to be a principal mediator for induction of NGF and p75 synthesis in Schwann cells (Heumann et al., 1987b,c; Lindholm et al., 1987). Since interleukin-1 is also produced in the CNS and has been reported to increase after tissue damage (Giulian and Lackman, 1985; Giulian et al., 1986), it seems possible that this factor could serve similar inductive mechanisms in both the PNS and CNS. Remarkably strong p75 immunoreactivity in Schwann cells aligned in the graft and the occurrence of biological responses from NGF-sensitive cells
in the host brain tend to confirm this postulate, which also agrees with the findings of Woolhead (1995) who demonstrated the production of NGF in the PN graft.

One striking effect of NGF on Schwann cells is to enhance the rate of their migration. Previous studies with Schwann cells and PC12 have demonstrated that NGF can upregulate the adhesion glycoprotein L1 in the absence of TrkA activation (Seilheimer and Schachner, 1988). These responses also depend on the appearance of p75 that may regulate the expression of such extracellular matrix proteins in Schwann cells and thereby influence their migration (Anton et al., 1994). Evidence of many columns of Schwann cells with strong p75 immunoreactivity invading striatal parenchyma was found in the present study, exclusively between 1 and 4wpo, and indicates that migration of these non-neuronal cells can also take place in the CNS environment. Moreover, expression of strong p75 in neuropil around the graft may help to achieve migration of Schwann cells by increasing the concentration of NGF in the surrounding parenchyma. Considering the rapid dissociation kinetics of NGF binding to p75 (Levi-Montalcini, 1987), it is possible that expression of p75 receptors either in neurons or in adjacent glial cells (see below) may also participate in increasing the local NGF concentration by binding to NGF diffusing from the graft. The evidence that p75-defective sensory and sympathetic neurons survive well in the culture in the presence of NGF, but need four times more NGF to achieve the same response as wild-type neurons (Barbacid, 1995) leads support to this function of p75. Therefore, the presence of Schwann cells in the surrounding tissue of the graft and upregulation of p75 in neuronal and non-neuronal cells as well as in neuropil around the graft could possibly result in high NGF concentration within this area and this would certainly attract regenerating axons to grow towards it. Woolhead (1995) who examined NGF level in and outside the graft implanted in the striatum by enzyme-linked immunoessay (ELISA) reported the much higher level of this neurotrophin in the graft than that in surrounding striatum. However, this assay was only done at 1 week after graft implantation. The observation that Schwann cell migration into the surrounding striatum occurs after 1 wpo suggests the possibility of the increase of the NGF synthesis in around the graft area over the time and resulting in alteration of the gradients of NGF concentration at the implantation site.
This paradigm possibly explains why more putative regenerating p75 immunopositive axons were found outside the graft. The postoperative time at which the number of columns of Schwann cells in the host brain parenchyma was significantly decreased, i.e., 4 wpo, corresponds to that at which an increase in retrogradely labelled cholinergic perikarya was found. It is not yet clear why the migrating Schwann cells were much less common at long survival times. A possible explanation is that the environment of CNS tissue is inappropriate for Schwann cells to survive, another is that they become undetectable because their marker, p75, is downregulated (in other words, the CNS environment induces a change in the Schwann cells' phenotype). Whatever is the cause, after 4 weeks the level of NGF inside the graft would become comparatively higher, which would attract regenerating axons growing into the graft. Nevertheless, the number of retrogradely labelled cholinergic neurons is still small in comparison with the number of labelled SNpc neurons. If it is the case that most of the regenerating axons found in the striatum, particularly at 1-4 wpo, emerge from cholinergic interneurons whose targets are within the striatum, some of the newly formed axons may establish synaptic connections with immediately neighbouring targets instead of growing into the graft. To verify this hypothesis, electrophysiological studies will be necessary to examine whether or not the injured interneurons established such connections.

3.4) Upregulation of TrkA and p75 immunoreactivity in non-cholinergic striatal neurons induced by a PN graft

At early survival times of 3-4 dpo, in addition to cholinergic interneurons, several small cells close to the brain/graft interface appeared to be immunopositive for TrkA and p75. Some of these small immunopositive cells were similar to small neurons, whereas others had the appearance of astroglia. Normally, astrocytes do not express p75 (Kumar et al., 1990) and cultured rat astrocytes contain no detectable NGF binding sites (DiStefano and Johnson, 1988). However, expression of p75 in reactive astrocytes induced under some circumstances has been reported. For example, p75 immunoreactivity was present in reactive astrocytes after transient forebrain ischemia in gerbil hippocampus (Lee et al., 1995). Gage et al. (1989) found hypertrophy of GFAP immunoreactive reactive astrocytes which showed a gradient with the largest and most
intensely stained cells in closest proximity to the damaged area in the striatum and these astrocytes were also presumably p75 positive. Reactive astrocytes can also synthesize NGF (Lindsay, 1979; Lorez et al., 1989; Shigeno et al., 1991; Hashimoto et al., 1992; Lee et al., 1995; Lee et al., 1998;) and other trophic factor such as CNTF (Rudge et al., 1995; Asada et al., 1995) after tissue injury, suggesting a possible role in promoting cell survival and axon regeneration, although many publications indicate inhibitory effects of astrocytes on CNS regeneration by the formation of a glial scar (see Chapter1: Introduction).

It remains uncertain whether reactive astrocytes participate in CNS axonal regeneration in vivo. Nevertheless, reactive astrocytes found in this study were unlikely to be helpful for the process of axonal regeneration of injured neurons in terms of providing a source of growth promoting factors such as NGF and CNTF, since such cells disappeared by 1 wpo. Moreover, Kordower et al. (1997) reported that numerous p75-expressing astrocytes which were found in the lesioned striatum of the rats, produced for a rodent model of Huntington's disease, would be absent if a source of NGF was provided. Therefore, the absence of p75 immunopositive reactive astrocytes in the present study at early survival times is possibly a consequence of the loss of p75 immunoreactivity in such cells. This phenomenon may occur by an inhibitory effect of NGF, which, in addition to being synthesized from the graft, could possibly be secreted by astrocytes themselves to act as both autocrine and paracrine factors in negative feedback control of p75 protein synthesis in these non-neuronal cells. It is uncertain whether NGF could also exert the same inhibitory effect on TrkA protein synthesis in reactive astrocytes; the parallel disappearance of TrkA and p75 immunoreactivity in these cells may lead one to postulate such an effect.

Another population of striatal cells which was moderately immunopositive for TrkA and p75 is likely to comprise small neurons. These neurons probably included medium aspiny and medium spiny striatal neurons, according to their sizes and shapes. However, without specific staining to detect phenotypic markers of these cells, determination of their cell types remains tentative. It is unclear whether NGF exerts its effect on the appearance of TrkA and p75 immunoreactivity in these non-cholinergic
neurons because there is no evidence to support these findings. This phenomenon is unusual since these neurons are not normally NGF-responsive. It is, however, possible that cytokine or neurotrophic factors rather than NGF derived from the graft induced p75 and TrkA immunoreactivity in these non-cholinergic neurons since the same responses were not detected in the animal implanted with freeze-killed grafts. The presence of p75 and TrkA immunoreactivity in small neurons was transient; no such immunopositive cells were present by 1 wpo. This probably reflects the poor regenerative response in this neuronal population, although a small number of medium aspiny interneurons were reported to be able to regenerate their axons into a PN graft (Woolhead et al., 1998).

3.5) NGF and colocalization of TrkA and p75: a trophic condition to promote axonal regeneration in cholinergic striatal interneurons after injury?

This present study has demonstrated that presumptive large, asiny cholinergic striatal interneurons undergo hypertrophy and reexpress the low affinity neurotrophin receptor, p75 in the presence of living grafts. Many axon outgrowths probably from cholinergic interneurons running along the graft and distributed in the surrounding area are also evident. That these regenerative responses were only mediated by the graft-derived factors was illustrated by the failure of these neurons to mount the same regenerative responses in the presence of freeze-killed, metabolically inactive grafts. The present study has also demonstrated the presence of TrkA and p75 in presumptive cholinergic interneurons whose axon had regenerated into the graft. Colocalization of immunoreactivity for TrkA and p75 in cholinergic interneurons can be good evidence to assume the co-existence of both receptors in these regenerating neurons. In contrast, other populations of striatal neurons did not show the same responses, as did large cholinergic neurons, although some small neurons transiently expressed TrkA and p75 (see Section 3.3). In the light of the present data, the correlated role of NGF, TrkA and p75 in promoting regeneration of cholinergic interneurons is accentuated.

A number of models have been proposed in an attempt to explain how p75 and TrkA interact to transduce neurotrophin signals and why this partnership is advantageous. Among various paradigms, a more generally accepted view is that p75
may facilitate NGF through TrkA receptors (Hempstead et al., 1991; Barbacid, 1993; Chao, 1994; Chao and Hempsted, 1995). A variety of reports have shown that p75 interacts and/or collaborates with TrkA to generate a “high affinity” binding site or to enhance cellular responses to low neurotrophin concentrations (Hempstead 1990, 1991; Barker and Shooter, 1994; Kahle et al., 1994; Mahadeo et al., 1994; Verdi et al., 1994; Twiss et al., 1998 and for reviews, see Chao, 1994; Bredesen and Rabizadeh, 1997) or that it mediates neurotrophin action on neurotransmitter release (Sala et al., 1998). On the other hand, the absence of either TrkA or p75 receptor may lead to a decrease in signal transduction of NGF or to apoptosis (Rabizadeh et al., 1993; Frade et al., 1996; Van der Zee et al., 1996). Mahadeo and his colleagues (1994) showed that when both TrkA and p75 receptors are coexpressed, the rate of association of NGF is increased 25-fold to produce a higher affinity binding site and the rate of internalization is also higher. In MAH cells, a neuronal progenitor cell line, coexpression of TrkA and a large molar excess of p75 substantially enhances the NGF-induced tyrosine autophosphorylation of TrkA, compared with cells expressing TrkA alone. Such cells expressing both TrkA and p75 stop dividing and acquire a mature neuronal morphology more rapidly and with greater efficiency than MAH cells expressing TrkA alone (Verdi et al., 1994). Interestingly, not only concomitant expression of TrkA and p75 but also the ratio of the two receptors are critical to the cellular responses (Chao, 1994; Twiss et al., 1998). Twiss et al. (1998) demonstrated that not only the presence of p75, but also the ratio of p75:TrkA determined the cellular responsiveness to NGF in PC12 or TrkA-tranfected Chinese hamster ovary cells (CHO); a 5:1 ratio of p75:TrkA cDNAs produced the greatest change in NGF-induced acid secretion in CHO cells. In the absence of TrkA expression, p75 cannot produce any signal of such extracellular acidification.

Ostensibly harmful effects can occur to cells which lack the appropriate combination of NGF and its receptors. The study of developing p75-deficient mice showing that p75 apparently mediates apoptosis of cholinergic neurons only in the absence of TrkA and TrkA counteracts this function of p75 (Van der Zee et al., 1996). Correspondingly, in mature rat oligodendrocytes which express p75 but not TrkA, NGF induces apoptosis by binding to p75 resulting in a sustained increase of intracellular
ceramide and c-jun amino-terminal kinase (JNK) activity, which is thought to participate in a signal transduction pathway leading to cell death (Casaccia-Bonnefil et al., 1996). Under some circumstances such as in the absence of NGF, unbound p75 can also cause apoptosis. Two recent in vitro studies show that Expression of p75 induces neural cell death constitutively when p75 is unbound; binding by NGF, however, inhibits cell death induced by p75 (Rabizadah et al., 1993; Barrett and Barrett, 1994). Similarly, transfection of p75 cDNA to a neuroblastoma cell line which lacks expression of both TrkA and p75 activates apoptosis in cultured cells; but, this effect could be greatly inhibited by NGF treatment (Bunone et al., 1997).

Taken together, these results suggest that coordinated binding to both receptors can maximize signal transduction. In other words, the presence of ligand, NGF, is also a prerequisite for TrkA and p75 to mediate signal transduction for cell survival and/or axonal regeneration. Thus, the presence of low and high affinity neurotrophin receptors and NGF produced from the graft can be considered as trophic factors particularly for promoting axon outgrowth in injured large, aspiny cholinergic striatal interneurons.

3.6) Critical period of time for successful axonal regeneration

The number of p75 immunoreactive and hypertrophic TrkA immunopositive neurons gradually declined by 4 weeks after implantation of a PN graft. Also, the intensity of p75 immunoreactivity in the neuropil surrounding the graft was much decreased by the same time. The loss of this NGF responsiveness indicates the reduction of NGF released from the following the times of graft implantation. According to the times of up- and downregulation of p75 immunoreactivity and hypertrophic response in TrkA immunopositive neurons, a high peak of NGF concentration produced from the graft and surrounding tissue is likely between 3 dpo-2 wpo. This duration of time may be considered as the critical period for cholinergic neurons to mount the regenerative response. Hagg et al. (1989), who examined the effects of delayed treatment of NGF on axotomized medial septal cholinergic neurons, also found that the maximal recoveries (i.e., restored cell size and phenotypic markers) of such neurons could be achieved if NGF treatment was given within 3-7 days after axotomy. Accordingly, Asada and colleagues (1996) demonstrated that the secretion
collected form striatal tissue at 1 week after being damaged provided the maximum effects for neuroprotective and neurite-promoting activities in cell culture, although they could not significantly detected the neurotrophic effect of NGF from the secretion. In agreement with these results, NGF should be available to injured neurons as early as possible after axotomy in order to generate effective biological responses in such cells and lead to successful axonal regeneration.

In conclusion, the present study provides strong evidence suggesting that the early presence of NGF and its receptors, TrkA and p75 are crucial for promoting axon regeneration in (presumptive) large, aspiny cholinergic interneurons, although it is very likely that other factors which are also involved in regulating regenerative process of this neuronal population (see also Chapters 4 and 5). Such factors are particularly required for axonal outgrowth in cholinergic striatal neurons but not in other CNS neurons which are not NGF-sensitive such as SNpc neurons.
Figure 3.1 TrkA immunoreactivity in the control striatum

3.1a,b) Low (3.1a) and high (3.1b) power photomicrographs of the corpus striatum illustrating strong TrkA immunoreactivity in presumptive aspiny cholinergic interneurons (a, e.g. arrows). These cells have a few short neurites extending from the cell body, which may be bipolar, fusiform or polymorphic (b). Scale bar = 100μm (3.1a); 50μm (3.1b).

3.1c) Presumptive cholinergic interneurons in the medial septum and nucleus of the vertical limb of the diagonal band also appear to be strongly TrkA immunoreactive. Scale bar = 250μm.

The strong TrkA immunoreactivity in both areas shown in a-c is in a consistent feature of normal animals and of control striatum and serves as a useful positive control for immunocytochemical procedure.

Abbreviations: MS, medial septum; VB, vertical limb of diagonal band.
Figure 3.2 TrkA immunoreactivity in the ipsilateral striatum at 3 days (3.2a-c) and 4 days (3.2d-f) after living PN graft implantation.

3.2a-c) Photomicrographs of the striatum at 3 dpo showing: the area of brain parenchymal border zone (PBZ), striatal parenchyma nearby (a) and the graft tip area (b). 3.2a is rotated 90° anticlockwise from the normal Dorsal-Ventral orientation so that dorsal is to the left. The graft is at the top of the picture. G indicates where the graft, which was lost during sectioning, had been located. Strongly TrkA-immunoreactive cholinergic interneurons can be seen near the graft area in a,b (solid arrows). Both TrkA-immunoreactive neurons, in particular the neuron shown in b,c, exhibit large, round perikarya extending long neurites. This morphology is different from that of TrkA-immunoreactive cells located further away from the graft, which show normal characteristics (open arrows in a,b). A thick process, probably an axon, containing very strong TrkA-immunoreactive, emerges from a large TrkA-immunoreactive perikaryon and is directed towards the graft, as clearly seen in b,c (arrowheads). In addition, small, round perikarya with moderate TrkA-immunoreactive are also present close to the graft/brain interface (a, small arrows). These cells are probably glia, and most likely to be astrocytes. Scale bar = 100μm (3.2a); 50μm (3.2b and inset in 5.2a); 25μm (3.2c).

3.2d-f) Similar to 3 dpo, heavily stained TrkA-immunoreactive neurons with large, round perikarya are seen near the graft in the striatum at 4 dpo (d-f, at arrows) and some of them also show unusually long immunoreactive processes (f, arrowheads). Additionally, numerous small, moderately TrkA-immunoreactive cells, most of which resemble small neurons, are present close to the graft/brain interface (d,e,f, small arrows). Scale bar = 50μm (3.2d and f); 100μm (3.2e).

The orientation marker (M, medial direction; L, lateral direction) in 3.2d applies also to 3.2c and d.

Abbreviation: G, PN graft; PBZ, parenchymal border zone
**Figure 3.3** TrkA immunoreactivity in the ipsilateral striatum at 1 week (3.3a,b) and 10 days (3.3c) following living PN graft implantation.

3.3a) Photomicrograph of the striatum at 1 wpo showing predominantly heavily stained TrkA-immunoreactive neurons near the graft (e.g., large arrows). The perikarya of these TrkA-immunoreactive neurons appear rounded in shape and appear to be more heavily immunoreactive than cells located further from the graft, which commonly display ovoid or spindle-shaped perikarya (small arrows). Scale bar = 100µm.

3.3b) Graft tip area of the striatum shown in 3.3a illustrating a strongly immunoreactive large cell body, roughly triangular in shape. A thin, long fibre, probably a regenerating axon, emerges from one pole of the perikaryon and extends very close to the graft tip (arrowheads). Scale bar = 50µm.

3.3c) Another example of a TrkA-immunoreactive neuron (arrow) near the graft tip from an animal at 10 dpo. Similar to those shown in 3.3a,b, the perikaryon of this neuron, which is located adjacent to the graft tip, contains stronger TrkA immunoreactivity than other TrkA-immunoreactive neurons further away from the graft in the ipsilateral striatum, or in the contralateral striatum (not shown). Scale bar = 50µm.

**Abbreviation:** G, PN graft.
Figure 3.4  TrkA immunoreactive neurons close to PN grafts at different survival times.

3.4a-d) High magnification photomicrographs of TrkA immunoreactive neurons near the graft at 4 dpo (a), 1 wpo (b,c) and 10 dpo (d). All of these neurons display large, rounded, triangular or polymorphic perikarya.

3.4e,f) TrkA immunoreactive neurons in the from contralateral striatum illustrating the normal characteristics of such neurons whose perikarya are usually elongate or bipolar in form (e) or oval (f).

Scale bar = 25 μm for all panels.
**Figure 3.5** TrkA immunoreactivity in the striatum at 4 weeks after living graft implantation.

3.5a,b) Photomicrographs showing a PN graft implanted in the caudal part of the striatum. At this survival time, there is no significant difference in the appearance of TrkA-immunoreactive cells near the graft and those further away in the striatal parenchyma (see 3.5b) or in the contralateral striatum (not shown). Scale bar = 500μm (3.5a); 100μm (3.5b).

3.5c) Enlargement of part of the graft tip area in 3.5a showing a fascicle-like group of TrkA-immunoreactive presumptive regenerating axons (*arrows*) running together to the proximal tip of the graft which is situated at the top of the picture. A long, thin axon running parallel to the fascicle for some distance then seems to loop back on itself as it approaches the graft and joins the fascicle (*arrowheads*). Scale bar = 10μm.

**Abbreviations:** CC, cerebral cortex; Cc, corpus callosum; G, PN graft; ST, corpus striatum
**Figure 3.6** p75 immunoreactivity in the normal striatum of adult rat.

3.6a) Control striatum immunostained for p75 showing no expression of this receptor protein in striatal neurons. Scale bar = 200μm.

3.6b) Strong p75 immunoreactivity in neurons of the medial septum (e.g. arrows). The staining of neurons in this region is used as a positive control for the staining technique. Scale bar = 500μm.

3.6c) An example of a medial septal neuron heavily immunostained for p75. Scale bar = 50μm.
Figure 3.7 p75 immunoreactivity in the striatum at 3 days (3.7a) and 4 days (3.7b,c) following living PN graft implantation.

3.7a) Photomicrograph of the area just below the graft tip in the striatum at 3 dpo showing a few small p75 positive cells, resembling glia, located very close to the graft/brain interface (small arrows), and two adjacent, large, heavily p75-immunoreactive cells, which are presumptive cholinergic interneurons (large arrow). Scale bar = 50µm.

3.7b) Area immediately adjacent to a graft (G) in the striatum at 4 dpo. Within the area, a large presumptive cholinergic interneuron with a cell body heavily immunostained for p75 is surrounded by a group of small and medium-sized cells which display much lighter p75 immunoreactivity (open arrows). Scale bar = 25µm.

3.8c) Area below the graft tip (G) in the striatum of the same animal shown in 3.8b illustrating a strongly p75 immunoreactive neuron situated close to the graft/brain interface. Two neurites from the triangular cell body of this neuron extend directly to the brain/graft interface (arrowheads). Scale bar = 25µm.

Abbreviation: G, PN graft.
Figure 3.8 Distribution of p75-immunoreactive striatal neurons in the ipsilateral striatum at 1 week (3.8a,b) and 4 weeks (3.8c,d) after living graft implantation.

3.8a,b) Photomicrograph of brain parenchyma near the tip of the graft (a) and an enlargement of part of this area (b) showing many strongly p75-immunoreactive neurons distributed within the zone of intensely p75 immunoreactive neuropil surrounding the graft tip (a,b, e.g., arrows). In this animal the graft is situated in the lateral part of the caudal stratum in which p75-immunoreactive neurons appear to be more numerous than in other parts of the striatum. Scale bar = 500μm (3.8a); 200μm (3.8b).

The orientation marker: D, dorsal direction; M, medial direction

3.8c,d) At 4 wpo, many p75-immunoreactive neurons are still present near the graft tip (c, arrows) although fewer than at earlier survival times (cf. 3.8a). A large p75-immunoreactive neuron from c is enlarged in d; this cell shows the characteristics of a healthy aspiny cholinergic striatal interneuron. No signs of cell death are seen in the PBZ around the graft. Schwann cells, aligned in columns within the graft are heavily immunostained for p75; invasion of the surrounding brain parenchyma by Schwann cells is apparent at the arrowheads in c. Scale bar = 500μm (3.8c); 50μm (3.8d).

Abbreviation: G, PN graft.
Figure 3.9  p75 immunoreactivity in the striatum 2 weeks after living PN graft implantation.

3.9a) Photomicrographic montage of the striatum with a living graft implanted showing numerous p75-immunoreactive neurons surrounding the graft, in particular in the brain parenchyma lateral to the graft tip. The boxed areas are enlarged in 3.9b,c,d. Scale bar = 125μm. The orientation marker: L = lateral direction; D = dorsal direction

3.9b,c) High magnification photomicrographs of neurons displaying heavy p75-immunoreactive from medial (b) and lateral sides (c) of the graft. These neurons show all of the characteristics of aspiny cholinergic interneurons. In b, one process of the immunopositive neuron, probably the axon, exhibits small bead-like varicosities (small arrows), a common feature of regenerating axons. A similar axon runs approximately parallel to the long axis of the immunopositive neuronal perikaryon and towards the brain/graft interface (3.9b, arrowheads). Scale bar = 25μm (3.9b,c).

3.9d) Beaded immunoreactive processes resembling regenerating axons, located close to the graft (large arrows); one process is seen extending across the brain/graft interface (small arrows). The neuronal perikarya from which these axons emanate are not present in this section. Scale bar = 25μm.

3.9e) Area below the graft tip and almost contiguous with the area shown in 3.9d (not seen in a). The asterisk indicates a blood vessel which also appears at the bottom of d (and is also indicated by an asterisk). Several p75-immunoreactive presumptive cholinergic interneurons are distributed within the area and among them are short unidentifiable immunoreactive fibres with either smooth (solid arrows) or beaded morphology (open arrows). Scale bar = 50μm.

Abbreviation: G, PN graft
Figure 3.10  p75 immunoreactive neurons in the striatum after living PN graft implantation at 5 days (3.10a,b), 10 days (3.10c) and 2 weeks (3.10d,e). All show common characteristics, i.e., spindle- or oval-shaped perikarya giving rise to a small number of neurites. These characteristics are also those of aspiny cholinergic striatal interneurons (to compare, see also Fig. 3.1b). However, some of these neurons show the unusual appearance of processes which appear to be sprouting from presumptive axons (d,e, arrows). In e, many smooth processes resembling those apparently sprouting from this neuron are present around it (small arrows). Scale bar = 25μm for all panels.
**Figure 3.11** p75 immunoreactive fibres in striatal parenchyma near a PN graft at 10 dpo (3.11b) and 2 wpo (3.11a).

3.11a) Photomicrograph of a massive array of fibres with beaded morphology, presumably regenerating axons, found near the graft tip (which is located at a distance approximately 100 μm from the top right corner of the picture) of the striatum at 10 dpo. These groups of fibres display strong p75-immunoreactivity; many of these fibres are beaded, some with comparatively large varicosities (*large arrows*) and others with small swellings (*small arrows*). The cell bodies giving rise to these fibres did not appear within the same section. Scale bar = 25 μm.

3.11b) Another type of strongly p75-immunoreactive fibre close to the graft resembles an axon sprout (*large arrow*) with numerous processes (*small arrows*) branching from it. This presumptive axonal sprout was found a few hundreds micrometers from the graft tip. Scale bar = 50 μm.
Figure 3.12  p75 immunoreactive neurons and presumptive regenerating fibres in the striatum at 10 dpo.

3.12a) Area below the tip of the graft showing strong p75-immunoreactive in the neuropil of the surrounding PBZ (seen as darker area). Within this area many strongly p75-immunoreactive neurons are widely distributed (e.g. arrows). Among the immunopositive cells, are p75 immunoreactive Schwann cells or short column of Schwann cells which appear to have penetrated the brain parenchyma (e.g. open arrows). Scale bar = 125μm.

3.12b) Detail of the boxed area in 3.12a showing a strong p75 immunoreactive process resembling a regenerating axon through the PBZ and approaching the brain/graft interface (arrows). Open arrows indicate immunopositive short column of Schwann cells in the brain parenchyma. Scale bar = 50μm.

Abbreviations: G, PN graft; PBZ, parenchymal border zone; ST, striatum.
Figure 3.13 p75 immunoreactivity in the striatum 4 days after implantation of a freeze-killed PN graft

3.13a,b) The striatum at 4 dpo following freeze-killed graft implantation shows a much lower level of immunoreactivity in neuropil around the graft than at the same stage after transplantation of a living graft. Only a few lightly immunopositive neurons (large arrows in b) and small cells, resembling glia, (small arrows) displaying faint p75-immunoreactive are seen near the graft, as shown in b (detailed of boxed area in a). G indicates the position of the graft, which was lost during the tissue processing. Scale bar = 250μm (3.13a); 50μm (3.13b).
Figure 3.14 Immunofluorescence staining for TrkA and p75 in the striatum 2 weeks after implantation of a living PN graft

3.14a-h) Pairs of colour photomicrographs (a,c and e,g in the left vertical row) of the PBZ taken through specific filters for Texas red (to view p75-immunoreactivity, seen in red), a,b and e,f, or for FITC (to view TrkA-immunoreactivity, seen in green), c,d and g,f. The pictures on the right are the enlargements of parts of the corresponding pictures on the left. The p75-immunoreactive neurons (a,e, arrows) found near the graft are also TrkA immunopositive (c,g, arrows); overlap of labelling in cell bodies is virtually complete in these figures. Scale bar = 100 μm (3.14a, b); 25 μm (3.14b, d, f, h); 50 μm (3.14e, g).

Abbreviation: G, PN graft.
Figure 3.15  Colocalization of TrkA and p75 receptors in neurons of the striatum 2 weeks following implantation of a living graft. The section was processed for double immunofluorescence staining for TrkA and p75, which appear as green and red colours respectively.

3.15a) Confocal images of double immunofluorescence staining for TrkA (green) and p75 receptors (red) in an area of brain parenchyma surrounding the graft. Three neurons double stained for TrkA and p75 (a, arrows) are located near the graft, which is devoid of TrkA but is very heavily immunopositive for p75. Scale bar = 50μm.

3.15b) High power photomicrograph of the uppermost of the three neurons in 3.15a illustrating more clearly the colocalization of TrkA and p75 in the cell body. Note that the signal of TrkA is more concentrated on the periphery of the perikaryon whereas that for p75 is concentrated more centrally. The presumptive axon of this neuron extends directly towards the brain/graft interface. A few linear arrays of p75-immunoreactive Schwann cells, which appear to have penetrated into the PBZ are also present nearby (3.15b, arrows). Scale bar = 10μm.

Abbreviations: G, PN graft; PBZ, parenchymal border zone.
**Figure 3.16** Confocal image of the same material as shown in Fig. 3.15 illustrating part of the graft tip and adjacent brain parenchyma. The Schwann cells within the tip of the graft at top centre of the picture are predominantly immunostained for p75. A few fibre-like structures, possibly regenerating axons, in this region of the graft tip appear to be TrkA immunoreactive (*small arrow*). Two neuronal perikarya below the tip of the graft are immunopositive for both TrkA and p75 (*large arrows*). Three putative regenerating axons, all with clearly beaded morphology are seen at the left of the picture, apparently heading towards the graft tip; one is p75-immunoreactive (*large arrowhead*) and the other two are TrkA-immunoreactive (*small arrowheads*). Scale bar = 20µm.

**Abbreviation:** G, PN graft.
Figure 3.17 Localization of TrkA receptor identified in regenerating neurons of the striatum 7 weeks (3.17a,b) and 12 weeks (3.17c,d) after graft implantation and 2 days after application of CT-HRP to the distal tip of the graft.

3.17a) Striatum at 7 wpo processed for TrkA-immunoreactive and TMB histochemistry for HRP visualisation. TMB product (identifying retrogradely transported HRP) is dark brown while TrkA-immunoreactive appears in light brown. Many regenerating axons containing HRP reaction product are apparent both inside the graft (small arrow) and at the graft tip (large arrows). A neuron whose cell body displays both HRP reaction product and with TrkA-immunoreactive is located close to the graft (open arrow). Numerous TrkA-immunoreactive neurons are widely distributed in the surrounding brain parenchyma (e.g., arrowheads). Scale bar = 200µm.

The orientation marker: D = dorsal direction; M = medial direction

3.17b) Enlargement of the double-labelled neuron in 3.17a. Particles of reaction product are mostly concentrated around the nucleus and sparsely scattered along the neuron’s processes. Scale bar = 10µm.

3.17c) Medium power photomicrograph of the striatum at 12 wpo showing part of the tip of a graft and the brain tissue lateral to it. A neuron with light HRP labelling and strong TrkA-immunoreactivity in its perikaryon (arrow) extends its axon directly towards the graft. Scale bar = 25µm.

3.17d) High magnification photomicrograph of the neuron shown in 3.17c. The plane of focus is through the axon and scattered granules of intra-axonal HRP reaction product (arrows). Scale bar = 25µm.

Abbreviation: G, PN graft.
Figure 3.18 Localization of p75-immunoreactivity in identified regenerating neurons of the striatum 7 weeks after graft implantation and 2 days after application of CT-HRP to the tip of the distal graft.

3.18a) Low power photomicrograph of a section of striatum immunostained for p75 and reacted by TMB histochemistry, showing two neurons heavily labelled with HRP located adjacent to the graft (arrows). Scale bar = 100μm. The orientation marker: D = dorsal direction; M = medial direction

3.18b) Enlargement of the lower of the two neurons seen in 3.18a. This neuron displays both strong p75-immunoreactive and HRP reactive product in its perikaryon and along its putative axon. Scale bar = 20μm.

3.18c,d) Medium power photomicrograph of another section from the same animal as the one illustrated in 3.18a, showing a large presumptive cholinergic interneurons with a spindle-shaped perikaryon situated adjacent to the graft (large arrow). This neuron contains dark brown particles of HRP reaction product and is also immunopositive for p75 as seen in the enlargement in 3.18d. A few axons containing HRP, possible regenerating axons are seen running parallel to and approaching the graft (c, small arrows). One of these axons appears to originate from the double labelled neuron; however the point of connection between the axon and cell soma (d, arrowheads) is not clear enough to be certain. Scale bar = 25μm (3.18c,d).

Abbreviation: G, PN graft.
Figure 3.19  Regenerating neurons in the substantia nigra ipsilateral to a living peripheral nerve grafted to the striatum 7 weeks previously.

3.19a,b) Substantia nigra from the same brain shown in 3.17a and 3.18, immunostained for p75-immunoreactive and reacted with TMB to detect HRP, showing three neurons strongly labelled with HRP in the SNpc. These neurons and all other neurons of the SNpc and SNpr are devoid of p75-immunoreactivity. Scale bar = 250μm (3.19a); 100μm (3.19b).

3.19c) Substantia nigra immunostained for TrkA and reacted with TMB histochemistry to detect HRP, showing a few SNpc neurons labelled with HRP. The HRP labelled neurons show no TrkA immunoreactive and neurons of the SNpc and SNpr normally contain very low to negligible level of TrkA receptor protein. Scale bar = 200μm.

Abbreviations: SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata.
CHAPTER 4

Expression of CHL1 mRNA in the striatum following peripheral nerve graft implantation

Some CNS neurons, which normally display little regenerative ability after injury, can regenerate their axons vigorously in the presence of a PN graft. These neurons include cholinergic striatal interneurons (Woolhead et al., 1998), TRN neurons (Benfey et al., 1985, Morrow et al., 1993) and DN neurons (Vaudano et al., 1998; also Anderson et al., 1998 for review). Nevertheless, not all CNS neurons demonstrate the same regenerative powers; in fact, CNS neurons show strikingly differential propensities in regenerating their axons into the graft. This may result from the cells having different capacities to express some molecules which are necessary for successful axonal regeneration. Although the molecular basis of the regenerative response to axotomy is still very poorly understood, a considerable amount of data suggests that the competence to upregulate particular genes, which are highly expressed during axonal growth at developmental stage but downregulated in adult, may play an important role in determining the intrinsic regenerative capability of CNS neurons. The upregulation of expression of genes for the transcription factor c-jun (Jenkins et al., 1993a-c; Vaudano et al., 1998), the growth-associated protein GAP-43 (Campbell et al., 1991; Vaudano et al., 1995; Chong et al., 1992, 1994a,b), and the cell recognition molecule L1 (Zhang et al., 1995) in injured CNS neurons has been reported to be associated with axonal regeneration of such neurons. CHL1, a close homologue of the cell recognition molecule L1, has been recently identified as a more powerful promoter than L1 of axonal outgrowth in vitro (Holm et al., 1996; Hillenbrand et al., 1999). As with other neuronal growth-associated molecules, CHL1 is predominantly expressed in the CNS during the later stage of fetal brain development, but is downregulated at the postnatal stage. Thus, CHL1 may be one of the key factors for promoting axon regrowth by CNS neurons. If so, neurons whose axons regenerate into the PN grafts are likely to express CHL1. To investigate this hypothesis, this chapter examines expression of CHL1 mRNA in the corpus striatum, a part of the CNS in which axons...
show different abilities to regenerate in the presence of a PN graft. The expression of this molecule in neurons of the SNpc which project to the striatum is also examined.

1. Summary of methods used
(See Chapter 2 for details)

Nineteen adult rats received autologous tibial nerve grafts implanted into the left striatum. At 1-4 days (n=6), 1 week (n=4), 2 weeks (n=4), 4 weeks (n=2), and 6-10 weeks (n=3) after grafting, the rats were deeply anaesthetized and then decapitated. Cryostat sections, cut at a neuronal thickness of 12 μm, of the striatum and substantia nigra, were processed for in situ hybridization using digoxigenin-labelled CHL1 cRNA probe. Some sections were also immunoreacted with antibody against GFAP or trkA, using the immunofluorescence histochemistry method.

As controls, 7 animals received freeze-killed grafts implanted into the striatum and were allowed to survive for 3 days to 4 weeks. The contralateral striatum of the operated animals and the striatum of unoperated animals were used as controls and to compare with the grafted striatum.

2. Results

2.1) Expression of CHL1 mRNA in the intact striatum and substantia nigra

Striatal neurons of both unoperated animals and contralateral brain expressed a low level of CHL1 mRNA. Some unidentified striatal neurons of various sizes expressed moderate CHL1 mRNA signals; such cells were distributed apparently randomly and were not conspicuous (Fig. 4.3). In contrast, neurons of the SNpc and SNpr displayed a moderate to strong level of CHL1 mRNA expression (Fig. 4.7b,d).

2.2) Expression of CHL1 mRNA in the striatum and substantia nigra following the implantation of a living PN graft

Striatal neurons showed a striking response to the implantation of the graft by upregulating CHL1 mRNA from the earliest survival time examined (1 dpo). Between 1 to 4 dpo, in situ hybridization identified a large population of striatal cells expressing a very strong level of CHL1 mRNA widely distributed in the ipsilateral striatum (Fig.
Cells containing CHL1 mRNA had both small and large perikarya; but large cells predominated among CHL1 mRNA positive cells. These large cells had perikaryal diameters of approximately 20-25 μm, similar to that of large, aspiny, cholinergic striatal interneurons. The neuronal characteristics of the large CHL1 mRNA positive cells were confirmed by thionin counterstaining (Fig. 4.4). Moreover, many of the large perikarya which expressed CHL1 mRNA were also clearly trkA immunoreactive (Fig. 4.5c). However, not all large CHL1 mRNA containing cells were trkA immunopositive; trkA immunoreactivity was not detected in very strongly CHL1 mRNA labelled cells. It is possible that such cells had virtually no trkA, but it is also possible that the strong signal for CHL1 message may have masked the presence of trkA expression. Some of the small CHL1 mRNA labelled cells were immunopositive for GFAP; these were exclusively concentrated within the parenchymal border zone (PBZ) indicating that they could be astrocytes (Fig. 4.5a,b). The other small CHL1 mRNA positive cells could not be precisely identified by the methods used but presumably included other types of glial cells and/or small striatal neurons. Presumptive CHL1 mRNA positive cholinergic interneurons were distributed widely in the striatal parenchyma, not only concentrated close to the graft; the longest distance between the graft tip and strongly CHL1 mRNA positive cells was approximately 1.5 mm.

At 1 wpo, strong CHL1 mRNA labelled cells were still numerous but there was a dramatic reduction in the population of small CHL1 mRNA positive cells. The number of CHL1 mRNA positive cells declined ever further by 7 wpo; only a small number of such cells were detected near the graft and almost all of them were presumptive aspiny cholinergic interneurons (Fig. 4.6e). At 10 wpo, CHL1 mRNA positive cells could be identified very rarely; one cell was found near the graft tip in one animal of this group. Interestingly, even though very few CHL1 mRNA containing cells were present at long survival times, CHL1 mRNA expression in these cells, nevertheless, was still remarkably high (Fig. 4.6d-f).
In the substantia nigra, there was no difference detectable in the visual intensity of signal for CHL1 mRNA in neurons of the SNpc and SNpr, before and after PN graft implantation, or at any survival times investigated (Fig. 4.7).

2.3) Expression of CHL1 mRNA in the striatum following implantation of a freeze-killed graft

A significant number of CHL1 mRNA containing cells was identified in the corpus striatum ipsilateral to the graft from 3 dpo to 2 wpo following freeze-killed graft implantation; the number of positive cells located around the graft, however was much smaller than around living grafts at the same survival times (Fig 4.8). Similarly, the types of cells upregulating CHL1 mRNA in response to freeze-killed graft insertion at 3-4 dpo included GFAP immunopositive astrocytes, unidentified small striatal neurons and presumptive aspiny striatal cholinergic interneurons. A few large CHL1 mRNA positive perikarya were still seen at 2 wpo (Fig. 4.8e and f). There was no evidence of CHL1 mRNA expression in striatal neurons at 4wpo.

There was no detectable change in the expression of CHL1 mRNA in neurons of the SNpc or of the SNpr following freeze-killed graft implantation (Fig. 4.9).

3. Discussion

The present study has shown that mRNA for the cell adhesion/recognition molecule CHL1 is generally present at low levels in striatal neurons of adult rats. In particular, negligible levels of CHL1 mRNA are expressed by cells with the characteristics of large, aspiny cholinergic interneurons. After injury alone (implantation of a freeze-killed graft), CHL1 mRNA was transiently upregulated by striatal neurons and glial cells including reactive astrocytes (which were GFAP immunopositive). This transient expression of CHL1 mRNA in striatal neurons and glia was downregulated again by 2 wpo. However, in the presence of a living PN graft, expression by striatal neurons was prolonged until 10 weeks, although the numbers of positive cells became gradually smaller after longer survival times. Interestingly, at early postgrafting survival periods (1dpo-1wpo), CHL1 mRNA-labelled cells seemed to
include more than one type of striatal neuron, one of which was characterized by a large perikaryon. Coexpression of trkA immunoreactivity by large neurons which expressed CHL1 supports the possibility that these CHL1 mRNA positive cells are large, aspiny cholinergic interneurons. With extended survival times, almost all of the CHL1 mRNA positive neurons found around the graft tip resembled large presumptive cholinergic neurons. In this study, the expression of CHL1 mRNA in neurons whose axons had regenerated along the graft was not demonstrated, due to some difficulties in retrograde labelling. However, upregulation of this cell adhesion molecule in identified regenerating neurons in the thalamus and cerebellum has been clearly shown in Chapters 6 and 7, respectively. Moreover, strong constitutive expression of CHL1 mRNA was found in neurons of the SNpc, which show a strong regenerative response to PN graft implantation into the striatum (Woolhead et al., 1998). Therefore, the results obtained from the present study strongly suggest a role for CHL1 in promoting axonal regrowth in striatal neurons, and demonstrate that cholinergic striatal interneurons have a capacity to upregulate this growth-related molecule in addition to GAP-43 (Woolhead et al., 1998) and c-jun (Chapter 5).

3.1) Expression of CHL1 mRNA in injured striatal neurons

Generally the level of CHL1 mRNA expression in the striatum is very low, although low to moderate levels of expression of this molecule are found in some intact cells. Apparently, CHL1 gene expression in cells of the striatum can be primarily induced by a mechanical lesion; the presence of many CHL1-positive cells following the implantation of a freeze-killed graft which produces no long distance axonal regeneration supports this view. After injury, the level of CHL1 mRNA in injured neurons is upregulated very rapidly, as evident in the appearance of intense expression of CHL1 mRNA in a large number of cells in the ipsilateral brain, 1 day after implantation of a PN graft. The distribution of positive cells was widespread, not restricted to regions near the graft, but also throughout the ipsilateral neostriatum. This probably indicates that the implantation of a graft into the striatum causes injury to a massive number of its intrinsic neurons which respond by expressing CHL1 mRNA. Cells showing hybridization signals for CHL1 mRNA, particularly those found at early survival times (between 1-4 dpo), displayed various sizes. The small positive cells,
mostly lining the graft/brain interface were also immunopositive for GFAP, indicating that they were probably astrocytes (see discussion below). Some large CHL1 mRNA expressing cells, which were shown to be large neurons by thionin counterstaining, were also immunopositive to trkA, a phenotypic marker for aspiny cholinergic interneurons (Steininger et al., 1993; Sobreviela et al., 1994). Other types of CHL1 mRNA positive cells were probably medium spiny and aspiny neurons, and/or other glia cells. However, with the techniques used in the present study, it is impossible to determine the exact types of these cells and the identification of other cell types made in this study remains tentative.

Even though many cell types of the striatum display a significant ability to express CHL1 mRNA in response to injury, different intensities of labelling were apparent among the CHL1 mRNA positive cells. The strongest signal for CHL1 mRNA was exclusively found in presumptive cholinergic interneurons. Transient expression of CHL1 mRNA in CNS neurons shortly (i.e., between 1dpo - 2 wpo) following axotomy is not a surprising phenomenon. Many studies have shown that a mechanical injury to the brain or spinal cord of the adult rat is a sufficient stimulus to elicit a temporary upregulation of growth-associated molecules in axotomized CNS neurons (Poltorak et al., 1993; Vaudano et al., 1995, 1998). For instance, a stab wound in the adult rat thalamus or cerebellar cortex causes the upregulation of expression of c-jun and GAP-43 mRNAs and increases the immunoreactivity for both molecules in neurons around the lesion, although these signals disappeared within 2 weeks (Vaudano et al., 1995, 1998). Similar results can be found in Chapters 5-8, which describe significant upregulation of c-jun mRNA in neurons of the striatum (Chapter 5), CHL1 mRNA in neurons of the thalamus and cerebellum (Chapters 6 and 7, respectively), and L1, c-jun and GAP-43 mRNAs in neurons of the cerebellum (Chapter 8) around the implantation site of freeze-killed grafts at early survival times. In mice, a lesion-induced change in expression of cell adhesion molecules in neurons of the dorsomedial striatum has also been reported by Poltorak et al. (1993). In their study, the immunostaining intensity of L1 and NCAM appeared to be enhanced in neurons within the most medial-periventricular and dorsomedial parts of the striatum after the removal of somatosensory and motor cortex of mice. However, it is readily apparent from the data
of Poltorak et al. (1993) that expression of growth-promoting molecules in axotomized adult CNS neurons is downregulated within a brief period of time after injury. The failure to maintain gene expression of these molecules, which may be essential to promote axonal growth in CNS injured neurons, indicates the lack of ability to mount a successful regenerative response in the absence of signals from a living nerve graft.

3.2) The effects of a living PN graft on the expression of CHL1 mRNA in injured striatal neurons and its correlation with axonal regeneration

While the expression of CHL1 mRNA in neurons of the striatum was downregulated and became insignificant by 2 weeks following the implantation of a freeze-killed graft, CHL1 mRNA expression was maintained for a longer period in the presence of a living nerve graft. Interestingly, the population of cells expressing CHL1 mRNA found after 2 wpo in animals implanted with a PN graft appeared to comprise predominantly presumptive cholinergic interneurons, most of which were located near the graft tip. Thus, a living nerve graft prolonged expression of CHL1 mRNA selectively in presumptive cholinergic neurons in the vicinity of the graft. As shown in the previous Chapter and elsewhere (Benfey and Aguayo, 1982; Woolhead et al., 1998) a living PN graft promotes axonal regeneration from axotomized striatal neurons, particularly cholinergic interneurons (Woolhead et al., 1998). Therefore, upregulation of CHL1 mRNA expression in striatal neurons is a response to injury of such cells and importantly, the ability to maintain high levels of CHL1 mRNA expression in the presence of a living PN graft is a characteristic of presumptive cholinergic interneurons and seems likely to reflect the higher regenerative capacity of this cell type. Furthermore, it has long been known that axonal injury close to the cell body results in more profound chromatolytic changes than following distant injury (Lieberman, 1974). Consequently, one possible explanation of the fact that following long survival times only those presumptive cholinergic neurons found near the graft display high level of CHL1 mRNA is that those neurons were subjected to a sufficiently proximal axotomy to initiate the necessary changes in gene expression at an adequate level to promote axon growth. The transient expression of CHL1 mRNA in cells situated at a distance (over 100μm away from the graft) supports this speculation. In SNpc neurons, the expression of CHL1 mRNA is normally high and its level in these neurons seems to be
unchanged following the implantation of a PN graft into the striatum even though they are known to be capable of regenerating axon into living nerve grafts (Woolhead et al., 1998). During the implantation of a graft, SNpc neurons, whose axons project to the striatum, would certainly be axotomized relatively close to their terminal fields. None the less, these neurons can successfully regenerate into grafts. These phenomena show that the relationship between regenerative success and the site of injury is not a simple one. However, besides CHL1, SNpc neurons constitutively express several other growth-associated molecules such as L1, NCAM and GAP-43 at high levels and the expression of these molecules is also apparently not altered after cell injury and the implantation of a PN graft (Woolhead et al., 1998). It is possible that the basic levels of growth-associated gene expression in SNpc neurons, together with trophic factors derived from Schwann cells in the graft, are already sufficient to lead to successful axon regeneration. However, since the basal level of CHL1 mRNA signal in nigral neurons was rather high, it is also possible that its strong expression masked the upregulation of this mRNA.

The number of CHL1 mRNA-positive neurons in the striatum was obviously decreased by 4 weeks after the implantation of a living nerve graft. As has been discussed in Chapter 3 (see section 3.3), between 2-4 wpo some axotomized striatal neurons, presumably cholinergic interneurons, may regenerate axons and establish synaptic contact with neighbouring neurons instead of sending axons along the graft. This might possibly occur due to the pressure of adequate amounts of trophic factor(s) (e.g., NGF) being available in the extracellular matrix surrounding the graft. There are several examples of growth-related molecules upregulated in regenerating neurons following injury being downregulated upon target innervation. For instance, following the implantation of NGF-producing fibroblasts in a lesion cavity in the fimbria-fornix, the expression of polysialic acid (PSA) and L1 is upregulated in regenerating cholinergic axons during axonal elongation and downregulated once axons reach the hippocampal formation (Aubert et al., 1998). Furthermore, in a study of CHL1 mRNA expression in the spinal cord and dorsal root ganglion (DRG) it was demonstrated that the upregulation of CHL1 mRNA in injured motor neurons and DRG neurons is prolonged when the sciatic nerve is cut and ligated to prevent regenerating axons.
reconnecting to their target tissues, but falls towards normal levels at the time of reinnervation following crush lesion (Zhang et al., submitted for publication). These examples suggest that one explanation of the reduction in the number of CHL1 mRNA-expressing neurons during 2-4 wpo is the downregulation of CHL1 mRNA in those neurons whose axons have successfully regenerated and reconnected to their targets. Alternatively, the neurons which downregulated CHL1 mRNA may be those which fail to receive a sufficient stimulus from the graft to initiate regrowth of their axons.

At survival times longer than 4 weeks, the number of CHL1 mRNA-labelled putative cholinergic neurons had steeply decreased; only a few such cells were found near the graft tips. This finding is very similar to that in Chapter 3, showing a few cholinergic interneurons immunopositive to trkA and p75 located adjacent to the graft tip at late survival times and that those neurons could be retrogradely labelled through the graft. The number of striatal neurons which can be retrogradely labelled from grafts (i.e., the number which grow axons through the grafts) is always very low (Woolhead et al., 1998). Moreover, there is strong evidence indicating that CHL1 mRNA is expressed by identified regenerating neurons of the thalamic reticular nucleus when grafts are implanted into the thalamus (Chapter 6) and the deep cerebellar nuclei when graft are implanted into the cerebellum (Chapter 7). Thus, it seems very likely that CHL1 mRNA-labelled presumptive cholinergic neurons found at late survival times correspond to neurons whose axons have regenerated through the graft.

3.3) Expression of CHL1 mRNA in non-neuronal cells

In the present study, one of the early responses to the implantation either of a living or freeze-killed graft was the appearance of small cells with strong CHL1 mRNA expression in a restricted area close to the brain/graft interface. Such small cells are also immunopositive for GFAP suggesting that they could be astrocytes. It is unclear whether expression of CHL1 in reactive astrocytes benefits axonal regeneration. It is well established that the glial scars which form following injury in the CNS mainly consist of hypertrophied astrocytes and their processes, and such scars have long been regarded as an important factor inhibiting axonal regeneration (Cajal, 1928; Reier et al., 1983, 1986, 1989). Nevertheless, positive effects of astrocytes on growing axons from
injured CNS neurons or on survival of such neurons have also been described both in vitro (Noble et al., 1984; Neugebauer et al., 1988; Ard and Bunge, 1988; Ard et al., 1991; Fawcett et al., 1992; Brown, 1999) and in vivo (Gage et al., 1988; Kliot et al., 1990; Kawaja and Gage, 1991). Recently, Chalmers and colleagues (1996) demonstrated intriguing data showing that sprouting cholinergic axonal processes that grew into grafts of NGF-producing genetically manipulated fibroblasts within the striatum of adult rats were fasciculated and followed the surface of astrocytic processes for long distances within the grafts. This result supports previous ultrastructural evidence that astrocytes may serve as a cellular substrate for sprouting cholinergic axons in vivo (Kawaja and Gage, 1991). Moreover, according to the study of Chalmers et al. (1996), the astrocytic processes in NGF-producing fibroblast grafts expressed the neural cell adhesion molecule (NCAM), suggesting that NCAM-mediated adhesion may be responsible for the close relationship between the axons and astrocytes within the grafts. Similarly, reactive astrocytes found near the PN grafts implanted in the thalamus (Zhang et al., 1995) and the striatum (Woolhead et al., 1998) expressed a high level of NCAM and in both cases there were significant numbers of regenerating axons from injured CNS neurons growing into the grafts. Accordingly, if astrocytes found near the brain/graft interface in the present study actually participate in supporting axons growing into the graft, CHL1 mRNA expressed by these non-neuronal cells possibly plays a specific role in cell-cell interaction between regenerating axons and astrocytic processes. However, the marked decrease in the number of CHL1 expressing reactive astrocytes at the graft/brain interface by 2 wpo leads to the conclusion that if such cells are really beneficial for regenerating CNS axons attempting to grow from the host brain into the graft, such axons would have a more difficult task after 2 wpo. Some CNS axons do regenerate their axons into nerve grafts prior to 2 weeks after injury (Campbell et al., 1992; Woolhead, 1995) and the downregulation of CHL1 mRNA after this time may explain the disparity between the number of sprouting axons around the graft and the number of neurons which can be retrogradely labelled (Woolhead et al., 1998)

CHL1 mRNA is strongly upregulated in presumptive Schwann cells in the graft. Likewise, NCAM and L1 mRNAs are also highly expressed by these non-neuronal
cells in the PN grafts (Zhang et al., 1995; Woolhead et al., 1998). The presence of these cell recognition molecules in Schwann cells suggests that in addition to providing some growth factors for promoting axon regeneration in axotomized CNS neurons of the host brain (see Chapter 1), these cells may function as a supporter to regenerating axons inside the graft by way of cell-cell contact.

The role of CHL1 in promoting regeneration of CNS axons into PN grafts and possible coordinated expression of CHL1 and other growth-related molecules are further discussed in Chapter 8 and 9.
Figure 4.1  Diagram showing the implantation site of a peripheral nerve graft in the striatum.

Figure 4.2  CHL1 mRNA expression in the corpus striatum at 1 dpo.

4.2a) Striatum containing a graft (G) and showing upregulation of CHL1 mRNA at 1 dpo. Cells displaying a strong hybridization signal are abundant in striatal parenchyma around the graft tip (e.g. arrows). Note that the CHL1 mRNA positive cells distributed around the graft are variable in size and show different intensities of labelling. Mostly, small-sized, moderately CHL1 mRNA labelled cells are concentrated along the glia limitans and within the PBZ. Scale bar = 250μm (4.2a,b)

4.2b) Striatum contralateral to 4.2a showing low expression of CHL1 mRNA in striatal cells. However, small- and medium-sized cells with moderate labelling for CHL1 mRNA are also seen (e.g. arrows).

Abbreviations: CC, cerebral cortex; Cc, corpus callosum; G, PN graft; JZ, junctional zone; PBZ, parenchymal border zone; ST, striatum.
**Figure 4.3** Expression of CHL1 mRNA in the control striatum

4.3a) Photomicrographs of the corpus striatum from an unoperated animal illustrating low expression of CHL1 mRNA in striatal neurons. Striatal neurons with a moderate signal for CHL1 mRNA are sparsely distributed (*e.g.* arrows). Scale bar = 250μm.

4.2b) Neurons in the globus pallidus express high level of CHL1 mRNA (*e.g.* arrows). Scale bar = 500μm (4.3b,c).

4.3c) Strong CHL1 mRNA signal is also present in neurons of the medial septum and vertical limbs of the diagonal band (*arrowheads*).

**Abbreviations:** CC, cerebral cortex; Cc, corpus callosum; G, globus pallidus; MS, medial septum; ST, striatum; VB, vertical limb of the diagonal band.
Figure 4.4 Striatum at 1 week following the implantation a living PN graft

4.4a,b) Area around the graft tip containing numerous strongly CHL1 mRNA labelled cells, most of which are large, presumptive aspiny cholinergic striatal interneurons (a, e.g. arrows). The positive cells in the boxed area of a are enlarged in b. The asterisk indicates a lesion area ventral to the graft tip which resulted from implantation of the graft. Scale bar = 500μm (4.4a); 20μm (4.4b)

4.4c) Striatum counterstained with thionin showing an intensely CHL1 mRNA expressing cell (encircled) containing a large vesicular nucleus which helps identify it as a neuron. Scale bar = 125μm

Abbreviations: G, PN graft.
Figure 4.5 Dual localization of CHL1 mRNA and GFAP immunoreactivity (4.5 a and b) and CHL1 mRNA and trkA immunoreactivity (4.5 c) in the striatum at 4 dpo. The sections were hybridized with probe for CHL1 mRNA and immunostained with antibody to GFAP or trkA (immunofluorescence). Immunoreactivity for GFAP and trkA appears as white area and CHL1 mRNA positive signals appears as black blobs.

4.5a,b) Photomicrographs of the brain/graft interface (a) and adjacent brain parenchyma (b) showing both small and large CHL1 mRNA containing cells within these areas. Many small, moderately CHL1mRNA-labelled cells appear to be GFAP immunopositive astrocytes (e.g. small arrows); but, large CHL1 mRNA containing cells near the graft are devoid of immunoreactivity for GFAP (large arrows). G indicates where the graft, which was lost during tissue processing, had been located.

4.5c) Area of brain parenchyma adjacent to that shown in 4.5b. Many large CHL1 mRNA-labelled cells, presumably cholinergic interneurons, display both CHL1 mRNA signal and trkA immunoreactivity (white arrow). TrkA immunoreactivity is not visible in some large neurons with exceedingly strong signals for CHL1 mRNA (open arrows).

Scale bar =25μm for all panels
Figure 4.6 Striatum at various survival times after implantation of a living graft: 1 day (4.6a), 4 days (4.6b), 2 weeks (4.6c), 4 weeks (4.6d), 7 weeks (4.6e) and 10 weeks (4.6f)

4.6a-f) Photomicrographs of the graft tip areas showing CHL1 mRNA positive neurons, the numbers of which decrease progressively with increasing survival time. Large arrows in a-e indicate large striatal neurons with strong expression of CHL1 mRNA. Intensely CHL1 mRNA-labelled presumptive cholinergic interneurons are few at 7 wpo (e) and only one is present (arrow) at 10 wpo (f). Note that numerous small CHL1 mRNA (both moderately and strongly) labelled cells are mainly concentrated within the PBZ at 1-4 dpo (small arrows in a and b) but are much less common by 4 weeks. Schwann cells within in the graft express high level of CHL1 mRNA although such expression is weaker at early survival times (a). The strong staining of the graft in b is a consequence of over-reacting the section with chromogen. Scale bar = 250μm for all panels

Abbreviations: G, PN graft; PBZ, parenchymal border zone.
**Figure 4.7** Substantia nigra at 1 week following the implantation of a living PN graft in the striatum.

4.7a-d) High levels of CHL1 mRNA expression in neurons of the SNpc (e.g., arrowheads in c,d) and SNpr (e.g., small arrows in c,d). No difference in CHL1 expression is apparent between the SNpc and SNpr ipsilateral to (a) and contralateral to the graft (b). The SNpc and SNpr are enlarged in c and d. Scale bar = 500μm (4.7a,b); 250μm (4.7c,d)

**Abbreviations:** SNpc, substantia nigra pars compacta; SNpr; substantia nigra pars reticulata
Figure 4.8. Expression of CHL1 mRNA in the striatum at 3 days (4.8a,b), 4 days (4.8c,d) and 2 weeks (4.8e,f) following the implantation of a freeze-killed graft into the striatum on one side. Fig. 4.8b,d,f are enlargements of Fig. 4.8a,c,e respectively.

4.8a-f) Photomicrographs of the freeze-killed graft tip area showing numerous CHL1 mRNA containing cells (e.g., arrows) around the graft at 3-4 dpo but very few at 2 wpo. G in 4.6 c and d indicates where the main part of the graft, which was lost during tissue processing, had been located. Scale bar = 500μm (4.8a,c,e); 250μm (b,d,f).

Abbreviation: G, PN graft
Figure 4.9 Substantia nigra at 3 days (4.9a,b) and 4 weeks (4.9c,d) following the implantation of a freeze-killed graft into the striatum.

4.9a-d) Strong expression in SNpc and SNpr neurons both ipsilateral (a,c) and contralateral (b,d) to the striatum containing the graft. No alteration in hybridization signal is detectable in response to freeze-killed graft implantation. Scale bar = 500μm for all panels

Abbreviations: SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata.
CHAPTER 5

Expression of c-jun mRNA in the striatum following peripheral nerve graft implantation

The proto-oncogene c-jun, an inducible transcription factor and a member of the immediate early gene family, has been shown to be upregulated by many types of axotomized CNS neuron (Jenkins and Hunt, 1991; Leah et al., 1991, 1993; Herdegen et al., 1992, 1993b; De Felipe et al., 1994; Jenkins et al., 1993 a,b,c; Brecht et al., 1994; Vaudano et al., 1998; also review, see Herdegen et al., 1997b). Although expression of c-jun may contribute to programmed cell death (e.g., Schlingensiepen et al., 1994; Ham et al., 1995), much evidence has shown that its expression is correlated to an even greater extent with regenerative events in injured neurons (for review, see Herdegen et al., 1997b). Following axon damage or block of axonal transport in peripheral sensory or motor neurons in the rat, there is delayed expression of c-jun protein and mRNA that is maintained until the nerve has fully regenerated (Jenkins and Hunt, 1991; Leah et al., 1991). Similar modifications in c-jun expression occur following axotomy in CNS neurons (Jenkins et al., 1993a,b), but these changes largely reverse by 2 weeks in parallel with the failure of these CNS neurons to regenerate. This molecule is upregulated in CNS neurons whose axons can regenerate very well into PN grafts such as retinal ganglion cells (Hüll and Bähr, 1994; Robinson, 1994, 1995), neurons of the thalamic reticular nucleus (Vaudano et al., 1998) and deep cerebellar nucleus neurons (Vaudano et al., 1993; and see Chapter 8). Moreover, coexpression of c-jun and the major growth-associated protein GAP-43 is observed in axotomized CNS neurons which regenerate axons through a PN graft (Robinson, 1995; Schaden et al., 1994). Similar results are demonstrated in Chapter 8. Taken together, these observations suggest that the expression of c-jun is in some way, if not essential, at least important for axonal regeneration of adult neurons and imply that differential expression of c-jun may be another main factor responsible for differential abilities of CNS neurons to regenerate axons. As shown in Chapters 3 and 4, neurons of the striatum display
differential responses to injury and to the presence of a PN graft, in particular with regard to the expression of growth-related molecules. The work described in this Chapter investigates the expression of c-jun in striatal neurons as well as in nigral neurons, in response to the implantation of PN grafts into the striatum.

1. Summary of methods used

(See Chapter 2 for details)

A segment of tibial nerve was autografted into the left striatum of adult rats. The animals were killed at 3 days (n=3), 1 week (n=2), 2 weeks (n=3), 4 weeks (n=3) and 6 weeks (n=2) after operation. Cryostat-cut sections through the region of the brain containing the graft, and of the midbrain, were used for c-jun mRNA hybridization. Some sections were selected for double staining with antibody against GFAP using the immunofluorescence method. In addition, some sections were counterstained with thionin to reveal the neuronal characteristics of c-jun positive cells.

As controls, in addition to unoperated animals, tissue was processed in the same way from animals implanted with a freeze-killed graft and killed at 3 days (n=2), 2 weeks (n=1), and 4 weeks (n=2) after the implantation of the graft.

2. Results

2.1) c-jun mRNA expression in intact striatum and substantia nigra

As seen in the striatum of unoperated animals and in the contralateral striatum of animals implanted with PN grafts, striatal neurons, by and large, showed very low level of c-jun mRNA expression. Only a small number of small- and medium-sized neurons displayed moderate expression of this gene; these positive cells were sparsely distributed throughout the striatum (Fig. 5.1a,b). In contrast, neurons of the globus pallidus and those of the medial septum and vertical limb of the diagonal band expressed comparatively high levels of c-jun mRNA (Fig. 5.1d,c).
In the substantia nigra, c-jun mRNA was expressed by SNpc neurons at low to moderate levels, whereas no expression could be detected in SNpr neurons (Fig. 5.5b,d,f).

2.2) c-jun mRNA expression in the striatum and substantia nigra following implantation of a living PN graft

The implantation of a living PN graft into the corpus striatum of adult rats had a modulatory effect on the expression of c-jun mRNA. Upregulation of c-jun mRNA expression was found in a large number of striatal cells around the graft at the earliest survival times examined, i.e., 3-4 dpo (Fig. 5.2a,c). However, unlike the pattern of CHL1 mRNA expression (see Chapter 4), the population of c-jun mRNA expressing cells was mostly concentrated along the brain/graft interface and in the parenchyma nearby (Fig. 5.2a). Cells expressing a strong c-jun mRNA signal spanned a range of perikaryal sizes; some of them were small, some were large. Small c-jun mRNA positive cells were not only concentrated at the proximal end of graft and its tip but were also aligned along the entire length of the graft including the more distal part traversing the cerebral cortex. Some of these small cells were immunopositive for GFAP, showing that they were presumptive reactive astrocytes (Fig. 5.3c). Cells positive for c-jun mRNA with large perikarya were intermingled with the small positive cells at the brain/graft interface but were more common in the striatal parenchyma surrounding the graft tip (Fig. 5.2, 5.4). Thionin counterstaining revealed that large and some small to medium sized c-jun mRNA labelled perikarya had neuronal characteristics and these cells were negative to GFAP antibody (Fig. 5.3). Perikarya of most large c-jun mRNA positive cells had a diameter range of 20-25μm which corresponded to that of large cholinergic striatal interneurons (Fig. 5.3).

Small and medium c-jun mRNA expressing cells with diameters of less than 20μm were probably other striatal cell types (e.g., medium spiny and aspiny neurons) or glial cells which, with the techniques used, could not be precisely categorized. After 2 wpo, the number of small c-jun mRNA positive cells at the brain/graft interface was dramatically decreased and the population of presumptive cholinergic interneurons displaying strong c-jun mRNA expression became prominent (Fig. 5.2b,d, 5.4a,b).
was noticeable that the longer the period of postoperative survival, the smaller were the numbers of small and medium c-jun mRNA positive cells found near the brain/graft interface.

Upregulation of c-jun mRNA expression was still apparent at 4 wpo. However, the number of heavily c-jun mRNA labelled cells, almost all of which were presumptive cholinergic neurons, had declined at this stage; only a small number of such cells was present near the graft tip (Fig 5.4c,d). The expression of c-jun mRNA by striatal neurons was significantly downregulated by 6 wpo: only a few labelled cells were seen in ipsilateral striatum at this survival time.

The implantation of living PN grafts also induced upregulation of c-jun mRNA expression in neurons of the substantia nigra. Between 3 dpo and 4 wpo, a significant number of SNpc neurons and a few SNpr neurons, ipsilateral to the grafted striatum, upregulated c-jun mRNA expression at a high level in comparison to the basal level of expression of this gene in neurons of the contralateral striatum (Fig. 5.5). Upregulation of c-jun mRNA expression was not further detected in neurons of the SNpc and SNpr of ipsilateral brain at survival times longer than 4 weeks. There was no evidence of alteration of c-jun mRNA expression in SNpc and SNpr neurons of contralateral brain at any survival time investigated.

2.3) c-jun mRNA expression in the striatum following implantation of a freeze-killed PN graft

Three days to 2 weeks after the implantation of freeze-killed grafts, c-jun mRNA expression was significantly upregulated by some striatal cells (Fig. 5.6). Similar to living graft implantation, c-jun mRNA was strongly expressed by both large and small cells, most of them distributed within the PBZ. At 3 dpo, the distribution and the numbers of c-jun mRNA containing cells were similar to what was found in animals implanted with living grafts after a similar survival time (Fig. 5.6a,b). However, the number of c-jun mRNA positive cells detected at 2 wpo was apparently smaller than the number of such cells seen at the same time following implantation of living grafts and the distribution of strongly c-jun mRNA labelled cells was confined to the
brain/graft interface. In addition, fewer presumptive cholinergic interneurons containing c-jun mRNA were found around the graft in comparison to the living graft cases. Striatal cells expressing c-jun mRNA were only very rarely found at 4 weeks after graft implantation.

In the substantia nigra, upregulation of c-jun mRNA was seen in a small number of SNpc neurons, but not SNpr neurons, at 3 days following the implantation of a freeze-killed graft in the ipsilateral striatum. Such expression was less common at 2 wpo; very few c-jun mRNA positive SNpc neurons were found at this survival time (Fig. 5.7) and the hybridization signals in these neurons appeared to be weaker than those in animals implanted with living grafts. No evidence of upregulation of c-jun mRNA by neurons of the SNpc and SNpr was apparent at 4 wpo.

3. Discussion

The results of this study have shown that striatal neurons upregulated the gene for the transcription factor, c-jun, in response to a mechanical injury (caused by implantation of freeze-killed grafts). The upregulation of expression of this gene in axotomized neurons was transient and was downregulated by 2 weeks after injury. However, in the presence of a living PN graft, expression of c-jun mRNA was prolonged until 4 wpo and was downregulated after that. The population of c-jun mRNA labelled cells was situated mostly adjacent to the brain/graft interface; these labelled cells included GFAP immunopositive presumptive reactive astrocytes and more than one type of striatal neuron, one of which was presumably the large cholinergic interneuron. In line with the patterns of expression of p75 and CHL1 following the implantation of a PN graft in the striatum (see Chapters 3 and 4, respectively), at survival times longer than 2 weeks, c-jun mRNA expression was detected mostly in presumptive cholinergic interneurons, most of which were located near the graft tip. Moreover, in the presence of a PN graft, neurons of the SNpc significantly upregulated c-jun mRNA and strong expression of this gene was maintained until 4 wpo, whereas such expression was downregulated by 2 wpo in the case of freeze-killed grafts. Since SNpc neurons and large, cholinergic interneurons of
the striatum can regenerate axons vigorously into PN grafts (Woolhead et al., 1998), the present data suggest the possibility that expression of c-jun may be involved in promoting axonal regeneration in such neurons.

3.1 Axotomy-induced expression of c-jun in striatal cells and SN neurons

As shown in the present study, strong expression of c-jun mRNA was upregulated in neurons of the striatum following axotomy. This finding is consistent with a substantial number of studies which have established that the inducible transcription factor c-jun is selectively expressed in axotomized CNS neurons (for review, see Herdegen et al., 1997b; Hughes et al., 1999). However, not all axotomized striatal neurons upregulated c-jun, only the group of cells located in the vicinity of the implantation site expressed strong levels of c-jun mRNA. When a graft is inserted into the striatum, an extensive field of axons of numerous neurons will be lesioned; upregulation of CHL1 mRNA in neurons widely distributed in the ipsilateral striatum after the implantation of a PN graft at early survival times supports this expectation (see Chapter 4). This indicates that c-jun expression in striatal neurons is induced only if axotomy occurs close to their cell bodies, but not if it is done distally. This speculation is in agreement with the results of many studies which have shown that c-jun/c-Jun expression after axotomy is dependent on the site of injury (Jenkins and Hunt, 1991; Jenkins et al., 1993a; Grass, et al., 1997; De Felipe and Belmonte, 1999 and for review, see Herdegen et al., 1997b). For example, distal axotomy of retina ganglion cells (RGCs), by intracranial transection of the optic nerve does not induce c-Jun (Robinson, 1995), whereas strong expression of c-Jun occurs following a proximal crush of the optic nerve (Isenmann and Bähr, 1997). These data obviously show that expression of c-jun is the response of neurons to severe injury, that is, when their axons are cut close to the cell bodies. This can explain the appearance of many c-jun mRNA expressing cells lying close to the brain/graft interface, along both the proximal and distal parts of the graft, that were found at early survival times in this study.

Expression of c-jun is the result of an extensive axotomy probably because of a disinhibition of gene repression initiated following deprivation of trophic factor(s) that normally arrive at the nucleus from the targets of the axotomized neurons, which signal
the integrity of the neurons target axis (Herdegen et al., 1991; Leah et al., 1993). In other words, unless most of the axons of neurons are completely cut, the neurons will continue to receive sufficient amounts of any necessary neurotrophic compounds via their remaining axons, to suppress the expression of c-jun. This hypothesis is supported by the experiments of Leah et al. (1993) which the microinjection of colchicine and vinblastine were used to produce a complete inhibition of axonal transport in a small volume of cortical and hippocampal tissue. This treatment was followed by the induction of Jun expression (including c-Jun and JunD) in cortical and hippocampal neurons. In contrast, the partial axotomy produced by commissural transection fails to induce expression of Jun (Leah et al., 1993).

In addition, when axotomy provokes very little functional response in the injured neurons, it also fails to induce c-jun and the extent of upregulation is related to the characteristics of the nerve injury. For example, following transection in the thoracic spinal cord, distally axotomized rubrospinal neurons neither die nor regenerate axons through the supportive environment of a PN graft, and they do not express c-Jun (Jenkins et al., 1993c; Tetzlaff et al., 1994). By contrast, more proximal lesions in the cervical spinal cord induce both cell death and regenerative competence as well as robust expression of c-Jun in the injured rubrospinal neurons (Jenkins et al., 1993c). These findings are consistent with the results of the present study that neurons which were situated far from the implantation site (both in freeze-killed and living graft cases) failed to express c-jun, although they were presumably axotomized by implantation of a PN graft. Also, such neurons were not found to regenerate axons through PN grafts (Woolhead et al., 1998 and see also Chapter 3). However, the failure to upregulate c-jun expression in distally axotomized neurons does not absolutely mean that such neurons would die. Hüll and Bähr (1994a) reported that c-Jun expression began later and declined faster after distal axotomy than after proximal transection of the optic nerve; however, at the same time significantly more retinal ganglion cells survived after distal than after proximal axotomy.

One disparity in the relationship between c-jun expression and the site of injury discussed above, appears in neurons of substantia nigra. It is well established that
ascending axons from SNpc neurons, forming the nigrostriatal pathway, collect in the rostral ventral tegmental area before projecting to the striatum via the medial forebrain bundle and internal capsule (Fallon and Loughlin, 1995). Therefore, implantation of a graft into the striatum would damage the very distal part of axons of SNpc neurons and the terminal fields of these axons. However, some SNpc neurons (which were presumably axotomized following implantation of nerve grafts into the striatum) showed strong upregulation of c-jun expression. This result is in line with those reported by Jenkins et al. (1993b) who induced axotomy of dopaminergic nigrostriatal neurons by injecting 6-hydroxydopamine (6-OHDA) into the striatum and found that SNpc neurons transiently upregulated c-jun to a maximal level at 4-8 days, followed by decline to control levels by 2 weeks post-lesion. Moreover, in this study, upregulation of c-jun mRNA expression was also found in a small number of SNpr neurons ipsilateral to the graft. Although the dopaminergic projections from SNpr neurons to the striatum are sparse in comparison with those from SNpc neurons (Fallon and Loughlin, 1995), it is likely that afferent fibers from SNpr neurons would be damaged by inserting a nerve graft into the striatum. Apart from SNpc neurons which can regenerate axons very well into the PN grafts (Woolhead et al., 1998), the evidence that SNpr neurons can occasionally be retrogradely labelled from a PN graft in the striatum has been also reported by Woolhead et al. (1998) indicating the ability of these neurons to regenerate their axons. Therefore, the ability to express c-jun after axotomy of SNpc and SNpr neurons, even though lesions to their axons are very distant from the cell bodies, may reflect the high regenerative propensity of these CNS population (Woolhead et al., 1998) (see also Section 3.2).

3.2 Effect of living graft implantation on the expression of c-jun

Expression of c-jun mRNA in neurons of the striatum and SNpc following freeze-killed graft implantation was transient; but, it could be prolonged if a living PN graft was inserted, suggesting that such a graft has a modulatory effect on c-jun mRNA expression. Examples demonstrating similar effect of a PN graft on c-jun expression are also available. In the thalamus, following stab wound lesions, axotomized TRN neurons briefly express c-jun (up to 7 days after lesions); on the other hand, in the presence of a living PN graft, c-jun is expressed in axotomized TRN neurons up to the
longest survival time studied (i.e., 86 days) and this gene has been shown to be expressed by TRN neurons whose axons regenerate into the graft (Vaudano et al., 1998). Hüll and Bähr (1994b) demonstrated that the number of c-Jun immunoreactive RGCs in animals with a PN graft attached to the retinal stump of the cut optic nerve, was significantly higher 2 and 3 weeks after the transection than in the animals with only axotomy (optic nerve transection), in which only a few c-Jun immunoreactive cells were found 2 weeks after lesion. Furthermore, in their experiment about 70% of the regenerating RGCs expressed c-Jun-immunoreactivity 2 weeks after grafting as compared to only 38% c-Jun immunopositive RGCs among the surviving but not regenerating RGCs. Interestingly, after thoracic spinal cord hemisections, axotomized brainstem neurons do not upregulate expression of c-Jun; but such expression is upregulated in animals treated with transplanted fetal spinal cord tissue and neurotrophins (Broude et al., 1999). As discussed in Chapter 3 and shown by C.L. Woolhead (1995), a living PN graft is a source of neurotrophic factors, which can encourage axotomized striatal neurons, predominantly presumptive cholinergic interneurons, and SNpc neurons (Woolhead et al., 1998) to regenerate axons into nerve grafts. Therefore, there can be no doubt that the prolonged expression of c-jun observed in SNpc neurons and striatal neurons (exclusively presumptive cholinergic interneurons) is attributable to the effect of living graft implantation.

It is known that the kinetics of c-Jun regulation in axotomized PNS neurons differs from that in axotomized CNS neurons (Leah et al., 1993), which do not normally reconnect with their targets after transection. In the PNS, expression of c-jun message or protein in neurons following axotomy is very prolonged, and in some cases it remains increased 100-300 days after nerve crush (Herdegen et al., 1991, 1992); it is supposed that c-jun expression is maintained until the nerve has fully regenerated. Similar modifications in c-jun expression occur following axotomy of CNS neurons, such as rubrospinal (Jenkins et al., 1993a) or nigrostriatal (Jenkins et al., 1993b) neurons, but it is downregulated within 14 days (which corresponds to the results of the present study) in parallel with the failure of these central neurons to regenerate. This may indicate that different signal transduction pathways are activated by CNS neurons, leading to a coordination of c-jun expression with other transcription factors that fail to
foster a regenerative response. Interestingly, although the cell bodies of sciatic nerve motor neurons reside in the CNS, they show a c-Jun response after peripheral axotomy resembling that of transected peripheral sensory ganglion neurons (Herdegen et al., 1992), and they regenerate their axon, with jun family protein levels declining following reinnervation of target tissue (Leah et al., 1991). This suggests that the peripheral environment provides unique signals that regulate the activity of transcription factors such as c-jun in a manner that promotes a growth response in PNS neurons, and that these signals may be absent or inhibited in the CNS (Jenkins et al., 1993a-c; Vaudano et al., 1996; Kenney and Kocsis, 1998).

Taken together, these results suggest that a segment of living PN graft implanted into the brain possibly modifies the CNS microenvironment around the implantation site in ways that benefit some CNS neurons in that area because the change enables them to overcome inhibitory factors and regain regenerative activity. The exogenous neurotrophic factors derived from living Schwann cells in the grafts, which promote sprouting and regrowth of CNS axons after lesion, may activate a cell body response by upregulating and sustaining expression of c-jun, the transcription factor which is likely to play a role in axonal regeneration in adult neurons. The failure to maintain expression of c-jun in neurons of the striatum and SNpc in the animals implanted with freeze-killed grafts, which lack living Schwann cells and do not promote axon regeneration, can be considered as persuasive evidence in support of this speculation.

Nevertheless, it is noteworthy that living graft implantation does not have positive effects on c-jun expression in every type of CNS neuron. For example, apart from putative cholinergic interneurons, c-jun expression in the population of other smaller cell types of the striatum, which manifest poor regenerative competency (Woolhead et al., 1998), were not further substantially detected after 2 weeks and became absent by 4 weeks after implantation of living grafts. In some animals, a very few medium sized striatal neurons, displaying strong levels of c-jun signals, were found near the graft, just as were presumptive cholinergic interneurons at late survival times (data not shown); these neurons probably belong to the medium aspiny striatal neuronal...
cell type, the one that has been reported to regenerate axons into the PN graft (Woolhead 1995). This implies that only particular types of CNS neuron can respond to the trophic factors from the graft, switch on expression of effector genes, such as c-jun, and consequently modulate gene expression relevant to axonal growth. In contrast, other cell types lack such ability, which results in failure of axonal regeneration.

3.3) Expression of c-jun and axon regeneration in presumptive cholinergic interneurons and SNpc neurons

Upregulation of expression of c-jun in axotomized neurons may activate a cascade of events in their cell bodies that can lead to apoptotic cell death or, paradoxically, vigorous regeneration of the injured neurons (Herdegen et al., 1997b). In particular, neurons or non-neuronal cells whose axons are cut close to their cell bodies (such as those found along the brain/graft interface in this study) are more likely to undergo cell death, since the extent of axotomy-induced cell death is greater with more proximal lesions (Lieberman, 1974). In this case, the physiological response in such cells probably permits c-jun to mediate cell death. In contrast, expression of c-jun in some striatal neurons located near the graft tip, particularly presumptive large, aspiny cholinergic interneurons, in the presence of a living graft, apparently initiates the process of axonal regeneration of those neurons rather than cell death. In this study, I did not attempt to demonstrate the expression c-jun in striatal neurons identified as regenerating by retrograde tracing as was done in the case of work in the cerebellum (see Chapter 8); however, the evidence that implantation of a living PN graft can promote axon regeneration in some axotomized cholinergic interneurons (Woolhead et al., 1998), supports the above expectation. Moreover, expression of c-jun in otherregenerating CNS neurons following the implantation of PN grafts has also been reported (Vaudano et al., 1998, see also Chapter 8). Likewise, expression of c-jun mRNA in neurons of the SNpc, which display a strong regenerative propensity (Woolhead et al., 1998) following living graft implantation, also indicates that expression of this mRNA is a sign of axon regeneration.

It was noticeable that the number of c-jun mRNA expressing cells was dramatically reduced at survival times longer than 2 weeks. At 4 wpo and after, fewer
c-jun mRNA positive cells were found and almost all of them appeared to be presumptive cholinergic interneurons, and were located near the graft tip. These findings are parallel to the patterns of the expression of p75 and CHL1 mRNA following the implantation of a PN graft into the striatum (see Chapters 3 and 4, respectively). It is unclear why some c-jun mRNA labelled presumptive cholinergic interneurons disappeared over the period 2-4 wpo, despite a presumption that these neurons would regenerate. In the PNS, there appears to be a correlation between the expression of c-jun and the ability of neurons to grow or regenerate. For example, after sciatic nerve lesion, c-Jun protein expression in DRG neurons remains upregulated until the regenerating cells has reached its target (Jenkins et al., 1991; Leah et al., 1991). In contrast, the increased levels of c-jun mRNA and protein are maintained if the damaged nerve is ligated, but return to basal levels if the peripheral nerve is allowed to regenerate (Jenkins and Hunt, 1991; Leah et al., 1991; Jenkins et al, 1993a; Kenney and Kocsis 1998). Kenney and Kocsis (1998) reported that following sciatic nerve transection, upregulation of AP-1 binding by c-Jun and elevation of c-Jun protein levels in DRG neurons persist for at least 30 days after axotomy if successful regeneration is blocked, but diminish over time if axons are permitted to proceed to target reinnervation after nerve crush. These examples suggest that c-jun is active in axotomized neurons while they are making regenerative attempts and that c-jun activity persists as long as such neurons have not reestablished contact with target tissue. Thus, one possible explanation for the reduction in number of presumptive cholinergic neurons expressing c-jun is that those interneurons have established synaptic contact with their targets in the vicinity (see also discussion in Chapters 3 and 4) and trophic factors derived from the target cells may suppress c-jun signals (Leah et al., 1993).

However, it is not the case that the decline of c-jun expression is always a sign of complete axon regeneration and target reinnervation. Expression of c-jun in axotomized SNpc neurons, whose axons mostly regenerate into the PN graft (Woolhead et al., 1998), was dowregulated by 4 wpo. Woolhead et al. (1998) reported that a substantial number of SNpc perikarya retrogradely labelled from the grafts in the striatum was present at 4 wpo and still numerous until 30 weeks (the longest survival time examined). In this case, it is obvious that regenerating axons from SNpc neurons
would not reach the targets and the process of regeneration still carries on after 4 weeks of graft implantation; therefore, downregulation of c-jun expression in SNpc neurons does not definitely result from target derived suppressors. One possible speculation for this phenomenon is that axon contact between regenerating axons and Schwann cell in the graft may somehow regulate and inhibit c-jun expression in parent SNpc neuron cell bodies. Alternatively, some intrinsic factors in SNpc neurons themselves may be involved in inhibitory regulation of c-jun expression.

In conclusion, it seems most likely that the induction of c-jun is part of a cell body reaction in response to axotomy and that the decision of individual injured neurons to regenerate or survive depends on additional signalling events that modulate the actions of c-jun.

The role of c-jun in promoting regeneration of CNS axons into PN grafts and possible coordinated expression of c-jun and other growth-related molecules are further discussed in Chapter 8 and 9.
Figure 5.1 Expression of c-jun mRNA in the control striatum (5.1a,b), medial septum (5.1c) and globus pallidus (5.1d,e).

5.1a,b) Photomicrographs of the striatum from an unoperated animal illustrating low expression of c-jun mRNA in striatal neurons. Moderate to strong levels of c-jun mRNA are, however, expressed by some small cells sparsely distributed throughout the corpus striatum (e.g., at arrows in a). An area in 5.1a is enlarged in 5.1b. Note that both strongly (b, arrows) and moderately (b, small arrows) c-jun mRNA-labelled cells are small in size. Scale bar = 500\(\mu\)m (5.1a); 125\(\mu\)m (5.1b).

5.1c) Strong c-jun mRNA expression in neurons of the medial septum and vertical limbs of diagonal band (e.g., arrowheads). Scale bar = 500\(\mu\)m.

5.1d,e) High level of c-jun mRNA expression in neurons in the globus pallidus (d and enlargement in e, arrows). Scale bar = 500\(\mu\)m (5.1d); 125\(\mu\)m (5.1e).

Abbreviations: CC, cerebral cortex; Cc, corpus callosum; GP, globus pallidus; ST, striatum; MS, medial septum; VB, vertical limbs of diagonal band.
Figure 5.2 Striatum at 3 days (5.2a,b) and 1 week (5.2c,d) following the implantation of a living graft

5.2a) Upregulation of c-jun mRNA expression by striatal cells at 3dpo showing c-jun mRNA containing cells of various sizes concentrated along the graft/brain interface and located close to the JZ (a, large arrowheads). Small c-jun mRNA-labelled cells (e.g. small arrows) are mostly confined within the PBZ and are also seen lying alongside the cortical part of the graft (small arrowheads) whereas large labelled cells (e.g. big arrows) are also found in the brain parenchyma. Scale bar = 500μm.

5.2b) An area at the graft/brain interface (boxed area in a) showing many large intensely c-jun mRNA-labelled perikarya (e.g., large arrows) intermingled with the smaller labelled perikarya (e.g., small arrows). Scale bar = 125μm.

5.2c,d) Numerous c-jun mRNA positive striatal cells around the graft, especially the graft tip at 1wpo. The graft tip area in c is enlarged in 5.2d. The population of large positive cells (e.g. thick arrows in c,d) is more widespread in the PBZ and brain parenchyma than that at 3dpo (see 5.2a). Many small c-jun mRNA containing cells are still aggregated within the PBZ, but the signal has declined to a moderate level (e.g., thin small arrows in d). Scale bar = 500μm (5.2b); 250μm (5.2d).

Abbreviations: CC, cerebral cortex; Cc, corpus callosum; G, PN graft; JZ, junctional zone; PBZ, parenchymal border zone.
**Figure 5.3** Expression of c-jun mRNA in presumptive cholinergic striatal interneurons in the striatum at 1 wpo.

5.3a,b) Graft tip area (5.3a) showing numerous c-jun mRNA containing striatal cells concentrated in the PBZ but sparingly distributed in surrounding brain parenchyma. Large striatal cells with intense c-jun mRNA labelling, presumptive aspiny cholinergic striatal interneurons, are prominent among labelled cells (e.g. arrows in 5.3a). A group of such cells within the boxed area in 5.3a are enlarged in 5.3b (large arrow). Scale bar = 250μm (5.3a); 25μm (5.3b).

5.3c) Area near the JZ double stained for c-jun mRNA and GFAP immunofluorescence showing that some small c-jun mRNA labelled perikarya are GFAP immunopositive astrocytes (small arrows) whereas large c-jun mRNA containing perikarya are not (large arrows). In this picture, c-jun mRNA-labelled perikarya appear as black blobs and GFAP immunoreactive product appears white. An open arrow indicates a GFAP immunopositive astrocyte devoid of c-jun mRNA expression. Scale bar = 25μm.

Abbreviations: G, PN graft; JZ, junctional zone; PBZ, parenchymal border zone.
Figure 5.4 Striatum at 2 weeks (5.4a,b) and 4 weeks (5.4c,d) following the implantation of a living PN graft

5.4 a,b) Many large c-jun containing cells, presumptive cholinergic interneurons, are still present around the graft at 2 wpo (e.g. large arrows in b), but the smaller positive cells form small aggregates close to the brain/graft interface (e.g., small arrows in b). The background of the section shown in a and enlargement in b is rather high due to overreaction with the chromogen. However, specific staining could be recognized because of the very strong hybridization signal from such cells. Scale bar = 500μm (5.1a); 50μm (5.1b).

5.4 c,d) At 4 wpo, presumptive large cholinergic interneurons containing high level of c-jun mRNA became the majority of c-jun-expressing cells (e.g. large arrows in d); very few small c-jun expressing cells appear near the graft/brain interface (d, small arrows). Note that the positive cells are widespread in surrounding brain parenchyma rather than concentrated in the PBZ as seen in 5.2d. Scale bar = 500μm (5.4c); 250μm (5.4d).

Abbreviation: Cc, corpus callosum; G, PN graft.
**Figure 5.5** Substantia nigra at 3 days (5.5a-d) and 1 week (5.5e,f) following the implantation of a living PN graft in the striatum

5.5a-d) Substantia nigra from the animal at 3 dpo showing upregulation of c-jun mRNA expression in neurons of the SNpc ipsilateral to the graft (a and enlargement in c, **arrows**). A few neurons of SNpr in the ipsilateral brain also upregulate c-jun mRNA expression to a high level (e.g., **arrowheads** in a,c). In contrast, neurons of the SNpc and SNpr on the contralateral side of the brain (b and d) express this gene at very low level. Scale bar = 500μm (5.5a,b); 250μm (5.5c,d).

5.5e,f) c-jun mRNA expression is upregulated in SNpc neurons but not in SNpr neurons in the ipsilateral brain at 1 wpo (5.5c, **arrows**). 5.5f illustrates insignificant level of c-jun mRNA in neurons of the SNpc and SNpr in the nigra contralateral to the graft. Scale bar = 500μm (5.5e,f).

**Abbreviations:** SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata.
Figure 5.6  Striatum at 3 days (5.6a,b) and 2 weeks (5.6c,d) following the implantation of a freeze-killed PN graft

5.6a,b) At 3 dpo, expression of c-jun mRNA in the striatum is more or less similar to that found in the living graft implantation as shown in Fig. 5.2a. Cells containing c-jun mRNA appear in various sizes and are confined within the PBZ. Many large, strongly c-jun mRNA-labelled perikarya are present close to the graft/brain interface as is clearly seen in b (arrows), the enlargement of the boxed area in a. Scale bar = 500μm (5.6a); 125μm (5.6b).

5.6c,d) A small number of presumptive c-jun mRNA-labelled cholinergic interneurons (e.g. large arrows in c,d) at 1 wpo appear around the lesion tract (a long line extending ventrally from the graft tip, seen in both pictures). This lesion tract is made during pushing the graft into the brain. A certain number of moderately c-jun mRNA-labelled cells are also present around the lesion and the graft tip (e.g. small arrows in d). Scale bar = 500μm (5.6c); 250μm (5.6b).

Abbreviations: CC, cerebral cortex; Cc, corpus callosum; G, (freeze-killed) PN graft; PBZ, parenchymal border zone; ST, striatum.
Figure 5.7 Substantia nigra at 3 days (5.7a,b) and 2 weeks (5.7c,d) following the implantation of a freeze-killed PN graft in the striatum

5.7a,b) At 3 dpo, c-jun mRNA expression is upregulated in neurons of the SNpc (e.g., arrows in a) and, although few in number, also in neurons of the SNpr (a, arrowhead) ipsilateral to the graft. In this section, the background is rather high. As a result, neurons of contralateral substantia nigra seem to be stained darker than normal. 5.7b is contralateral to 5.7a. Scale bar = 500μm (5.7a,b).

5.7c,d) Downregulation of c-jun mRNA expression in neurons of the SNpc and SNpr at 2 wpo ipsilateral to an implanted graft. Only moderate levels of c-jun mRNA are expressed by SNpc neurons (e.g., arrows in c) and by none of SNpr neurons (c). 5.7d is contralateral to 5.7c. Scale bar = 500μm (5.7c,d).

Abbreviations: SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticularata.
CHAPTER 6
Expression of CHL1 mRNA in the thalamus following peripheral nerve graft implantation

CHL1, a cell recognition molecule, is a powerful promoter of neurite outgrowth in vitro, more so even than L1 (Holm et al., 1996; Hillenbrand et al., 1999). In vivo, this molecule is expressed by some neurons and is upregulated by motor and some sensory neurons during peripheral nerve regeneration (Roslan et al., 1998). The results in Chapter 4 showed that following the implantation of a PN graft in the striatum, CHL1 mRNA is upregulated in presumptive cholinergic striatal interneurons, a CNS neuronal population showing a high ability to regenerate axons into nerve grafts (Woolhead et al., 1998). These data strongly suggest a possible role of CHL1 in promoting axonal regeneration of CNS neurons. The thalamus is a part of the CNS where one particular population of neurons shows a very strong propensity for axon regeneration in response to injury, particularly in the presence of a PN graft. The majority of the neurons which regenerate axons into such grafts have cells bodies in the thalamic reticular nucleus (TRN); by comparison only a few thalamic projection neurons regenerate axons into the same grafts (Benfey et al., 1985; Morrow et al., 1993; Vaudano et al., 1993a; Vaudano et al., 1998). This is one of the best documented examples of differential regenerative response of CNS neurons (Anderson et al., 1998).

In rats, the TRN is a thin sheet-like nucleus which lies over the dorsolateral and anterior aspects of the thalamus, intercalated between the latter and the internal capsule (Ohara and Lieberman, 1985). This nucleus is traversed by the thalamo-cortical and cortico-thalamic fibre systems (Ohara and Lieberman, 1985), giving it the reticulated appearance that helps identify this nucleus at the light microscopic level. Neurons of the TRN are GABAergic (Hourser et al., 1980) and receive topographically organized excitatory inputs from both dorsal thalamus and cerebral cortex and project to nuclei of the ipsilateral dorsal thalamus to which they provide powerful inhibitory input, part of
the mechanism for regulating the transmission of information to the cerebral cortex (e.g., Ohara et al., 1983; de-Biasi et al., 1986).

During regeneration, TRN neurons upregulate several growth-related molecules such as L1 (Zhang et al., 1995), GAP-43 (Vaudano et al., 1995) and c-jun (Vaudano et al., 1998). Other thalamic neurons which fail to express those growth-related molecules fail to regenerate axons even into the favourable environment provided by a PN graft (for review, see Anderson and Lieberman, 1999). These findings suggest that CHL1, a putative promoter for axonal growth of CNS neurons, may be upregulated by TRN neurons whose axons regenerate well into a nerve graft but would not upregulated in other thalamic nuclei whose axons fail to regenerate. To test this hypothesis, this Chapter investigates the expression of CHL1 mRNA in the thalamus following the implantation of a PN graft. The relationship between the expression of CHL1 mRNA and neurons whose axons regenerate into the graft is also demonstrated.

1. Summary of methods used
(See Chapter 2 for details)

Twenty-one rats were examined for expression of CHL1 in the thalamus after PN graft implantation by in situ hybridization. Fifteen of these received a living PN graft implanted into the left thalamus with survival periods of 3 days (n=3), 1 week (n=3), 9 days (n=2), 2 weeks (n=2), 4 weeks (n=2), 6 weeks (n=2) and 10 weeks (n=2). Six rats received a freeze-killed PN grafted into the left thalamus with survival periods of 2 weeks (n=2), 4 weeks (n=2) and 6 weeks (n=2). All animals, including two unoperated animals, were processed for in situ hybridization for CHL1 mRNA. Thionin counterstaining was done in some sections to reveal neuronal characteristics. In addition, a few sections from the 1 wpo group were selected for combined in situ hybridization for CHL1 mRNA and immunofluorescence for GFAP.

Five animals implanted with living grafts from 4, 6 and 10 wpo groups were injected with CT-HRP solution 2 days before being killed. Cryostat sections from the brains of these animals were collected as two parallel series of alternate sections and
processed either by the TMB method for visualisation of HRP or hybridized with CHL1 cRNA probe.

2. Results

In all animals, the graft tips were embedded in the thalamus. The location of the graft tip and thus the area of damage to the thalamic parenchyma is summarised for all the animals in this group in Table 6.1.

2.1) CHL1 mRNA expression in the intact thalamus

In control (unoperated animals; contralateral thalamus of experimental animals) very low to negligible levels of CHL1 mRNA were expressed by neurons of most thalamic nuclei, especially the TRN. However, a moderate to strong level of CHL1 mRNA was detected in some small cells, presumably glia, and medially located nuclear groups in the central region of the thalamus (Fig. 6.3). In the caudal thalamus, neurons of the lateral geniculate nucleus, particularly those in the ventral part of this nucleus also expressed CHL1 mRNA moderately.

2.2) CHL1 mRNA expression in the thalamus following implantation of a living PN graft

Following the implantation of a living PN graft into the thalamus, many small cells resembling glia around the brain/graft interface, within the area described by Campbell et al. (1992) as the parenchymal border zone (PBZ) upregulated CHL1 mRNA at early survival times up to 2 wpo (Figs 6.4b; 6.5b,c). Some of these small CHL1 mRNA positive cells were also immunopositive for GFAP, suggesting that they were probably reactive astrocytes (not illustrated). At 2 wpo GFAP positive astrocytes as well as presumptive glial cells containing CHL1 mRNA were still detectable (Figs. 6.5b,c) but were much less common than at earlier survival times. The numbers of such cells were insignificant at survival times longer than 2 weeks. Unlike expression of CHL1 mRNA in presumptive glia, high levels of CHL1 mRNA were also present in neurons of the thalamus ipsilateral to the graft at all survival times examined, i.e., from 3 dpo to 10 wpo. Neuronal cell bodies displaying strong signal for CHL1 mRNA were
found located both close to the graft tip and up to 1 mm away from it. The position of
the labelled thalamic neurons in individual animals varied according to the precise
position of the graft. For example, in the animal in which the graft passed through the
lateral dorsal nucleus (LD), ventral posteromedia nucleus (VPM), thalamic reticular
nucleus (TRN) and posterior nucleus (PO) of the thalamus, the labelled cells were in
the ventral posterolateral nucleus (VPL), VPM, TRN and PO; whereas in the case of
the graft ending in the TRN and VPL, the labelled cells were found only in the TRN
(see also Table 6.1). However, the upregulation of CHL1 mRNA by neurons in the
TRN was more conspicuous than upregulation of this molecule in the other nuclei
(Figs. 6.2a, 6.3, 6.4a,b, 6.5): a strong signal for CHL1 mRNA was detected in TRN
neurons at all survival times and in every animal with a living graft implanted in the
thalamus even in animals in which grafts did not encroach on the TRN itself (Figs
6.5a,d). Furthermore, the intensity of CHL1 mRNA labelling in TRN neurons was still
strikingly heavy even at the longest survival time examined, i.e., 10 wpo (Fig 6.5e). In
contrast, CHL1 mRNA expression in other thalamic nuclei such as the PO, VPM, VPL,
ZI and LD was observed between 3 dpo and 2 wpo (Figs. 6.5a,b,c), but was very rarely
seen at the longer survival times (Figs. 6.2a, 6.5e).

2.3) CHL1 mRNA expression in identified regenerating TRN neurons

Five rats, which had received living grafts 4, 6 and 10 weeks previously, were
chosen for an investigation of whether CHL1 mRNA was expressed in neurons whose
axons could be shown to have regenerated along the PN graft. To identify the
regenerating neurons, CT-HRP was injected into the distal end of the graft 2-3 days
before the animals were killed. In all cases, retrogradely labelled neurons containing
HRP reaction product were found in the TRN ipsilateral to the graft. When sections
immediately adjacent to those processed for HRP were hybridized with the CHL1
riboprobe, strong signal for CHL1 was apparent in many neurons of the TRN, and the
pattern of CHL1 mRNA expression was very similar to the pattern of retrograde
labelling found in adjacent sections (Fig. 6.6). Although it was not always possible to
identify the same cells on adjacent sections, by comparing the location of individual
labelled neurons in immediately adjacent sections, using landmarks such as blood
vessels to ensure that sections were correctly aligned, it was possible to show that many of the retrogradely labelled neurons had also upregulated CHL1 mRNA (Fig 6.7).

2.4) **CHL1 mRNA expression in the thalamus following implantation of a freeze-killed graft**

Six animals into which freeze-killed grafts had been implanted were investigated for CHL1 mRNA expression 2, 4 and 6 wpo. There was no detectable expression of CHL1 mRNA in either glia or neurons of the thalamic nuclei ipsilateral to the grafts in one of the two animals at 2 wpo (Fig. 6.8) or in any of the 4 and 6 wpo animals, even though the grafts encroached onto the TRN. However, in the other animal at 2 wpo in which the graft encroached into the dorsal part of TRN, upregulation of CHL1 mRNA was detected in a small number of presumptive glia and in a few neurons of the ipsilateral TRN around the graft. However, the number of CHL1 mRNA positive neurons found in this freeze-killed graft case was only a fraction of the number found at the same survival time in animals in which a living graft had been implanted.

3. **Discussion**

In keeping with the results of Chapter 4, the present study has shown that following a mechanical injury (as represented by the implantation of a freeze-killed graft), the expression of CHL1 mRNA is transiently upregulated by both neurons and smaller cells, believed to be reactive glia, close to the lesion. Such expression was seen up to 2 wpo and was significantly downregulated at later survival times. In the presence of a living PN graft, the expression of CHL1 mRNA in thalamic neurons was prolonged, mainly in the neurons of the TRN, up to 10 wpo (the longest survival time examined). This study also confirmed that when segments of living, autologous tibial nerve are implanted into the dorsal thalamus of adult rats, CNS neurons, predominantly in the TRN, grow axons into the grafts. By comparing immediately adjacent sections processed for HRP and those hybridized with the CHL1 riboprobe, it was shown that individual retrogradely labelled regenerating TRN neurons had upregulated CHL1 mRNA. Expression of CHL1 mRNA in other axotomized thalamic nuclei was downregulated and absent by 4 weeks; also these neurons were not apparently
retrogradely labelled for HRP through the graft. These results suggest that the cell recognition molecule CHL1 can therefore be placed among the family of neuronal growth-associated molecules whose expression distinguishes cells which regenerate axons into PN grafts from those which do not.

3.1) A transient increase in CHL1 mRNA expression in cells of the thalamus after axotomy and the effects of living PN grafts

The implantation of a PN graft into the thalamus caused the appearance of high levels of CHL1 mRNA in a variety of cells, including neurons in the TRN, neurons in other thalamic nuclei around the graft tip, and smaller cells near the brain/graft interface. This was apparent three days after grafting (the earliest postoperative survival time studied), and occurred irrespective of whether the graft was living or freeze-killed. Since freeze-killed grafts lack Schwann cells and are not a conducive substrate for axonal regeneration (Anderson et al., 1983; Berry et al., 1988), the initial upregulation of CHL1 mRNA around the graft tip is most likely a general response to injury rather than a sign of axonal regeneration (also see Chapter 4: section 3.1). This speculation is supported by the absence of signals of CHL1 mRNA in cells of the thalamus in animals implanted with non-living grafts longer than 2 weeks. In contrast, in the presence of a living graft, a growth permissive substrate for axonal regeneration in CNS neurons (Aguayo, 1985; Anderson et al., 1998), strong expression of CHL1 mRNA was sustained, exclusively in TRN neurons, up to the longest survival time examined (10 wpo). In other words, the implantation of a living PN graft can extend expression of CHL1 mRNA in neurons of the TRN, but not in other groups of thalamic neurons. It is now well established that not all thalamic nuclei can regenerate axons into the PN graft (Benfey et al., 1985; Morrow et al., 1993; Vaudano et al., 1995). For example, if a graft is inserted in the thalamus, a good regenerative response is obtained from neurons of the TRN and to a much lesser extent, from neurons of the posterior nucleus and the medial geniculate complex, but other neurons of dorsal thalamus regenerate very poorly into such grafts (Benfey et al., 1985; Morrow et al., 1993). Therefore, long term expression of CHL1 mRNA induced by implantation of living grafts may indicate or relate to a sign of axonal regeneration, as shown by neurons of the TRN (see Section 3.2). These findings closely parallel the pattern of expression of GAP-43 and c-jun...
following graft implantation in the thalamus (Vaudano et al., 1995, 1998). Following a stab wound, mRNAs and proteins of c-jun (Vaudano et al., 1998) and of GAP-43 (Vaudano et al., 1995) were transiently expressed (up to 2 weeks for c-jun and 1 week for GAP-43) by several groups thalamic nuclei; but such expression was prolonged, predominantly in TRN neurons, when a living PN graft was implanted into the dorsal thalamus.

The small cells near the brain/graft interface almost certainly include reactive astrocytes; they are similar in size and distribution to GFAP positive astrocytes around grafts which have been shown in previous studies to express N-CAM (Zhang et al., 1995). In double labelling experiments on grafts implanted into the striatum (Chapter 4) and in the cerebellum (Chapter 7), many of these transiently CHL1 mRNA positive cells are also immunoreactive for GFAP. These cells may play a role in allowing axonal regeneration into grafts during the first 2 weeks after graft implantation: axonal sprouts have to pass across the brain/graft interface and CHL1 is a potent promoter of neurite growth (see Chapter 4: Section 3.3). If so, then the disappearance of cells expressing CHL1 mRNA from the brain/graft interface after 2 weeks may make it more difficult for axonal sprouts to enter the grafts, and explain why axonal sprouts are much more numerous in the parenchymal border zone than in the grafts themselves (Campbell et al., 1992).

3.2) Expression of CHL1 and the regeneration of thalamic neurons into PN grafts

The present study provides evidence to support the belief that neurons in the TRN have a special propensity to regenerate their axons into PN grafts, and that most other thalamic nuclei have much more limited capacities for regeneration (Benfey et al., 1985; Morrow et al., 1993; Vaudano et al., 1995). The TRN neurons which regenerate into grafts in the thalamus are found at the level of and close to the graft tip, and rostral to the graft tip (Morrow et al., 1993). It was in these same locations that neurons containing CHL1 mRNA were found in experiments with living grafts at survival times of 4 weeks or more and when retrograde labelling was combined with in situ hybridization, it was shown that the CHL1 mRNA positive cells included many and perhaps all neurons which had regenerated their axons into the grafts. Because of the
practical difficulties of identifying cells on adjacent sections it cannot be categorically stated that all the TRN neurons which regenerated their axons into the grafts had upregulated CHL1 mRNA, but certainly most had done so.

Some axotomized neurons probably upregulated CHL1 mRNA even though they failed to regenerate axons into the grafts. Such cells would have included all the CHL1 mRNA positive neurons in the experiments with freeze-killed grafts, and most of the non-TRN thalamic neurons which contained CHL1 mRNA in living graft experiments. In both cases, expression of CHL1 mRNA in these non-TRN neurons after axotomy occurs only when their cell bodies are situated close to the lesion. In contrast, TRN neurons, even though the cell bodies are located at a distance from the lesion site (in this study, up to 1mm away from the graft) still strongly upregulate CHL1 mRNA. Therefore, one possible explanation for transient expression of CHL1 mRNA in non-TRN neurons (which show a poor regenerative capacity) is that axonal injury which occurs very close to the cell bodies of such neurons could intrinsically induce expression of CHL1 mRNA. In line with this conclusion, previous reports have been shown that GAP-43 levels do increase in some axotomized CNS neurons, particularly if the lesion is close to the cell body (Doster et al., 1991; Tetzlaff et al., 1991). However, it is unclear why these non-TRN neurons are unable to maintain CHL1 mRNA signal in the presence of a living graft, whereas TRN neurons are. It was noticeable that all the neurons which had upregulated CHL1 mRNA in response to a freeze-killed graft, and the non-TRN thalamic neurons which upregulated CHL1 expression in response to living grafts, had downregulated CHL1 mRNA expression again by 4 weeks. It is possible that to maintain expression of CHL1 in CNS neurons, coexpression of other factors/transcription genes are needed, but such factors are missing in those neurons which display poor regenerative response. Expression in TRN neurons in animals receiving a living graft was always noticeably higher than that found in other neurons and remained strong for up to 10 weeks, the longest survival time examined in this study. It is obvious that a factor or factors provided from a living graft can exert specific effects on the expression of CHL1 in neurons which regenerate very well such as TRN neurons, but not in those which regenerate poorly. The differential capacity of different types of thalamic neurons to respond to trophic factors
from a living PN graft is likely to reflect intrinsic differences in the regenerative potential of these neurons.

3.3) Growth-associated molecules and regeneration of the axons of TRN neurons

Previous studies from this laboratory have shown that the ability of TRN neurons to regenerate their axons can be correlated with their ability to upregulate the expression of mRNAs for a variety of molecules. These include GAP-43 (Campbell et al., 1991; Vaudano et al., 1995; Vaudano et al., 1998) and L1 (Zhang et al., 1995), which might be expected to play important roles in axonal growth and pathfinding (Strittmatter et al., 1995; Caroni, 1997; Dahme et al., 1997), and c-jun (Vaudano et al., 1998) which may regulate the expression of other growth-related genes. CHL1 can now be added to this group of molecules. It is curious that, in the intact brain, the mRNAs for these regeneration-relevant molecules are more strongly expressed in dorsal thalamic nuclei than in the TRN. In response to injury, however, the TRN neurons rapidly upregulate expression of mRNAs for these molecules to much higher levels than do neurons in most dorsal thalamic nuclei, and the level of expression remains high for prolonged periods (at least several months). In the case of GAP-43, L1 and now CHL1, it is difficult to prove that the levels of the proteins coded for by the mRNAs also increase, because GAP-43 and L1 proteins do not usually accumulate in the cell bodies and, in the case of CHL1, antibodies which would enable us to investigate the protein by immunohistochemistry are not available. None the less GAP-43 protein becomes more abundant in some TRN neurons following graft implantation (Campbell et al., 1991) and both GAP-43 and L1 are present in or on regenerating axons within living grafts (Campbell et al., 1991; Vaudano et al., 1995; Zhang et al., 1995). The protein product of c-jun, being a transcription factor, accumulates in the nucleus of neurons which are expressing the mRNA and has been shown to be present in increased amounts in regenerating TRN neurons (Vaudano et al., 1998).

The role of CHL1 in promoting regeneration of CNS axons into PN grafts and possible coordinated expression of CHL1 and other growth-related molecules are further discussed in Chapter 8 and 9.
Table 6.1. Distribution of neurons expressing high levels of CHL1 mRNA following PN graft implantation in the thalamus

<table>
<thead>
<tr>
<th>Survival times</th>
<th>Animals</th>
<th>Regions damaged by insertion of the graft</th>
<th>Location of CHL1 positive neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>VP 203</td>
<td>LD, VPM, VPL</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 226</td>
<td>DLG, LP, VPM, VPL</td>
<td>TRN, VPM, VPL</td>
</tr>
<tr>
<td>7 days</td>
<td>VP 130</td>
<td>LD, VPM, VPL, PO</td>
<td>TRN, ZI</td>
</tr>
<tr>
<td></td>
<td>VP 169</td>
<td>B, TRN</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 174</td>
<td>TRN, VPL</td>
<td>TRN</td>
</tr>
<tr>
<td>9 days</td>
<td>VP 231</td>
<td>LD, PO, VPM, ZI</td>
<td>TRN, ZI, LD, VPM, VPL, PO</td>
</tr>
<tr>
<td></td>
<td>VP 232</td>
<td>LD, TRN, VPM, PO</td>
<td>TRN, VPL, VPM, PO</td>
</tr>
<tr>
<td>2 weeks</td>
<td>VP 171</td>
<td>TRN</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 201</td>
<td>TRN, LD, AV, VL</td>
<td>TRN, VL</td>
</tr>
<tr>
<td>4 weeks</td>
<td>VP 244</td>
<td>TRN</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 176</td>
<td>TRN</td>
<td>TRN</td>
</tr>
<tr>
<td>6 weeks</td>
<td>VP 233</td>
<td>LP, DLG, VPL, VPM, ZI</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 158</td>
<td>TRN</td>
<td>TRN</td>
</tr>
<tr>
<td>10 weeks</td>
<td>VP 170</td>
<td>DLG, TRN</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 177</td>
<td>TRN, AV</td>
<td>TRN</td>
</tr>
<tr>
<td>2 weeks Freeze-killed grafts</td>
<td>VP 242</td>
<td>TRN</td>
<td>TRN</td>
</tr>
<tr>
<td></td>
<td>VP 245</td>
<td>LD, LP, PO, VPM</td>
<td>---</td>
</tr>
<tr>
<td>4 weeks</td>
<td>VP 167</td>
<td>TRN</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>VP 243</td>
<td>TRN</td>
<td>---</td>
</tr>
<tr>
<td>6 weeks</td>
<td>VP 206</td>
<td>TRN, LD</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>VP 207</td>
<td>LP, PO, VPM</td>
<td>---</td>
</tr>
</tbody>
</table>

Abbreviations: AV, anterior ventral nucleus; B, basal nucleus on Meynert; DLG, dorsal lateral geniculate nucleus; LD, lateral dorsal nucleus; LP, lateral posterior nucleus; VL, ventral lateral nucleus; VPL, ventral posterolateral nucleus; VPM, ventral posteromedial nucleus; PO, posterior nucleus; TRN, thalamic reticular nucleus; ZI, zona incerta.
Figure 6.1 Diagram showing the implantation site of a PN graft in the thalamus.

Figure 6.2 Thalamus 6 weeks after the implantation of a living PN graft

6.2 a) Upregulation of CHL1 mRNA in the thalamus after insertion of a living PN graft. In this picture the graft tip ends near the dorsal part of the TRN (the boundaries of which are indicated by the dashed lines). Strong expression of CHL1 mRNA in neurons of the dorsal part of the TRN is seen near the graft tip (arrows). Some of the CHL1 positive cells (from an adjacent section) are enlarged in the inset. Scale bar = 500\( \mu \text{m} \) (6.2a); 250\( \mu \text{m} \) (inset).

6.2 b) Photomicrograph of the thalamus contralateral to 6.2a showing very low level of CHL1 mRNA expression in thalamic neurons including TRN (outlined by dashed lines). Scale bar = 500\( \mu \text{m} \).

Abbreviations: G, PN graft; H, hippocampus; F, fimbria; TRN, thalamic reticular nucleus; V, lateral ventricle
Figure 6.3 Thalamus at 9 days following the implantation of a PN graft, showing upregulation of CHL1 mRNA in the brain parenchyma around the graft tip. Negligible level of CHL1 mRNA is seen in the thalamus contralateral to the graft. A small group of very faintly CHL1 mRNA-labelled cells of uncertain identity is present in the dorsal part of the TRN (open arrow). Neurons of the VPM, VPL, PO and especially of the TRN (e.g., arrows) express high levels of CHL1 mRNA. Scale bar = 500 μm

Abbreviations: CTh, contralateral thalamus; F, fimbria; G, PN graft; H, hippocampus; PO, the posterior nucleus TRN, thalamic reticular nucleus; V, lateral ventricle; VPL, ventral posterolateral nucleus; VPM, ventral posteromedial nucleus.
**Figure 6.4** Thalamus 9 days following the implantation of a living PN graft

6.4a) Thalamus contralateral to a PN graft inserted 9 days previously at a more rostral level than shown in Fig. 6.3. Note that no CHL1 mRNA expression can be detected in neurons of the TRN. Scale bar = 250μm.

6.4b) CHL1 mRNA expression in the thalamus ipsilateral and close to the graft in the same animal. Note that strong upregulation of CHL1 mRNA expression in neurons close to the graft (e.g., *large arrows*). However, the most conspicuous CHL1 mRNA expression appears in the neurons of the TRN (*arrowheads*), although in this case the graft tip did not encroach directly on this nucleus. Many small, intensely labelled cells, probably glia (reactive astrocytes), are situated at the brain/graft interface, abutting the parenchymal border zone (e.g. *small arrows*). Scale bar = 500μm.

6.4c) Higher magnification photomicrograph of the TRN from 6.4b illustrating the labelled TRN neurons more clearly. Scale bar = 100μm.

**Abbreviations:** F, fimbria; G, PN graft; H, hippocampus; TRN, thalamic reticular nucleus.
Figure 6.5 Expression of CHL1 mRNA in the thalamus 9 days (6.5a), 2 weeks (6.5b,c), 6 weeks (6.5d) and 10 weeks (6.5e) after implantation of a living PN graft.

6.5a-e) Strong upregulation of CHL1 mRNA expression is seen in the TRN (arrowheads) at all survival times. Other groups of thalamic neurons upregulate CHL1 mRNA only if they are located within the lesion area near the graft tip (a, b, c, large arrows). At survival times longer than 2 weeks, CHL1 mRNA-containing cells are only detected in the TRN (d,e). A large number of small CHL1 mRNA labelled cells, presumably reactive astrocytes and/or other glia, are seen lining the brain/graft interface and also scattered among larger cell bodies, probably of neurons (b,c, small arrows). Such cells at the brain/graft interface are no longer detected at survival times longer than 2 weeks (d,e). Scale bar = 500μm (6.5a,b,d); 250μm (6.5c,e).

Abbreviations: F, fimbria; G, PN graft; H, hippocampus; TRN, thalamic reticular nucleus; V, lateral ventricle.
Figure 6.6 Adjacent sections of the thalamus from an animal 6 weeks after implantation of a PN graft. The sections were processed for CHL1 mRNA *in situ* hybridization (a) and for TMB histochemistry (b) to show the corresponding patterns of CHL1 mRNA expression and retrogradely labelled perikarya in TRN (e.g. *arrow*). Regenerating axons running along the graft in 6.6b are seen as delicate white streaks or lines. Scale bar = 125μm (6.6a,b).

Abbreviations: F, fimbria; G, PN graft; v, small blood vessels.
**Figure 6.7** Expression of CHL1 mRNA in identified regenerating TRN neurons in the thalamus from two animals at 10 weeks following the implantation of a living graft.

6.7a-d) High magnification photomicrographs of identical areas from immediately adjacent sections (a adjacent to b and c adjacent to d), showing CHL1 mRNA expressing perikarya (a,c) and retrogradely HRP-labelled perikarya (b,d) in the TRN. Corresponding labelled perikarya in a,b and c,d are matched and numbered. The asterisks in both pairs of pictures indicate corresponding blood vessels. The photomicrograph in 6.7d was taken in bright field to reduce background and improve the visualization of retrogradely labelled cells. The needle-like structures emerging from the labelled perikarya in 6.7d are TMB crystals which result from deliberately over-reacting the section in TMB solution.

Scale bar = 100μm for all panels.

**Abbreviation:** G, PN graft.
Figure 6.8 Thalamus at 2 weeks following the implantation of a freeze-killed graft.

6.8a, b) Brain parenchyma surrounding the graft (a) and enlargement of this area (b) showing no evidence of CHL1 mRNA expression. Only a certain number of small presumptive CHL1 mRNA labelled glia are present at the boundary of the graft (e.g. arrows in b). G indicates the position of the freeze-killed graft, the main part of which had been lost during tissue preparation. Scale bar = 500μm (6.8a); 250 (6.8b).

Abbreviations: G, PN graft; H, hippocampus; LD, lateral dorsal nucleus; LP, lateral posterior nucleus; VPM, ventral posteromedial nucleus; PO, posterior nucleus.
CHAPTER 7

Expression CHL1 mRNA in the cerebellum following peripheral nerve graft implantation

The cerebellum is a part of the CNS in which neurons display differential regenerative responses after injury and in the presence of a peripheral nerve graft. Previous reports have shown that axotomized neurons of the cerebellar cortex, such as Purkinje cells, never grow axons into nerve grafts, whereas neurons in the deep cerebellar nuclei (DN) regenerate successfully into the same grafts (Dooley and Aguayo, 1982; Vaudano et al., 1993a,b; Zhang et al., 1997). As shown in Chapters 4 and 6, the cell recognition molecule CHL1 is expressed by CNS neurons which regenerate axons into peripheral nerve grafts, but not in those which fail to do so. This implies that following implantation of a PN graft, neurons of the cerebellum may show differential expression of CHL1, depending on the ability in regenerating axons of each type of neurons. This Chapter investigates expression of CHL1 mRNA in cerebellar neurons following PN graft implantation. In addition, the relationship between neurons expressing CHL1 mRNA and neurons which regenerate axons into nerve grafts, is also examined.

1. Summary of methods used

(See Chapter 2 for details)

Seventeen adult rats were implanted with living PN grafts into the deep cerebellar region of the left cerebellum and had survival periods of 3 days (n=1), 1 week (n=4), 2 weeks (n=4), 3 weeks (n=1), 4 weeks (n=3), 6 weeks (n=2) and 7 weeks (n=2). In another group of animals, freeze-killed grafts were inserted into the cerebellum instead of living grafts, with postoperative survival periods of 3 days (n=1), 1 week (n=2), 2 weeks (n=2), 4 weeks (n=1) and 6 weeks (n=2). All animals were processed for non-radioactive in situ hybridization for CHL1 mRNA. In some animals
a selection of sections was made for double staining fluorescence *in situ* hybridization (FISH) for CHL1 and GFAP immunofluorescence staining.

Three animals from the living graft implantation groups at 2, 4 and 7 wpo were injected with tracer CT-HRP to the distal end of the graft 2 days before being sacrificed to identify neurons whose axons have regenerated along the graft. Alternative sets of cryostat brain sections were stained either for *in situ* hybridization for CHL1 mRNA or for TMB method of HRP visualisation.

2. Results

In the 27 animals used in this study, 18 contained grafts which encroached on the deep nuclear region of the cerebellum; 8 contained grafts which their tips also went deeply into the junction between the cerebellum and brainstem; and 1 contained the graft embedded in the cerebellar cortex (see Chapter 8: Table 8.2). Since, in several cases, implantation of PN grafts induced upregulation of CHL1 mRNA expression in neurons of the brainstem, the following results will include the observation from those neurons.

2.1) **Expression of CHL1 mRNA in the intact cerebellum and brainstem**

In the intact cerebellum, CHL1 mRNA was found at low levels throughout the granule cell layer and at moderate to strong levels in scattered cells within this layer which may have been Golgi cells, but was not expressed by Purkinje cells (Holm *et al.*, 1996). However, some cells in the molecular layer, possibly glia, expressed moderate to strong levels of CHL1 mRNA. Neurons of DN and brainstem expressed low to moderate levels of this molecule (Fig. 7.2b, 7.3).

2.2) **Expression of CHL1 mRNA in the cerebellum following implantation of a living PN graft**

Implantation of a PN graft could induce CHL1 mRNA expression in DN. At the early stage of graft implantation (3 dpo-1 wpo), a large number of peculiarly strong CHL1 mRNA containing cells were distributed around the graft; these positive cells
appeared both in the cerebellar cortex and DN region, close to where the graft passed through (Fig. 7.5a). A group of small CHL1 mRNA positive cells, resembling glia, were located close to the junctional zone (Fig. 7.2a, 7.5a-c, 7.6a,b); some of these cells were identified as reactive astrocytes since there were GFAP-immunoreactive (Fig. 7.8a). Presumptive CHL1 mRNA expressing glia mostly disappeared by 2 wpo; however, in rare cases such cells were still detected after this period of time (Fig. 7.6a,b). Strong CHL1 message was also found in Golgi cells and other interneurons in the granular layer near the graft. Such cells in the granular layer were visible at 3 dpo and 1 wpo (Fig. 7.3, 7.5a,b, 7.8a) and declined by 2 wpo. However, at all survival time examined, upregulation of CHL1 mRNA expression was not detectable in Purkinje cells, or in cells of molecular layer, even in the animal in which the graft tip was situated in the cerebellar cortex (killed at 1 wpo).

In the DN region, cells which upregulated CHL1 mRNA were found in all of the ipsilateral DN (Fig. 7.1a, 7.3-7.6). Most of the cells in DN containing CHL1 mRNA had the appearance of neurons (not illustrated) and were not immunoreactive for GFAP (Fig. 7.8b). At 3 dpo and 1 wpo, strong CHL1 mRNA labelled DN neurons were widespread around the graft tip as shown in Fig. 7.3 and 7.5a-d. In many cases, upregulation of CHL1 mRNA expression were seen in some neurons of the medial group of DN on the contralateral cerebellum (Fig. 7.3). These neurons probably belonged to the group of DN neurons which had projected their axons across the midline and the axons was cut by the graft. Strong signal of CHL1 mRNA was detectable in many DN neurons until 4 wpo (Fig. 7.5e, 7.6a,b). By the 6 and 7 wpo, many CHL1 positive DN neurons were still found near the graft; nevertheless, the signal in DN neurons was weaker than at earlier survival times (Fig. 7.6c,d).

In animals in which graft tips terminated the brainstem, there was usually some upregulation of CHL1 mRNA in the brainstem nuclei, including the medial lateral and spinal vestibular nuclei, and dorsal cochlear nucleus (Fig. 7.4).
2.3) **Expression of CHL1 mRNA in identified regenerating DN neurons**

In all three animals (survival times of 2, 4 and 7 weeks) used for this experiment, HPR-labelled neurons were found in the DN region close to the graft tip (Fig.7.9, 7.10). Most regenerating neurons which could be identified on immediately adjacent sections used for *in situ* hybridization, were found to give strong signals for CHL1 mRNA. Although it was not always possible to compare all DN neurons containing significant level of CHL1 mRNA to those whose axons have regenerated along the graft on adjacent sections, most of them could undoubtedly be distinguished as the corresponding neurons (Fig.7.10). The number of retrogradely labelled neurons, and neurons that had detectably upregulated CHL1 mRNA within the same nuclear groups were broadly similar.

In all animals, neither Purkinje cells nor any cells in cerebellar cortex were retrogradely labelled with HRP.

2.4) **Expression of CHL1 mRNA in the cerebellum following implantation of a freeze-killed graft**

Similar to implantation of a freeze-killed graft into the striatum (see Chapter 4) and thalamus (see Chapter 6), this type of graft failed to bring about conspicuous upregulation of CHL1 mRNA expression in the cerebellum, particularly in DN neurons. At 3 days following insertion of a freeze-killed graft, expression of CHL1 mRNA was significant in some cells of the granular layer, DN neurons and astrocytes situated close to the graft. However, the number and intensity of these positive cells were not as distinct as those detectable in the living graft case. There was no evidence of CHL1 mRNA message in DN neurons at survival times longer than 3 dpo (Figs. 7.7, 8.22b). CHL1 mRNA expression was not detected in Purkinje cells in any case.

3. **Discussion**

The results of this study have shown that following axotomy, cells of the cerebellum highly upregulated CHL1 mRNA expression. Such cells included neurons of DN and some small neurons in the granular layer, probably Golgi cells but not
Purkinje cells. In addition, when the tip of grafts impinged the brainstem, axotomized brainstem neurons, mostly belonged to sensory nuclei such as cochlear and vestibular nuclear complex, also upregulated CHL1 mRNA expression. Glia and GFAP immunopositive presumptive astrocytes close to the brain/graft interface also expressed CHL1 mRNA, but expression in these cells was downregulated by 2 wpo, as observed in graft implantation in the striatum and thalamus. In keeping with the expression of CHL1 mRNA in the striatum and thalamus (Chapters 4 and 6, respectively), such expression in DN and brainstem neurons which regenerated axons vigorously in PN grafts, could be prolonged in the presence of a graft containing living Schwann cells. Importantly, this study has confirmed that CHL1 mRNA was expressed by some regenerating DN and brainstem neurons. This can be another example to support the evidence that CHL1 is a growth-related molecule and may be a key molecule promoting axon growth in CNS neurons.

Since this study is related to that in Chapter 8, further discussion is integrated in the discussion of Chapter 8 to avoid repetition.
Figure 7.1 Diagram showing the site of implantation of a peripheral nerve graft into the cerebellum. The proximal end of the graft terminates in the region of nucleus interpositus of the DN (see contralateral side of the diagram for nuclear locations).

Figure 7.2 Photomicrographs of a frontal section of the cerebellum, 1 week following the implantation of a living graft.

7.2a) Upregulation of CHL1 mRNA expression in neurons of the interpositus nucleus of the DN (large arrows) around the graft tip. Small CHL1 mRNA positive cell, resembling glia, are also present near the graft/brain interface (small arrows). No evidence of CHL1 mRNA expression is seen in neurons of the molecular layer or in Purkinje cells.

7.2b) Photomicrograph of the control contralateral cerebellum from the same section as shown in 7.2a, showing absence of expression of CHL1 mRNA in the corresponding DN neurons. A few small cells with moderate CHL1 labelling are detected in the granular layer (arrows); similar cells are also seen but not labelled in the cerebellar cortex ipsilateral to the graft (Fig. 7.2a).

Scale bar = 500μm for all panels.

Abbreviations: BS, brainstem; CT, cerebellar cortex; DN, deep cerebellar nuclei; G, PN graft; Gr, granular layer; I, interpositus nucleus; L, lateral nucleus; M, medial nucleus; Mo, molecular layer; IV, fourth ventricle.
Figure 7.3 Photomicrographic montage of a coronal section through the brainstem and cerebellum, showing upregulation of CHL1 mRNA expression at 1 wpo. Many strongly CHL1 mRNA-labelled neurons in the interpositus nucleus of the DN are distributed around the graft tip (e.g., at large arrows). Note that a few neurons in the medial nucleus of the DN in the contralateral cerebellum also express CHL1 mRNA (open arrows). Some cells in the granular layer close to the graft apparently express this gene at strong level (small arrows). Scale bar = 500μm.

Abbreviations: DN, deep cerebellar nuclei; G, PN graft; I, interpositus deep cerebellar nucleus; L, lateral nucleus; M, medial nucleus.
Figure 7.4 Cerebellum at 1 wpo containing a graft (G) with its tip terminating between deep cerebellar nucleus (DN) and brainstem (BS) regions. Upregulation of CHL1 mRNA is seen in neurons of both regions, i.e., DN neurons (e.g. arrows) and pre-cerebellar neurons (e.g. open arrows). Scale bar = 125μm.

Abbreviations: BS, brainstem; DC, dorsal cochlear nucleus; DN, deep cerebellar nuclei.
Figure 7.5 Expression of CHL1 mRNA in the cerebellum at 3 days (7.5a-c), 1 week (7.5d) and 4 weeks (7.5e) following the implantation of a living PN graft.

7.5a-c) Cerebellum at 3 dpo showing upregulation of CHL1 mRNA expression in numerous cells around and close to the graft (G). Heavily labelled DN neurons are located both close to the graft/brain interface (e.g., solid arrows in a,c) and in surrounding brain parenchyma (e.g., open arrows in c). The framed areas in a are shown at higher magnification in b and c. A large number of CHL1 mRNA labelled cells, most of which are probably glia, are seen at the brain/graft interface along the entire length of the proximal part of the graft (e.g. thin arrows in b,c). Some cells in the granular layer near the graft also upregulate CHL1 mRNA (e.g. arrowheads in a, b). Scale bar = 500μm (7.5a); 100μm (7.5b,c).

7.5d) Medium power photomicrograph of a section adjacent to that shown in Fig. 7.2a, showing the tip of a living graft embedded in the DN of the cerebelum 1 week after graft insertion. Numerous DN neurons near the graft tip are heavily labelled with CHL1 mRNA (e.g. arrows). However, only small numbers of presumptive glia at the graft/brain interface display CHL1 mRNA signal at this survival time (e.g. small arrows). Scale bar = 250μm.

7.5e) At 4 wpo many neurons in the DN still express CHL1 mRNA (arrows). Note that at this survival time there are no CHL1 mRNA-labelled cells (presumptive glia) at the brain/graft interface. Scale bar = 500μm.

Abbreviations: DN, deep cerebellar nuclei; G, PN graft; Gr, granular layer; Mo, molecular layer.
**Figure 7.6** Cerebellum at 4 weeks (7.6a,b) and 6 weeks (7.6c,d) following implantation of a living PN graft.

7.6a,b) Low power photomicrograph of the cerebellum at 4 wpo (a) and an enlargement of the framed graft tip area (b) showing upregulation of CHL1 mRNA in the region of DN close to the graft tip. A small number of large neurons, strongly labelled for CHL1 mRNA in the interpositus nucleus of the DN are seen near the tip of the graft (e.g., *large arrows* in a,b). A few presumptive glia expressing CHL1 mRNA also appear close to the junctional zone in this animal (b, *small arrows*). There is no sign of CHL1 mRNA upregulation in neurons of the cerebellar cortex at this survival time. Scale bar = 500µm (7.6a); 250µm (7.6b).

7.6c) Photomicrograph of ipsilateral cerebellum close to the tip of a graft at 6 wpo, illustrating CHL1 mRNA-labelled neurons in the lateral nucleus of the DN (e.g., *arrows*). Scale bar = 500µm.

7.6d) Upregulation of CHL1 mRNA in DN neurons in another animal at 6 wpo. This picture illustrates a region of DN caudal to the graft tip in which there are many strongly CHL1 mRNA positive neurons in the interpositus nucleus (e.g., *arrows*). The *asterisk* indicates an area of damage in the region between the DN and the brainstem produced by the graft tip. Scale bar = 100µm.

**Abbreviations:** BS, brainstem region; DN, deep cerebellar nuclei; G, PN graft.
Figure 7.7 Cerebellum at 1 week (7.7a) and 6 weeks (7.7b) following the implantation of a freeze-killed PN graft.

7.7a,b) Low power photomicrographs of the ipsilateral cerebellum at 1 week (a), containing a graft with its tip embedded in the DN, and at 6 weeks (b), containing a graft with its tip embedded in the brainstem. In both cases, there is no upregulation of CHL1 mRNA in the vicinity of the graft. Neurons of the brainstem nucleus located close to the fourth ventricle (IV) normally express CHL1 mRNA at moderate levels as seen in a; however, neurons of the same nucleus seen in b seem to express high levels of this gene because the section has been over-reacted with the probe. Scale bar = 250μm.

Abbreviations: BS, brainstem; DN, deep cerebellar nuclei; G, PN graft; IV, fourth ventricle.
**Figure 7.8** Dual fluorescence to visualize CHL1 mRNA (labelled with FITC, which appears green) and GFAP immunoreaction (labelled with TRITC, which appears red) in the cerebellum of an animal implanted with a living PN graft at 1 wpo.

7.8a) Confocal image showing a region of the cerebellar cortex near the graft (G). Many CHL1 mRNA positive cells are located in the granular layer close to the graft (G). Some of the CHL1 mRNA-expressing cells are immunopositive for GFAP, showing that they are presumptive astrocytes (appear yellow because of colocalization/overlap of fluorescence, *large arrows*) whereas others are probably small interneurons and Golgi cells (seen as green spots of various sizes). A small group of CHL1 mRNA labelled cells lying close to the junctional zone can be seen near the top left corner of the picture (e.g., *small arrows*). CHL1 mRNA signal within the graft area (*arrowheads*) is presumably expressed by Schwann cells in the graft. Scale bar = 25μm.

7.8b) Confocal image of the graft tip area from the section shown in 7.8a showing three large, strongly CHL1 mRNA-labelled DN perikarya. Part of the graft tip is located at the top of the picture (G). Note the absence of overlap of GFAP immunofluorescence with CHL1 signal in the DN neurons. Scale bar = 10μm.

**Abbreviations:** DN, deep cerebellar nuclei; G, PN graft; Gr, granular layer; Mo, molecular layer.
Figure 7.9 Expression of CHL1 mRNA and distribution of retrogradely labelled neurons in immediately adjacent sections of the cerebellum 6 weeks after the implantation of a living PN graft.

7.9a,c) Low (a) and high (b) magnification photomicrographs of the section hybridized with probe for CHL1 mRNA showing upregulation of expression in DN neurons to the right of the graft (G). Scale bar = 250μm (7.9a); 125μm (7.9c).

7.9b,d) Corresponding low power dark-field illumination photomicrograph (b) of the same area shown in a and bright field enlargement (d) showing retrogradely labelled DN perikarya located in the same area as the labelled cells in a and c (see also Fig. 7.10). Scale bar = 250μm (7.9b); 125μm (7.9d).

Abbreviation: G, PN graft
Figure 7.10 Comparison between CHL1 mRNA expressing cells and retrogradely labelled neurons in immediately adjacent sections of the cerebellum at 6 wpo following the implantation of a living PN graft. These photomicrographs are taken from the same animal illustrated in Fig. 7.9.

7.10a-d) Bright-field (a,c) and phase contrast photomicrographs (b,d) of the DN region showing localization of CHL1 mRNA labelled DN perikarya and retrogradely HRP-labelled DN perikarya, respectively. Corresponding labelled perikarya are matched and indicated by identical Arabic numbers (1-8). c and d are enlargements of a and b respectively but are rotated 90° clockwise with respect to photomicrographs a and b. All of the in situ positive cell bodies in c contain HRP in d. The two unnumbered HRP labelled cell bodies at the bottom right of d have no counterparts in c but may have displayed hybridization signal in the other adjacent sections (not probed for CHL1 mRNA). Scale bar = 100μm (7.10a,b); 50μm (7.10c,d).

Abbreviation: G, PN graft.
CHAPTER 8

Differential expression of L1, CHL1, c-jun and GAP43 mRNAs in the cerebellum following peripheral nerve graft implantation

Although axons do not normally regenerate within the CNS of adult mammals, many types of CNS neuron will regenerate axons into segments of peripheral nerve implanted into the brain or spinal cord (e.g., David and Aguayo, 1981; Benfey et al., 1985; Anderson et al., 1998). However, not all CNS neurons show equal propensities for regenerating their axons, even into nerve grafts. The most thoroughly studied example of such differential axonal regeneration involves peripheral nerve grafts in the rat thalamus. Such grafts axotomize many types of neuron, but >90% of the axons which regenerate into the grafts originate from a single source, the thalamic reticular nucleus (TRN) (Benfey et al., 1985; Morrow et al., 1993). Peripheral nerve grafts in other regions of the CNS also induce differential regeneration. For example, most of the neurons which can be retrogradely labelled from grafts in the caudatoputamen arise from the substantia nigra pars compacta, although the small population of striatal cholinergic interneurons may also have a relatively good capacity for axonal regrowth (Woolhead et al., 1998). Perhaps the most clear-cut model for studying differential axonal regeneration is provided by nerve grafts implanted into the cerebellum. Such grafts are invaded by axons from the DN and precerebellar nuclei in the brainstem, but never by the axons of Purkinje cells (Dooley and Aguayo, 1982; Vaudano et al., 1993a, 1998; see also Chapter 7).

The CNS neurons which regenerate axons into grafts do not share obvious neuroanatomical or neurochemical characteristics, but successfully regenerating neurons have been shown to either upregulate, or have high levels of constitutive expression of, mRNAs for several molecules, the expression of which has been correlated with axonal regeneration in the peripheral nervous system (PNS) and with
neurite outgrowth in culture. These molecules include the growth-associated phosphoprotein GAP-43, the cell recognition molecule L1, its close homologue, CHL1 (see Chapters 4, 6 and 7), and the transcription factor, c-jun (reviewed by Anderson et al., 1998).

In situ hybridization studies have demonstrated that mRNA for GAP-43 is upregulated by neurons in the TRN (Vaudano et al., 1995), neostriatum (Woolhead et al., 1998) and retina (Jung et al., 1997) when they regenerate their axons into nerve grafts. Neurons of the TRN also upregulate L1 (Zhang et al., 1995), c-jun (Vaudano et al., 1998), and CHL1 (see Chapter 6) when they regenerate their axons. Nigral neurons show a moderate to high level of constitutive expression of GAP-43, L1 (Woolhead et al., 1998) and CHL1 mRNAs (see Chapter 4), but do not appear to upregulate them during regeneration. In the case of grafts in the thalamus and striatum, those classes of CNS neurons which fail to regenerate their axons neither upregulate mRNAs for GAP-43, L1 and CHL1, nor show high levels of constitutive expression of those molecules. Those CNS neurons which regenerate axons into grafts upregulate c-jun (Hull and Bähr, 1994; Vaudano et al., 1998; and see Chapter 5), but so do other neurons around the graft tip. This may be because c-jun is involved in controlling other processes such as apoptosis, as well as axonal regeneration. Although DN neurons upregulate GAP-43 and c-jun when they regenerate their axons into peripheral nerve grafts (Vaudano et al., 1993a,b), various studies of GAP-43 and c-jun expression by the non-regenerating Purkinje cells following axotomy, show apparent contradictions. Buffo et al., (1997), in a study of transgenic mice in which GAP-43 is overexpressed in Purkinje cells, did not report the presence of GAP-43 or c-jun expression in Purkinje cells in non-transgenic animals, even following axotomy and the implantation of cultured Schwann cells at the site of axotomy. In addition, Vaudano et al., (1998) found no GAP-43 expression in Purkinje cells following the implantation of peripheral nerve grafts into the cerebellum of adult rats. On the other hand, Dusart et al., (1997) reported that Purkinje cells upregulated GAP-43 mRNA after axotomy. Similarly, although Vaudano et al. (1988) found no evidence of c-jun expression by Purkinje cells following the implantation of nerve grafts into the cerebellum. Zagrebelsky et al. (1998) reported that a relatively small number of Purkinje cells express c-jun after being axotomized very
close to their cell bodies. If the fundamental processes which limit regeneration in the CNS are to be understood and rational strategies for repair are to be developed, it is important to identify which genes are expressed by regenerating CNS neurons but not by those cells which cannot regenerate their axons.

In Chapter 7, the results of a systematic study of CHL1 expression in the cerebellum following PN graft implantation showed that CHL1 was upregulated in DN neurons which regenerate axons along the graft but was not expressed by non-regenerating cells such as Purkinje cells. In parallel with the studies of CHL1 expression, I have also examined the expression of L1, GAP-43 and c-jun, and in this Chapter, I bring the CHL1 findings into juxtaposition with the L1, GAP-43 and c-jun findings, with the particular aim of comparing the responses of these 4 molecules and determining whether or not all behave identically in the different populations of neurons examined. In fact two of these mRNAs, GAP-43 and c-jun have previously been examined in earlier studies carried out in our laboratory, mostly using radioactive oligonucleotide \textit{in situ} probes and focusing primarily on the cerebellar cortex and to much lesser extent on the DN (Vaudano \textit{et al.}, 1993a,b, 1998). I shall therefore compare the new findings on GAP-43 and c-jun with older findings. Finally, in this Chapter, I investigate if there is correlated expression among these 4 molecules in individual neurons which regenerate or fail to regenerate, their axons after graft insertion. For the purposes of direct comparison with the pattern of expression of L1, GAP-43 and c-jun, some of the data and illustrations of CHL1 expression from Chapter 7 are presented again in this Chapter.

1. \textbf{Summary of methods used}

\textit{(See Chapter 2 for details)}

Living and freeze-killed autologous tibial nerve grafts were implanted into the left cerebellum of adult Sprague Dawley rats. Animals were left to survive between 3 days and 16 weeks. They were then reanaesthetized, killed and processed as described in Chapter 2.
**LI:** Sixteen rats received living grafts and had survival times of 3 days (n=1), 1 week (n=2), 2 weeks (n=3), 3 weeks (n=2), 4 weeks (n=1), 5 weeks (n=1), 6 weeks (n=2), 8 weeks (n=2). Nine rats received freeze-killed grafts and had survival times of 1 week (n=3), 2 weeks (n=3), 4 weeks (n=1), and 6 weeks (n=2).

**CHL1:** see Chapter 7 for details.

**c-jun:** Eighteen rats received living grafts and had survival times of 3 days (n=1), 1 week (n=3), 2 weeks (n=4), 3 weeks (n=1), 4 weeks (n=3), 6 weeks (n=2), 7 weeks (n=2), 13 weeks (n=1) and 16 weeks (n=1). Eight rats received freeze-killed grafts and had survival times of 3 days (n=1), 1 week (n=2), 2 weeks (n=2), 4 weeks (n=1) and 6 weeks (n=2).

**GAP-43:** Thirteen rats received living grafts and had survival times of 3 days (n=1), 1 week (n=3), 2 weeks (n=1), 3 weeks (n=1), 4 weeks (n=2), 6 weeks (n=2), 7 weeks (n=1), 13 weeks (n=1) and 16 weeks (n=1). Six rats received freeze-killed grafts and had survival times of 3 days (n=1), 2 weeks (n=2), 4 weeks (n=1) and 6 weeks (n=2).

Cryostat sections of unfixed cerebellum, cut at 12 μm, were processed for in situ hybridization using digoxigenin labelled cRNA probes made against c-jun, GAP-43, L1 or CHL1. Pairs of adjacent sections were selected to investigate co-expression of these molecules, as follows: c-jun/GAP-43, c-jun/CHL1, GAP-43/CHL1, c-jun/L1 or CHL1/L1.

To investigate if neurons which expressed high levels of, L1 CHL1 c-jun, and GAP-43 were the ones which had regenerated their axons into the grafts, a group of animals from postoperative survival periods between 3 and 8 weeks were injected with CT-HRP into the distal end of the grafts 2 days before the animals were killed. Adjacent cryostat sections of the cerebellum from these animals were selected for processing either by the TMB method to reveal transported CT-HRP or for in situ hybridization to detect mRNAs for L1, CHL1, c-jun, or GAP-43.
To compare expression among the four mRNAs and also to compare cells which show colocalization of markers (in situ signal and retrograde HRP labelling), labelled cells were counted. Recognisable profiles of neuronal cell bodies displaying L1, CHL1, c-jun or GAP-43 hybridisation signals, or containing CT-HRP reaction product were counted in every section taken through the region containing cells labelled with the marker under investigation. Fragments of neurons were not counted. Means were derived by considering only sections containing labelled neurons of a particular group. For example, the mean number of L1 mRNA positive DN neurons per section in any given animal was determined as follows. The most rostral and the most caudal sections through DN containing L1 expressing neurons were identified. L1 expressing neurons were counted in all available sections containing labelled neurons between these levels and the total number of labelled cells divided by the number of sections containing such cells to give an average number per section. Although such counts were carried out separately for individual groups of DN and brainstem, they are aggregated in the results presented here for the three DN nuclear groups, the lateral, interposed (interpositus) and medial nuclei and for all brainstem nuclear groups.

2. Results

Animals used in this study contained grafts with their tips embedded in different regions of the cerebellum, i.e., the cerebellar cortex, the DN region, the junction between the DN region and brainstem, and also in the brainstem. The details of all graft tip positions are summarised in Tables 8.1 (for L1), 8.2 (for CHL1), 8.3 (for c-jun) and 8.4 (for GAP-43).

2.1) L1
A) Expression of L1 mRNA in the intact cerebellum

In unoperated animals and on the contralateral side of animals with nerve grafts in the left cerebellar hemisphere, L1 mRNA was expressed at a weak to moderate level throughout the granule cell layer and at a moderate to strong level by a few, evenly scattered, granule cells. In the molecular layer, stellate and basket cells also exhibit weak to moderate levels of expression. There was no hybridization signal for L1
mRNA over Purkinje cells (Fig. 8.2b, see also Holm et al., 1996). Neurons in all the cerebellar deep nuclei and neurons in certain brainstem nuclei expressed moderate to high level of L1 mRNA (Fig. 8.2b). Only sections passing through or close to the nerve graft were examined in detail. In these sections, neurons in the ventral cochlear nucleus, posterior part, medial vestibular nucleus (main nucleus and ventral part), lateral vestibular nucleus, spinal trigeminal nucleus, dorsal cochlear nucleus, mesencephalic trigeminal nucleus, nucleus hypoglossus prepositus, facial nucleus, caudal interstitial nucleus, gigantocellular reticular nucleus and locus ceruleus were found to express L1 mRNA.

B) Expression of L1 mRNA in the cerebellum following the implantation of a living PN graft

Three days after graft implantation into the cerebellum, the hybridization signal in neurons in the cerebellar cortex and DN on the side receiving the graft was no stronger than on the unoperated side, irrespective of the position of the graft tip, with the exception of 4 neurons in 3 sections of the medial deep nucleus close to the graft tip which showed a stronger signal suggesting the onset of upregulation of L1 mRNA (Fig. 8.20). From 1 to 8 wpo in animals with the graft tips ending in the DN region, there was not much difference in the level of expression or the number of neurons which had upregulated L1 mRNA (Figs. 8.2a, 8.19b and Table 8.1). The neurons which had upregulated L1 mRNA were found in all the DN groups (medial, interpositus and lateral) and were ipsilateral to the graft tip except for some animals with medially positioned grafts, in which neurons in the contralateral medial deep nucleus showed sign of upregulation. The labelled neurons were mainly within 1 mm of the graft tip except for those in contralateral medial DN, which were up to 2 mm from the graft. There was, however, no evidence for the upregulation of L1 mRNA expression by any cells in the cerebellar cortex. In no case did Purkinje cells express detectable levels of L1 mRNA (Figs. 8.2a, 8.21b), although the course of the grafts indicated that Purkinje cells axons must have been injured by graft implantation. Furthermore, even in those animals, in which the graft terminated in the cerebellar cortex, L1 message was not detected in the Purkinje cells. In most of the animals surviving from 1 to 8 weeks after implantation of a graft which entered the brainstem (see Table 8.1), neurons in several
nuclei close to the graft tip, including the dorsal and ventral cochlear nuclei, medial and lateral vestibular nuclei, had also upregulated L1 mRNA (Fig. 8.4a,b).

C) Expression of L1 mRNA in identified regenerating DN neurons

Sections from 5 animals were processed for both L1 mRNA and retrograde labelling. The number of retrogradely labelled neurons, and neurons expressing high levels of L1 mRNA within the nuclear groups examined were broadly similar. In all nuclei that contained retrogradely labelled neurons, neurons expressing strong signal for L1 mRNA were present in the same region of adjacent sections. Furthermore, the great majority of individual neurons retrogradely labelled from the grafts (both in the DN and in the brainstem, including the medial, lateral and spinal vestibular nuclei, and dorsal cochlear nucleus) which could be identified in the immediately adjacent sections used for in situ hybridization, also expressed high levels of L1 mRNA (compare Fig. 8.3b with 8.3a,c and Fig. 8.4b with 8.4c and Table 8.6).

D) Expression of L1 mRNA in the cerebellum following the implantation of a freeze-killed PN graft

Freeze-killed PN grafts seem to be unable to induce upregulation of L1 mRNA in neurons of the cerebellum. Only a few neurons of DN in one of the animals killed after 1 week and a small number of DN and brainstem neurons in one animal at 6 wpo (in which the graft tip was located in the brainstem) showed upregulation of L1 mRNA (not illustrated). No purkinje cells or other cells in the cerebellar cortex showed detectable upregulation of L1 mRNA (Tables 8.1 and 8.5).

2.2) CHL1

The detailed findings are presented and illustrated in Chapter 7. Here, selected illustrations from Chapter 7, showing expression of CHL1 mRNA in cerebellar neurons (Fig. 7.5a,d) and in identified regenerating neurons (retrogradely CT-HRP-labelled neurons) (Fig. 7.10) after graft implantation, are shown again in Figs. 8.5-8.7, in order to allow easier comparison of patterns of CHL1 mRNA expression with patterns of expression of the mRNAs for L1 (Figs. 8.2,8.3), c-jun (Figs. 8.8-8.10) and GAP-43 (Figs. 8.11-8.13).
2.3) c-jun

A) Expression of c-jun mRNA in the intact cerebellum

In the intact cerebellum, c-jun mRNA was expressed at low level by cells throughout the granule cell layer and some cells in the molecular layer, but was not expressed by Purkinje cells (Fig. 8.9b). Neurons in the DN expressed low or undetectable levels of c-jun mRNA (Fig. 8.9b) as did most brainstem nuclei except for the medial and lateral vestibular nuclei which showed moderate levels of expression; and the facial nucleus and the nucleus of the solitary tract which showed strong expression. Scattered cells in the granule cell layer of uncertain identity showed moderate to strong levels of c-jun mRNA expression.

B) Expression of c-jun mRNA in the cerebellum following implantation of a living PN graft

Three days to 16 weeks after graft implantation, many neurons in all the deep nuclei around the tip of the graft and in the contralateral medial nucleus of the DN and in the brainstem when the graft impinged on the region, showed a strong signal for c-jun mRNA (Figs. 8.8, 8.9a, 8.20 and Table 8.3). The numbers of c-jun positive DN neurons and the strength of expression appeared to be maximal at 3 dpo, but there was a high level of expression throughout the period 3 dpo to 4 wpo (Figs. 8.8, 8.9a). The c-jun signals in DN and brainstem neurons ipsilateral to the graft were weaker at 6 wpo and longer survival times; the numbers of positive cells found at these survival times were much smaller than at earlier survival times (Fig. 8.21d). Expression of c-jun was still detected in a few DN neurons at 16 wpo, but the signal was relatively weak.

In keeping with the results of c-jun expression in the striatum (Chapter 5), at early survival times (3 dpo to 1 wpo), a large number of cells, situated close to the brain/graft interface and along the course of the graft, upregulated strong expression of c-jun. (Fig. 8.8); some of these positive cells resembled reactive astrocytes (or other glia) whereas others appeared to be neurons (Fig. 8.8). The latter group of c-jun positive cells which were small in size appeared to comprise a population distinct from that of the c-jun positive cells in the DN and other nuclei at greater distances from the graft. The number of small c-jun mRNA labelled cells, both presumptive astrocytes and
neurons, at the brain/graft interface gradually declined and they were very few at 6 wpo.

Interestingly, some Purkinje cells located in the folium near the graft also expressed c-jun at strong to moderate levels at 3 dpo-3 wpo (Figs. 8.8, 8.20) and at low levels at 4 -16 wpo (Fig. 8.14a). The numbers of c-jun-expressing Purkinje cells also declined following the increasing survival times.

C) Expression of c-jun mRNA in identified regenerating DN neurons

In the 3 animals with living grafts used for retrograde labelling combined with in situ hybridization for c-jun mRNA, HRP-labelled neuronal perikarya were found in the DN and in some brainstem nuclei. In nearby sections, neurons in the same regions showed strong signals for c-jun mRNA. The numbers of retrogradely labelled neurons, and the numbers of neurons that had high levels of c-jun in the same nuclear groups were similar (Table 8.6). When cells expressing c-jun could be identified in the immediately adjacent sections reacted by the TMB method for visualisation of HRP, they were usually found to be retrogradely labelled from the graft (compare Fig. 8.10a with 8.10b).

D. Expression of c-jun mRNA in the cerebellum following the implantation of a freeze-killed PN graft

Following freeze-killed graft implantation into the cerebellum, c-jun mRNA expression was found in a small number of DN neurons close to the graft in some animals at 3 dpo to 4 dpo. Six week after graft implantation, the numbers of c-jun positive cells in the DN region were very few; only one such cell was found in one animal of this group, but not in the other. Strong expression of c-jun in Purkinje cells was found at all survival time examined; however, the number of c-jun expressing Purkinje cells was reduced as survival time increased. In addition, a few cells in the granular layer and molecular layer close to the graft also expressed strong signals for c-jun from 3 dpo to 6 wpo, and a small number of brainstem neurons expressing c-jun was detected in one animal at 1 wpo (Tables 8.3 and 8.5).
2.4) GAP-43

A) Expression GAP-43 mRNA in the intact cerebellum

In the intact cerebellum, GAP-43 mRNA was expressed at low to moderate levels by cells throughout the granular layer, and at low levels by some cells in the molecular layer, but was not expressed by Purkinje cells (Fig. 8.12b). Scattered cells in the granule cell layer expressed moderate levels of GAP-43 mRNA. Neurons of the DN expressed low or undetectable levels of GAP-43 mRNA (Fig. 8.12b). In the brainstem, high levels were found in the C1 group of adrenergic cells and the N4 group of noradrenergic cells, gigantocellular reticular nucleus and facial nucleus; moderate levels were observed in the hypoglossus prepositus nucleus. Negligible levels of GAP-43 mRNA were found in vestibular nuclei.

B) Expression GAP-43 mRNA in the cerebellum following the implantation of a living PN graft

Similar to the expression of other molecules at 3 days after graft implantation described above, many neurons of the DN expressed high levels of GAP-43 mRNA at this early survival time. Strikingly, cells expressing GAP-43 mRNA found around the graft were mostly large, presumably neurons, and there were rarely small cells situated along the brain/graft interface as seen in the sections hybridized with c-jun probe (Fig. 8.11). Strong upregulation of GAP-43 mRNA expression in DN neurons was detected at all survival times examined (3dpo-16 wpo) (Figs. 8.11, 8.12a, 8.21e and Table 8.4), although the number of the positive cells declined as survival time increased. In addition to ipsilateral DN neurons, upregulation of GAP-43 mRNA was also found in the contralateral medial group of the DN in animals with a medially positioned graft. However, GAP-43 mRNA was not detected in Purkinje cells in any animal studied from 3 days to 16 weeks following graft implantation, although Purkinje cells expressed c-jun mRNA in the adjacent sections (compare Fig. 8.14a with 8.14b), with the single exception of one Purkinje cells in one animal at 6wpo. There was no evidence of upregulation of GAP-43 mRNA expression in other neurons of the granular and molecular layers.
C) Expression of GAP-43 mRNA in identified regenerating DN neurons

Two rats, killed at 6 and 7 weeks after the implantation of a living nerve graft, were used for this study. Several neurons around the graft in the DN were found to be retrogradely labelled. In nearby sections, neurons in similar regions were found to express high levels of GAP-43 mRNA. The numbers of GAP-43 expressing and retrogradely labelled neurons were closely matched (Table 8.6). Where retrogradely labelled regenerating neurons could be identified on the immediately adjacent hybridized sections, they could usually be shown to co-express GAP-43 mRNA (compare Fig. 8.13a with 8.13b).

D) Expression GAP-43 mRNA in the cerebellum following the implantation of a freeze-killed graft

After implantation of a freeze-killed graft into the cerebellum, strong GAP-43 mRNA was found in many DN neurons close to the graft at 3 dpo, and the number of such cells declined as the survival times increased (Tables 8.4 and 8.5). GAP-43 mRNA labelled cells were still found in the DN region until 4 wpo (Fig 8.22d). By 6wpo, GAP-43 mRNA was found in a few DN neurons in one animal and in a few neurons in the medial vestibular nucleus of the other animal. However, GAP-43 mRNA was not detectable in Purkinje cells around the graft in any of the animals (Tables 8.4 and 8.5).

2.5) Co-expression of CHL1, L1, c-jun and GAP-43 mRNAs in DN and brainstem neurons

Immediately adjacent sections from animals at different survival times were used for this study. The co-localization of more than one of the investigated mRNAs in the DN and/or brainstem nuclei was consistently observed when sections from individual animals were examined for the expression of more than one mRNA. In this study, co-expression of L1 and CHL1, CHL1 and c-jun, CHL1 and GAP-43, c-jun and L1, c-jun and GAP-43, and L1 and GAP-43 in the same neurons were examined (Table 8.7). When immediately adjacent sections were hybridized with different mRNA probes, co-localisation of more than one mRNA within individual neurons was shown.
This is referred to as co-expression and the findings are described below, for every combination of mRNA pairs.

A) Co-expression of L1 and CHL1 mRNAs

Two animals at 4 and 6 weeks after graft implantation were examined for the co-expression of L1 and CHL1 mRNAs. Immediately adjacent sections were used for L1 and CHL1 *in situ* hybridization. Cells displaying strong L1 message were found in the same regions of DN as cells expressed strong levels of CHL1 mRNA on adjacent sections. Some of the cells could be shown to co-express both L1 and CHL1 mRNAs (compare Fig. 8.15a with 8.15b).

B) Co-expression of L1 mRNA and c-jun mRNAs

In one animal at 2 weeks after the implantation of a living nerve graft, immediately adjacent sections were hybridized for L1 mRNA and c-jun mRNA. Cells displaying strong signal for L1 mRNA were found in the same regions of the deep nuclei as cells displaying strong signal for c-jun mRNA on adjacent sections. Furthermore, a few neurons expressing strong L1 mRNA could be identified on both sections and could be shown also to express strong c-jun mRNA (compare Fig. 8.16a with 8.16b). There were more neurons in the DN expressing strong signal for c-jun mRNA than strong signal for L1 mRNA.

C) Co-expression of c-jun and CHL1 mRNAs

Immediately adjacent sections in one animal at 1 week after the implantation of a living nerve graft were used for CHL1 and c-jun *in situ* hybridization. Cells containing CHL1 mRNA and cells containing c-jun mRNA were found in the same region of the DN. Some of the DN neurons could be shown to express both c-jun and CHL1 mRNA (compare Fig. 8.17a with 8.17b). From 1 to 3 weeks after graft implantation there were more neurons in the deep nuclei expressing strong signals for c-jun mRNA than for CHL1 mRNA, but at 6 weeks the numbers were broadly similar (Table 8.7).
D) Co-expression of GAP-43 mRNA and c-jun mRNAs

Six animals killed 1, 2, 4, 13 and 16 weeks after the implantation of a living nerve graft were examined for the co-expression of GAP-43 and c-jun mRNAs. Immediately adjacent sections were used for GAP-43 and c-jun in situ hybridization. In all six animals, cells containing GAP-43 mRNA were found in the same regions where cells containing c-jun mRNA were also present. Some of these cells were shown to co-express both GAP-43 and c-jun mRNAs (compare Fig. 8.18a with 8.18b).

E) Co-expression of GAP-43 and CHL1 mRNAs

Two animals at 1 week and 6 weeks after the implantation of a living nerve graft were used for this study. Where DN neurons expressing GAP-43 mRNA could be identified on adjacent sections they were found to co-express high levels of CHL1 mRNA (compare Fig. 8.19b with 8.19a,c).

3. Discussion

The main findings of the work reported in this chapter showing that implantation of a living PN graft into the cerebellum induced axonal regeneration into and along the graft from neurons of the DN and brainstem neurons projecting to the cerebellum, predominantly from neurons in the vestibular nuclei. Such grafts also induced upregulation of GAP-43, L1, CHL1 and c-jun mRNAs in the same neuronal population. Furthermore, by comparing in situ hybridization and retrograde labelling it was possible to show that the neurons upregulating these molecules have regenerating axons extending into the graft. Finally, when adjacent sections were hybridized with different probes, it is apparent that most individual regenerating neurons probably upregulated all 4 of the molecules investigated. None of the molecules was upregulated in Purkinje cells or other cells of the cerebellar cortex with the exception of c-jun, which were upregulated in Purkinje cells in animals both implanted with living and freeze-killed graft.
3.1) **Differential expression of L1, CHL1, GAP-43 and c-jun in the cerebellum following axotomy and the influence of a living graft**

When a PN graft was inserted into the DN region or encroached on the brainstem, strong expression of the mRNAs for 4 growth-associated molecules, L1, CHL1, GAP-43 and c-jun was observed in DN neurons and in some brainstem neurons. However, the patterns of expression of each of these four mRNAs were distinct. Following axotomy (caused by the implantation of freeze-killed grafts), expression of mRNAs for the cell recognition molecules, L1 and CHL1 was transiently upregulated in small numbers of DN neurons and was undetectable after 1 wpo, with the exception of one animal at 6 wpo in which a small number of L1 mRNA labelled DN and brainstem neurons was found. In contrast, expression of c-jun and GAP-43 mRNAs was detectable in a small number of DN and brainstem neurons at almost all survival times examined (see Table 8.5). However, the upregulation of GAP-43 and c-jun mRNAs in DN neurons following freeze-killed graft insertion can not be considered as a sign of successful axon regeneration in these neurons, because such grafts do not provide a favourable environment in which injured CNS neurons can regenerate (Berry *et al.*, 1988); further discussion on this matter appears in Section 3.4. The presence of PN grafts containing living Schwann cells enhanced the expression of all the genes examined and increased the number of labelled cells (see Tables 8.1-8.4) and these cells were shown to regenerate axons along the graft. Such grafts also exert common effects on the expression of the same genes in other CNS neurons (Vaudano *et al.*, 1995; 1998; and see Chapters 4-6). The possible role of a living graft in promoting expression of the investigated genes in axotomized CNS neurons is also discussed elsewhere (Chapters 4-6) and below (Section 3.4).

The patterns of gene expression of c-jun and GAP-43 and the number of neurons expressing both genes found in individual animals were similar; so too were the patterns of L1 and CHL1 expression. However, there were subtle differences between L1 and CHL1 expression patterns and between GAP-43 and c-jun expression patterns and more obvious differences between L1/CHL1 pattern on the one hand and GAP-43/c-jun pattern on the other hand. Expression of L1 and CHL1 was generally induced only by living graft and both expression declined after 4 wpo, whereas
expression of GAP-43 and c-jun was induced both by living and freeze-killed grafts. When the numbers of neurons expressing the four mRNAs were compared in individual animals at each survival time, it was shown that the numbers of cells expressing c-jun and GAP-43 mRNAs were usually larger than the numbers expressing L1 and CHL1 mRNAs (see Table 8.7). It is noteworthy that the mean numbers of labelled neurons derived in these studies cannot be taken to represent actual numbers or to serve as a reliable guide to the relative number expressing each mRNA because the counts for each mRNA were made on different sections. Nevertheless, the sections selected to be hybridized with all four probes (to detect L1, CHL1, GAP-43 and c-jun mRNAs) were in consecutive series, and many of the labelled cells which appeared in adjacent sections processed for different probes, could be shown to coexpress two mRNAs. The numbers of labelled neurons presented here, therefore, can serve as a rough way of comparing expression of the four mRNAs in individual animals.

Ample evidence has been provided to show that DN neurons of the cerebellum display a high regenerative propensity by growing axons into peripheral nerve grafts (Dooley and Aguayo, 1982; Vaudano et al., 1993a). This study has confirmed this capacity of DN neurons. Additionally, when immediately adjacent sections were probed for L1, CHL1, c-jun or GAP-43 mRNA and reacted for visualization for HRP the evidence that regenerating DN neurons upregulated expression of at least two of the investigated genes was unequivocal. Due to the technical limitations, namely that in sections cut 12μm thick through neurons approximately 15 -20μm in diameter, it is not usually possible to reliably identify profiles in more than two (occasionally three) adjacent sections, as parts of an individual neuron. Thus, it was difficult to determine if all neurons which could be retrogradely labelled from the graft displayed expression of more than two mRNAs.

In parallel with DN neurons, strong expression of L1, CHL1, GAP-43 and c-jun mRNAs was seen in some brainstem neurons, particularly in neurons of the cochlear nuclei, vestibular nuclei and hypoglossal prepositus nucleus, even when the graft tip did not encroach on the brainstem. Neurons of these nuclei are known to project their axons to the DN and cerebellar cortex where they end as mossy fibers (for review, see Palay
and Chan-Palay, 1974; Voogd, 1995). Recently, a number of studies have shown that some neurons of vestibular and hypoglossal prepositus nuclei have synaptic terminations on at unipolar brush cells, a novel type of small neuron mostly distributed in the granular layer of the cerebellum (e.g., Dino et al., 1999). Thus there seems to be no doubt that these brainstem neurons, much like neurons of the DN, upregulated all four growth-associated markers in response to axotomy. Moreover, the same groups of brainstem neurons also regenerated axons vigorously into the PN graft. These finding promote additional evidence that there is a close relationship between the expression of the growth-related molecules, L1, CHL1, GAP-43, and c-jun, and the regenerative capacity of CNS neurons.

In contrast to DN and brainstem neurons, neurons of the cerebellar cortex did not upregulate L1, CHL1 and GAP-43 mRNAs after axotomy or implantation of living PN grafts; only a small number of cells close to the graft, resembling Golgi and small granule cells transiently upregulated CHL1 mRNA expression. Such cells were however not detectable at survival times longer than 2 wpo. Similar to CHL1, expression of c-jun was also upregulated by a small group of cells, probably including astrocytes and neurons, in animals implanted with both living and freeze-killed grafts. These cells were located along the brain/graft interface and gradually declined by 6 wpo. Moreover, these c-jun-expressing cells were apparently never retrogradely labelled with CT-HRP (data not shown). As c-jun expression can induce apoptosis (Ham et al., 1995; Herdegen et al., 1997b; Watson et al., 1998), these cells might be considered to be undergoing cell death (see discussion below, Section 3.4). Interestingly, strong expression of c-jun mRNA was upregulated by some Purkinje cells near the graft at all survival time examined. The expression of c-jun in Purkinje cells could not be considered to indicate activation of the same intracellular signalling pathways that it is assumed to be activated in DN and brainstem neurons, since such expression was consistent even in the animals implanted with freeze-killed grafts (see discussion in Section 3.5). Neurons of the cerebellar cortex, particularly Purkinje cells do not regenerate axons into nerve grafts, even when the grafts are embedded in the cerebellar cortex rather than in the DN region (Vaudano et al., 1993a,b). Thus, the
failure to upregulate expression of these growth-associated markers in CNS neurons following injury may indicate a poor regenerative potential of these neurons.

3.2) Expression of LI and possible role in axonal regeneration

LI mRNA expression by DN neurons was more slowly upregulated following graft implantation than the other regeneration-related molecules investigated in the present study; there was little expression in the first week whereas the other molecules were highly upregulated by 3 dpo. This is one significant difference in the expression patterns of L1 and CHL1. The delayed upregulation of L1 mRNA expression could be related to the following possibilities. First, the induction of L1 may need a prerequisite or priming signal(s) from other factors, so the induction of gene transcription for L1 in injured neurons occurs more slowly than that of other genes. Second, the basal level of L1 mRNA expression in DN and brainstem neurons is normally rather high; small increases in the expression of this following experimental manipulation would therefore not be easy to distinguish from the level of constitutive expression. The presence of moderate levels of L1 mRNA in normal DN and brainstem neurons may likewise mask modest levels of upregulation following graft implantation and could explain why fewer L1 mRNA-upregulated neurons were detected, at all survival times, than neurons upregulating the other investigated genes, especially after short survival times. This hypothesis is supported by the delayed expression of L1 mRNA in TRN neurons, which normally express L1 mRNA at very low levels; strong upregulation of L1 mRNA expression in TRN neurons is not seen until 2 weeks following the implantation of a living graft, although a moderate level of such expression can be detected from 3 dpo (Zhang et al., 1995). In some CNS neurons which display a strong propensity for axon regeneration, strong L1 gene expression seems to be one of their characteristics. The neurons of the SNpc do not upregulate L1 mRNA when they regenerate axons into nerve grafts in the striatum but they express moderate to high levels even in the unoperated state (Woolhead et al., 1998). In this respect they resemble spinal motor neurons and small diameter DRG neurons both of which also regenerate axons vigorously after peripheral nerve injury or grafting, and constitutively express high levels of L1 mRNA. Expression of L1 by neurons may enhance the regeneration of their axons but it is clearly not essential for regeneration within the environment provided by injured peripheral nerves: large diameter DRG neurons do not express
even moderate levels of L1 mRNA (Roslan et al., 1998) yet regenerate their axons vigorously following injury. There are probably other recognition molecules which are expressed by large DRG neurons and enable their regenerating axons to interact successfully with Schwann cells.

The cell recognition molecule L1 (NILE) is important for the growth of neurites on the surfaces of Schwann cells in culture (Kleitman et al., 1988), acting principally via homophilic interactions between similar molecules on the adjacent surfaces of Schwann cells and axons. Such interactions are not solely and not necessarily primarily adhesive, since there is now considerable evidence that L1 is able to stimulate neurite outgrowth via second messenger systems (Doherty et al., 1996; Burden Gulley et al., 1997; Heiland et al., 1998), even when present in a soluble form (Sugawa et al., 1997; Walsh et al., 1997). The expression of L1 by neurons is therefore potentially important for their ability to extend their axons into nerve grafts (Zhang et al., 1995; Dezawa and Nagano, 1996). Previous studies from this lab have shown that the TRN neurons which regenerate into PN grafts upregulate L1 mRNA (Zhang et al., 1995) whereas thalamocortical projection cells, which fail to regenerate their axons, do not upregulate L1. In the present study the correlation between L1 expression and the regenerative potential of cerebellar neurons was as obvious as that for thalamic neurons; Purkinje cells did not express L1 and did not regenerate axons into grafts, whereas DN and brainstem neurons both expressed L1 mRNA and regenerated their axons. The finding that L1 is upregulated on regenerating cholinergic axons during axonal elongation and downregulated upon target innervation (Aubert et al., 1998) is another piece of evidence attesting to the functional role of L1 in axon regeneration. In addition, the present study has shown that, at least some of the cells which upregulated L1 were the same cells that upregulated CHL1, c-jun and probably also GAP-43 (data is not available) and could be retrogradely labelled from the grafts. At present, there is no clear evidence for the role played by L1 and other growth-promoting molecules such as c-jun or CHL1 in CNS regeneration. However, the close correlation between the expression of mRNAs for GAP-43 and L1 by injured neurons may be functionally significant because recent evidence suggests that the stimulation of axonal growth by neural cell-recognition molecules requires the presence of GAP-43 in growth cones.
(Meiri et al., 1998) and soluble the form of these molecules can enhance phosphorylation of the GAP-43 protein in growth cones (Walsh et al., 1997).

3.3) CHL1 and possible role in axonal regeneration

Like GAP-43 and c-jun, CHL1 is upregulated by neurons which display a high regenerative propensity manifested by the regeneration of axons into grafts, such as DN neurons, TRN neurons (see Chapter 6) and cholinergic striatal interneurons (see Chapter 4), but is not expressed by neurons which fail to regenerate their axons, such as Purkinje cells. There was a good correlation between the DN neurons which could be retrogradely labelled from grafts in the cerebellum and those which expressed CHL1, GAP-43, L1 and c-jun. In some cases I have shown that the same DN neurons which express CHL1 also express GAP-43, L1 or c-jun. Similar to GAP-43 and c-jun mRNAs, CHL1 mRNA was more rapidly upregulated by the regenerating DN neurons than was L1. Similar to L1, CHL1 mRNA was downregulated in DN and brainstem neurons, to control levels, sooner than c-jun and GAP-43. Like GAP-43, CHL1 mRNA is upregulated by motor neurons following sciatic nerve injuries, but its expression is apparently downregulated earlier than that of GAP-43 (Zhang et al., submitted for publication). In the same experiment, when ligation is applied to cut sciatic nerve to prevent reconnection with targets the downregulation of CHL1 and GAP-43 mRNAs by injured motor neurons is significantly delayed, indicating the correlation of expression of CHL1 as well as GAP-43 in axon regenerating process. CHL1 mRNA expression is also upregulated by a subpopulation of DRG neurons in the same animals (Roslan et al., 1998). However, like L1 but in contrast to GAP-43 and c-jun, CHL1 does not appear to be expressed by the largest DRG neurons even after axotomy (Roslan et al., 1998). Taken together, expression of CHL1 mRNA is involved in promoting axon growth in injured CNS neurons and its expression is consistently correlated with the ability to regenerate axons in those CNS neurons investigated in this study, although CHL1 mRNA expression cannot be essential for axonal regeneration of peripheral neurons.

It is not clear what the role of neuronal CHL1 in axonal regeneration may be. CHL1, presented as a substrate or in solution, is a more powerful promoter of neurite outgrowth in vitro than even L1, but it does not act through homophilic binding or by
interactions with L1 or N-CAM (Hillenbrand et al. 1999). The function of neuronal CHL1 must depend on whether it is expressed on the surface of growth cones or secreted by the neuron. If CHL1 is secreted by growing axons it could act as a paracrine or autocrine growth promoter, and if it is expressed on the surface of regenerating axons it could promote the growth of adjacent axons. Another possibility is that CHL1 expressed on growth cones binds to unidentified receptors on the surfaces of Schwann cells and the interaction causes the transduction of a growth-promoting signal into the axon. Immunohistochemical studies are necessary to clarify this situation. Recent evidence suggests that GAP-43 may be necessary for the ability of cell recognition molecules such as L1 (and presumably CHL1) to enhance neurite outgrowth (Meiri et al. 1998). This would make the coordinated expression of these molecules a considerable advantage for CNS neurons faced with regenerating their axons into peripheral nerve grafts.

### 3.4) Expression of GAP-43, c-jun and possible role in axonal regeneration

GAP-43 and c-jun mRNAs showed remarkable similarities in the pattern of upregulation by neurons in the DN when their cell bodies were close to the tip of a living nerve graft. These neurons, which had presumably been axotomized by the implantation, corresponded in location to the retrogradely labelled cell bodies detected following application of tracer to the distal end of the graft. When retrograde labelling was combined with *in situ* hybridization, it was shown that the GAP-43 mRNA positive cells were the same cells as many and perhaps all neurons which had regenerated their axons into the grafts. Because of the practical difficulties of identifying cells on adjacent sections it cannot be categorically stated that all the DN neurons which regenerated their axons into the grafts had upregulated GAP-43 but certainly most had done so. Some axotomized neurons probably upregulated GAP-43 even though they failed to regenerate axons into the grafts (as was the case in the experiments with freeze-killed grafts—see below), but such cells had probably downregulated GAP-43 by the time retrograde labelling was carried out, which was necessarily several weeks after grafting. The same populations of DN neurons which upregulated GAP-43 also upregulated c-jun, and in some cases, when individual cells could be identified in
adjacent sections, the same cells were shown to express both molecules. Retrogradely labelled neurons in the DN were also shown to express c-jun mRNA.

There are striking similarities in the pattern of expression of GAP-43 and c-jun by the successfully regenerating neurons in the DN following graft implantation into the cerebellum and also by those in the TRN after graft implantation into the thalamus (Vaudano et al., 1995, 1998). With grafts in the cerebellum or thalamus, GAP-43 and c-jun expression in the populations of neurons capable of regeneration (mainly those in the DN and TRN), was upregulated from a very low (or undetectable) base, but the increase in expression was transient unless prolonged axonal growth was allowed through a living graft (the present study; Vaudano et al., 1995, 1998). The strong expression of c-jun and GAP-43 mRNAs in neurons of DN and TRN was apparently maintained for several months if a living graft was implanted into the brain. When freeze-killed grafts, which cannot support axonal regeneration (Berry et al., 1988), were placed in the cerebellum or thalamus, or stab wounds were made, fewer cells in the DN or TRN upregulated GAP-43 and c-jun (Vaudano et al., 1995, 1998); the expression of these molecules declined by 1-2 weeks in such conditions, particularly in the thalamus and was weaker at later survival times, particularly in the DN of the cerebellum. In the latter at 4-6 wpo in animals receiving freeze-killed peripheral nerve grafts, the numbers of neurons expressing c-jun or GAP-43 appeared smaller than similar neurons expressing the same molecules at the same survival times in animals receiving a living peripheral nerve graft. The expression of c-jun and GAP-43 mRNA by DN neurons observed several weeks after implantation of freeze-killed graft was probably a response to disconnection from neuronal targets (Jenkins et al., 1993a-c; Schreyer and Skene, 1993; Chong et al., 1992, 1994b). Vaudano et al. (1995) also showed that mechanical injury alone was a sufficient stimulus to induce the transient expression of GAP-43 mRNA in thalamic neurons, especially those were located near the graft. In the case of GAP-43 expression, it might contribute to the pathfinding signals from the growth cone (Strittmatter et al. 1995) of axotomized DN neurons, but without support from the living grafts, these signals were finally withdrawn. Therefore, it seems that an unknown signal from living Schwann cells is required both to induce expression of the molecules in some cells and to maintain expression thereafter.
The expression of c-jun and GAP-43 by neurons of the DN (and also the TRN) differed, however, from the pattern of expression of the same molecules by motor and primary sensory neurons after peripheral nerve injury. When PN trunks are crushed, motor and sensory axons regenerate vigorously and c-jun and GAP-43 mRNAs are upregulated by the injured neurons for a period which is correlated with the time taken for the regenerating axons to reconnect with their targets (Jenkins and Hunt, 1991; Leah et al., 1991; Jenkins et al., 1993a-c; Chong et al., 1992, 1994b). It is clear that injury-associated up-regulation of GAP-43 expression in DRG neurons is triggered only when the peripheral branch is cut, but not the central branch, supporting the hypothesis that GAP-43 induction in injured neurons is caused by disconnection from peripheral target tissue, not by axon injury per se (Schreyer and Skene, 1993; Chong et al., 1994a). Therefore, the main factor which brings about the downregulation of GAP-43 in injured peripheral neurons is probably the reconnection of the successfully regenerating axons with their (peripheral) targets. The expression of c-jun and GAP-43 by motor and dorsal root ganglion (DRG) neurons remains elevated for longer periods after PN trunks are cut and ligated (to prevent long distance regeneration) than after crush injuries, which allow regeneration and reconnection with targets (Jenkins and Hunt, 1991; Chong et al., 1992, 1994b; Leah et al., 1991; Herdegen et al., 1991; De Filipe et al., 1993). Furthermore, when freeze-killed grafts were attached to the sciatic nerve in adult rats, the upregulation of GAP-43 by motor and sensory neurons was more prolonged than after sciatic nerve crush (Chong et al., 1994b), even though regeneration was abortive, a striking contrast with the effects of killed grafts on CNS neurons. However, injured axons in the proximal stumps of PN trunks remain in contact with Schwann cells, irrespective of the type of lesion, whereas CNS axons injured by stab wounds or implantation of a freeze-killed graft have no contact with Schwann cells. Signals from Schwann cells in PN grafts can also increase the expression of growth-related molecules in peripheral neurons under some circumstances: when tibial nerve grafts were attached to the ganglionic stumps of severed lumbar dorsal roots, a subpopulation of DRG neurons which does not express GAP-43 after dorsal rhizotomy alone, was induced to upregulate GAP-43 mRNA (Chong et al., 1996).
Taken together, the differences in the patterns of expression of c-jun and GAP-43 between CNS and PNS neurons upon axon regeneration after injury may relate to distinct intrinsic characteristics in the two types of neuron, as well as to the diversity of extrinsic factors in the surrounding environment of different parts of the nervous system. However, expression of c-jun and GAP-43 in injured CNS neurons, such as DN and TRN neurons, in response to the implantation of a living graft is certainly involved in molecular cascades leading to successful axon regeneration in those neurons. It is likely that trophic factors released from living Schwann cells in the graft are essential to stimulate this process, probably by inducing upregulation of other intrinsic genes which may be required to functionally cooperate with GAP-43 and c-jun. The failure of DN neurons to regenerate through acellular (freeze-killed) grafts, despite the fact that they express both c-jun and GAP-43 under these conditions, would be taken as evidence to support this hypothesis.

Since the GAP-43 gene has a functional AP-1 site in its regulatory region and transcriptional control by Jun depends on the binding of Jun homo or heterodimers to the AP-1 site, it is possible that c-jun is directly involved in the regulation of GAP-43 expression during axonal regeneration. Following the implantation of a PN graft into the cerebellum, the number of neurons which upregulate c-jun is very similar to the number which upregulate GAP-43, and in many cases the same cells express both molecules, a correlation which clearly supports the hypothesis that c-jun plays a role in controlling GAP-43 expression (Bisby et al., 1995; Tetzlaff et al., 1994; Schreyer and Skene, 1993; Schaden et al., 1994; Vaudano et al., 1995, 1998; for review see Herdegen et al., 1997b). However, c-jun is a transcription factor with many possible targets and has been implicated in regulating other activities of neurons, including apoptosis (Ham et al., 1995; Hardegen et al., 1997; Watson et al., 1998). In culture of cerebellar granule neurons, c-jun mRNA and protein increase rapidly after survival signal withdrawal (i.e., by reducing concentration of KCl in the serum-free medium) phosphorylation of c-Jun appears to induce apoptosis (Watson et al., 1998). It is not the case that all c-jun-expressing cells found in this study also express GAP-43. Additionally, the study of Watson and colleagues (1998) has shown that AP-1 activity of c-Jun is required for cell death in cerebellar granule neurons in vitro. Thus, cells
displaying c-jun signal without a concomitant GAP-43 signal (and/or expression of L1 and CHL1), particularly granule cells and cells of molecular layer which situated close to the brain/graft interface may have been cells in the early stages of apoptosis rather than representing cells primed to regenerate. The absence of such cells at longer survival period of time could be considered as evidence supporting this hypothesis.

3.5 Expression of c-jun in axotomized Purkinje cells

In this context it is noteworthy that in animals with nerve grafts in the cerebellum (the present study), or direct injury to the cerebellum (Zagrebelsky et al., 1998), Purkinje cells near the site of injury express c-jun, but none express GAP-43 (except a single cell in the present study), CHL1 or L1. It is appropriate to point out, that in a previous study from this laboratory, in which grafts were inserted into the cerebellum, predominantly superficially in the cortex, it was reported that Purkinje cells did not upregulate c-jun mRNA (Vaudano et al., 1998). There is a straightforward explanation for this discrepancy. In the study of Vaudano et al. (1998) an oligonucleotide probe was used. Such probes are less sensitive than the riboprobes used in my study and it is probable that the c-jun upregulation was not detected for this reason.

Clearly, neuronal c-jun expression, even for periods of several weeks (up to 16 weeks—the longest time examined), does not invariably lead to upregulation of expression of the other three molecules, especially GAP-43. However, the finding that injured Purkinje cells do not upregulate GAP-43 contradicts the findings of Dusart and colleagues (1997), who reported that some Purkinje cells express GAP-43 mRNA at similar long periods after axotomy. Purkinje cells in the two animals with living grafts which survived for such long periods in the present study (13 and 16 wpo) did not express GAP-43 mRNA but a few Purkinje cells expressed c-jun mRNA. The reason for the difference between the present study and that of Dusart et al., (1997) with regard to Purkinje cells and GAP-43 expression at 13 and 16 wpo is not clear but it could relate to differences in probes and/or other methodological differences or to the fact that, in this study, relatively few sections through the lesioned area were processed for GAP-43 in situ hybridization. Nevertheless, the results of this study, showing that
injured, c-jun-expressing Purkinje cells did not express GAP-43, is probably a reliable observation, since when immediately adjacent sections, probed for c-jun and GAP-43 mRNAs, were compared, there was no evidence of the presence of both mRNAs in the same cells.

The observation that a large number of Purkinje cells expressing c-jun mRNA were still present in long-term freeze-killed graft experiments, in which c-jun had been downregulated by DN neurons, suggests that the expression of c-jun by injured Purkinje cells is not a specific response of this cell type to the trophic factors from the living grafts, as occurs in DN neurons. Instead, expression of c-jun in Purkinje cells is a prolonged and fundamentally different phenomenon from its expression by cells which can regenerate their axons into the graft. It is well established that mature Purkinje cells do not regenerate their axons after axotomy (Dusart and Sotelo, 1994; Dusart et al., 1997), even in the presence of living peripheral nerve grafts (Vaudano et al., 1998; the present study) or other permissive environments for axonal growth (Bravin et al., 1997 Rossi et al., 1995, 1997). In the case of severe axotomy, some regenerative attempts (i.e., axon sprouting) occur in axotomized Purkinje cells; nevertheless, they do not regenerate axons, and are unable to reestablish the connection with their targets in the DN (Dusart and Sotelo, 1994). Whether or not the expression of c-jun in injured Purkinje cells, without co-expression of GAP-43 (and probably also related to the absence of CHL1 or L1) is responsible for suppression of regenerative process in such cells is still ambiguous. In this study, there was no obvious evidence of death of Purkinje cells near the graft detectable and when thionin counterstaining was applied to some in situ hybridization sections, it was shown that some Purkinje cells labelled with c-jun probe seemed to be healthy cells (data not shown). Thus, in the case of Purkinje cells, it could not be simply concluded that upregulation of c-jun expression in axotomized Purkinje cells is associated with cell death, a role for c-jun that has been strongly suggested (e.g., Schlingensiepen et al., 1994; Ham et al., 1995; Watson et al., 1998; for review see Herdegen et al., 1997b), as discussed below.
Apparently, Purkinje cells, even though they do not regenerate their axons, survive axotomy; those with axons severed in the white matter at distances varying from 100μm to 3 mm remain alive for at least 6 months after axotomy (Dusart and Sotelo, 1994). Moreover, the same group has evidence that the axons of Purkinje cells start to sprout 3 months after axotomy (Dusart and Sotelo, 1994; Dusart et al., 1999). In fact, Purkinje cell displays unique characteristics compared to other cerebellar neurons; it has recurrent collateral branches, which originate from the main trunk of the axon before it enters the white matter, and these recurrent collaterals display extensive ramifications that make up the deep plexuses in the granular layer in which their terminals establish synapses with Golgi cells, neighbouring Purkinje cells, Lugaro cells and probably granule cells (Palay and Chan-Palay, 1974). The existence of the recurrent collateral system of the Purkinje cell may in part account for the ability of this cell type to resist axon injury. When implanting a graft, most injured Purkinje cells would probably retain an intact collateral arborization, if the graft did not injure directly the collateral branches close to their cell bodies. In this case, trophic factors derived from target cells, retrogradely transported via axon collaterals to the cell bodies of injured Purkinje cells are probably enough to ensure survival of the Purkinje cells. Although the evidence linking cell survival with intact axon collaterals is rather weak (Lieberman, 1974), this speculation does offer a possible explanation for the remarkable resistance to axotomy of this cell type.

It is still uncertain if there is any relationship between sprouting and c-jun expression in Purkinje cells at long survival periods, but there is no doubt that such expression is not a signal of axon regeneration in these cells. The evidence that axotomized Purkinje cells can sprout, whereas they never regenerate axons under the same conditions, supports the hypothesis that sprouting and regenerating axons may differ in their requirements for growth after injury (Bernstein et al., 1997). There is strong evidence suggesting that upregulation of c-jun expression in axotomized neurons is a result of disinhibition of gene repression following deprivation of trophic factor(s) that normally arrive at the nucleus from the neurons’ targets (Herdegen et al., 1991; Leah et al., 1993). The induction of expression of c-jun in Purkinje cells, therefore, may occur because the main neuron-target axis of these cells is cut off. However,
trophic factors from the graft seem to be unable to activate a cascade of events leading to axon regeneration regulated by c-jun in Purkinje cells, as those factors are supposed to do in other CNS neurons which regenerate axons very well along nerve grafts (Vaudano et al., 1993a,b, 1998, the present study; and see also Chapter 5).

Interestingly, a traumatic lesion or the implantation of a living PN graft into the cerebellum can induce strong and prolonged expression of p75 in injured Purkinje cells, but not in other cerebellar neurons (Dusart et al., 1994; Vaudano et al., 1998; Martinez et al., 1998). The expression of this neurotrophin receptor is maintained in axotomized Purkinje cells for up to one year (Dusart et al., 1994). This response might also be related to the high resistance of Purkinje cells to axotomy (Dusart and Sotelo, 1994; Dusart et al., 1997). Much evidence has suggested a specific role of p75 in NGF mediated cell protection against induced apoptosis (Rabizadah et al., 1993; Barrett and Barrett, 1994; Cortazzo et al., 1996; Bunone et al., 1997). As discussed in Chapter 3, under some circumstances such as tissue damage and inflammation, NGF is produced and its synthesis modulated by non-neuronal cells such as reactive astrocytes at the site of injury (Lindsay, 1979; Lorez et al., 1989; Shigeno et al., 1991; Yoshida and Gage, 1991; Hashimoto et al., 1992; Lee et al., 1995, 1996, 1998). This means that after injury to the cerebellum, the local level of NGF would be significantly increased, particularly around the site of injury, and even higher when a living PN graft is implanted (Woolhead et al., 1998). Therefore, in the case of Purkinje cells, high level of p75 expression probably allow them to take advantage of locally produced neurotrophins, especially neurotrophins produced in the graft (Woolhead, 1995). These factors may in turn promote survival and probably also promote axon sprouting in injured Purkinje cells. However, these effective factors, though they may be in promoting cell survival and axonal sprouting, do not support axon regeneration in these cells. Recently, Buffo et al. (1997) reported that after axotomy, Purkinje cells of mice, in which GAP-43 overexpression was driven by the Purkinje cell-specific L7 promoter, showed profuse sprouting along the axon and its severed ends, but were not able to regenerate, indicating that the presence of GAP-43 is not sufficient to enable these neurons to accomplish a full program of axon regeneration. These data suggest that the
regeneration process in Purkinje cells may involve some specific intrinsic factors which may differ from those operating in other neurons.

In conclusion, expression of c-jun in axotomized Purkinje cells, without coordinated expression of other growth-promoting molecules, i.e., GAP-43, CHL1 and L1, is not sufficient to induce axonal regeneration in such cells. As an alternative function of c-jun is suggested to be an involvement in programmed cell death (e.g., Schlingensiepen et al., 1994; Ham et al., 1995; Watson et al., 1998), it could be considered that expression of c-jun by the relatively small numbers of Purkinje cells close to the graft is more likely to represent the partial activation of an apoptotic pathway than it is to signify attempted regeneration. If it is the case, other (intrinsic) factors in Purkinje cells, such as those discussed above, may somehow suppress or rescue them from cell death. However, expression of c-jun alone does not trigger the molecular cascade(s) leading to apoptotic cell death (Herdegen et al., 1997b). Buschman et al. (1998) reported that a pivotal program of c-jun, involving regeneration or degeneration, might be determined by the presence or absence of the activator transcription factor ATF-2. Further study is, therefore, needed to elucidate the role of c-jun expressed by injured Purkinje cells.

In contrast to Purkinje cells, which are endowed with poor regenerative capabilities, axotomized DN and brainstem neurons, which display high regenerative ability by regenerating axons into a living nerve graft, express all four molecules: c-jun, GAP-43, L1 and CHL1 mRNAs. This suggests that expression of these genes can be distinctive markers indicating the strength of regenerative potential of CNS neurons. The findings of coexpression among these growth-associated molecules suggest that gene expression of at least 2 of them is involved in, and may even be necessary for promoting axon regeneration in injured CNS neurons.
Table 8.1 Pattern of expression of L1 mRNA in the cerebellum and brainstem following PN graft implantation

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>Position of graft tip</th>
<th>Cells expressing L1 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>3 d</td>
<td>YZ 202</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>1 w</td>
<td>YZ 114</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>YZ 221</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td>2 w</td>
<td>YZ 209</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>YZ 218</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>YZ 219</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td>3 w</td>
<td>YZ 226</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>YZ 229</td>
<td>DN/BS</td>
<td>+/-</td>
</tr>
<tr>
<td>4 w</td>
<td>YZ 210</td>
<td>CT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 210</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>YZ 232</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>5 w</td>
<td>YZ 230</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 235</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 236</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>8 w</td>
<td>YZ 233</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>YZ 234</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>1 w</td>
<td>YZ 227</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>YZ 229</td>
<td>CT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>YZ 238</td>
<td>CT</td>
<td>-</td>
</tr>
<tr>
<td>2 w</td>
<td>YZ 228</td>
<td>CT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 246</td>
<td>DN</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 247</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td>4 w</td>
<td>VP 237</td>
<td>DN</td>
<td>-</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 239</td>
<td>DN</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 241</td>
<td>DN/BS</td>
<td>+</td>
</tr>
</tbody>
</table>

The records of the number of labelled neurons summarized in this Table (and also applied to Tables 8.2, 8.3, 8.4) are derived from the mean number of labelled neurons per section, calculated by dividing the total number of neurons (counted in all available sections through the region containing labelled cells) by the number of sections in which the counts were made (see also Section 1; Summary of methods used).

**Abbreviations:** BS, brainstem; CT, cerebellar cortex; DN, deep cerebellar nuclei

+++, labelled cells were found; +, a few labelled cells were found;
+/-, one or two labelled cells were found; -, no labelled cells detected

*, animal injected with CT-HRP
Table 8.2 Pattern of expression of CHL1 mRNA in the cerebellum and brainstem following PN graft implantation

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>Position of graft tip</th>
<th>Cells expressing CHL1 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>3 d</td>
<td>VP 224</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>1 w</td>
<td>VP 125</td>
<td>CT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 129</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VP 172</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 130</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>2 w</td>
<td>VP 184</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 194*</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 196</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>YZ 242</td>
<td>BS</td>
<td>+/-</td>
</tr>
<tr>
<td>3 w</td>
<td>YZ 258</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>4 w</td>
<td>YZ 253</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>YZ 256</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 210</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 235*</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 236</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td>7 w</td>
<td>VP 195*</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VP 229</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td>3 d</td>
<td>VP 225</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>1 w</td>
<td>VP 190</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 205</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td>2 w</td>
<td>VP 246</td>
<td>DN</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 247</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td>4 w</td>
<td>VP 237</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 239</td>
<td>DN</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VP 241</td>
<td>DN/BS</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations:** BS, brainstem; CT, cerebellar cortex; DN, deep cerebellar nuclei

+++ many labelled cells were found; +, a few labelled cells were found;

+-/+, one or two labelled cells were found; -, no labelled cells detected

*, animal injected with CT-HRP
Table 8.3 Pattern of expression of c-jun mRNA in the cerebellum and brainstem following PN graft implantation

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>Position of graft tip</th>
<th>Cells expressing c-jun mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>3 d</td>
<td>VP 224</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>1 w</td>
<td>VP 129</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 172</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 208</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td>2 w</td>
<td>VP 184</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VP 194</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 196</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>YZ 209</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>3 w</td>
<td>YZ 258</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td>4 w</td>
<td>YZ 253</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VP 210</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>YZ 256</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 235*</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 236</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td>7 w</td>
<td>VP 195*</td>
<td>DN/BS</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 229</td>
<td>DN</td>
<td>-</td>
</tr>
<tr>
<td>13 w</td>
<td>YZ 254</td>
<td>DN/BS</td>
<td>+</td>
</tr>
<tr>
<td>16 w</td>
<td>YZ 263</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>3 d</td>
<td>VP 225</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>1 w</td>
<td>VP 190</td>
<td>DN/BS</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 205</td>
<td>DN/BS</td>
<td>-</td>
</tr>
<tr>
<td>2 w</td>
<td>VP 246</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VP 247</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>4 w</td>
<td>VP 237</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 239</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 241</td>
<td>DN/BS</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations:** BS, brainstem; CT, cerebellar cortex; DN, deep cerebellar nuclei

++ , many labelled cells were found; + , a few labelled cells were found;
+-/ , one or two labelled cells were found; - , no labelled cells detected
*
, animal injected with CT-HRP
Table 8.4 Pattern of expression of GAP-43 mRNA in the cerebellum and brainstem following PN graft implantation

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>Position of graft tip</th>
<th>Cells expressing GAP-43 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>Living graft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 d</td>
<td>VP 224</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>1 w</td>
<td>VP 129</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 172</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 208</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>2 w</td>
<td>VP 196</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>3 w</td>
<td>YZ 258</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>4 w</td>
<td>YZ 253</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 210</td>
<td>DN/BS</td>
<td>++</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 235&lt;sup&gt;+&lt;/sup&gt;</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>VP 236</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>7 w</td>
<td>VP 195&lt;sup&gt;+&lt;/sup&gt;</td>
<td>DN</td>
<td>+/-</td>
</tr>
<tr>
<td>13 w</td>
<td>YZ 254</td>
<td>BS</td>
<td>+</td>
</tr>
<tr>
<td>16 w</td>
<td>YZ 263</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td>Freeze-killed grafts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 d</td>
<td>VP 225</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>2 w</td>
<td>VP 246</td>
<td>DN/BS</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>VP 247</td>
<td>DN</td>
<td>++</td>
</tr>
<tr>
<td>4 w</td>
<td>VP 237</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td>6 w</td>
<td>VP 239</td>
<td>DN</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VP 241</td>
<td>DN/BS</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations:** BS, brainstem; CT, cerebellar cortex; DN, deep cerebellar nuclei  
+++, many labelled cells were found; +, a few labelled cells were found;  
+/-, one or two labelled cells were found; -, no labelled cells detected  
*, animal injected with CT-HRP
Table 8.5 Comparison location and number of neurons upregulating L1, CHL1, c-jun or GAP-43 following implantation of a freeze-killed graft into the cerebellum

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>Graft tip</th>
<th>L1 mRNA+ neurons</th>
<th>CHL1 mRNA+ neurons</th>
<th>c-jun mRNA+ neurons</th>
<th>GAP-43 mRNA+ neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DN</td>
<td>PC</td>
<td>BS</td>
<td>DN</td>
</tr>
<tr>
<td>3 d VP 225</td>
<td>DN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>2w VP 246</td>
<td>DN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DN/BS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>4w VP 237</td>
<td>DN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6w VP 239</td>
<td>DN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>DN/BS</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

The numbers shown are mean numbers of neurons per section. All neurons displaying a strong hybridization signal were counted as described in Section 1; Summary of methods used in this Chapter. Note the larger number and large range of postgraft survival times of DN neurons upregulating c-jun and GAP-43 compared with those upregulating L1 and CHL1 and also that c-jun mRNA is expressed by Purkinje cells under these conditions. This Table summarizes expression data from animals, in which at least three mRNAs were probed.

Abbreviations: BS, brainstem; DN, deep cerebellar nuclei; PC, Purkinje cells
-, data is not available
Table 8.6 Comparison of patterns of mRNA expression and retrograde labelling in DN and brainstem neurons in individual animals.

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>mRNA probes</th>
<th>Graft tip</th>
<th>Deep cerebellar neurons</th>
<th>Purkinje neurons</th>
<th>Brainstem neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 w</td>
<td>VP 194</td>
<td>CHL1, c-jun, CT-HRP</td>
<td>DN</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>3 w</td>
<td>YZ 229</td>
<td>L1, CT-HRP</td>
<td>DN/BS</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>4 w</td>
<td>YZ 232</td>
<td>L1, CT-HRP</td>
<td>DN</td>
<td>2</td>
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<td>5 w</td>
<td>YZ 230</td>
<td>L1, CT-HRP</td>
<td>DN</td>
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<tr>
<td>6 w</td>
<td>VP 235</td>
<td>L1, CHL1, c-jun, GAP-43, CT-HRP</td>
<td>DN</td>
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<td>7 w</td>
<td>VP 195</td>
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<td>DN/BS</td>
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<td>0</td>
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<tr>
<td>8 w</td>
<td>YZ 233</td>
<td>L1, CT-HRP</td>
<td>DN/BS</td>
<td>3</td>
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<td>5</td>
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<td>YZ 254</td>
<td>c-jun, GAP-43, CT-HRP</td>
<td>BS</td>
<td>5</td>
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</table>

The numbers of labelled neurons presented in this Table are the mean numbers of labelled neurons obtained as described in Section 1; Summary of methods used. In general, there is correspondence between in situ and retrograde labelling data, reflecting colocalization of mRNA expression and CT-HRP labelling within the same nuclear groups. On further analysis in these animals, colocalization of markers in some individual cells could be identified.

Abbreviations: BS, brainstem; DN, deep cerebellar neurons
Table 8.7 Numbers and distribution of DN and brainstem neurons expressing three or four mRNAs in individual animals.

<table>
<thead>
<tr>
<th>Survival time</th>
<th>Animal number</th>
<th>mRNA probes</th>
<th>Graft tip</th>
<th>Deep cerebellar neurons</th>
<th>Purkinje neurons</th>
<th>Brainstem neurons</th>
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</thead>
<tbody>
<tr>
<td>3 d</td>
<td>VP 224</td>
<td>CHL1, c-jun, GAP-43</td>
<td>DN/BS</td>
<td>9 24 20 0 4</td>
<td>0 24 7 1</td>
<td></td>
</tr>
<tr>
<td>1 w</td>
<td>VP 129</td>
<td>CHL1, c-jun, GAP-43</td>
<td>DN/BS</td>
<td>3 5 12 0 0</td>
<td>0 4 1 0</td>
<td></td>
</tr>
<tr>
<td>1 w</td>
<td>VP 172</td>
<td>CHL1, c-jun, GAP-43</td>
<td>DN/BS</td>
<td>5 13 17 0 0</td>
<td>0 5 3 0</td>
<td></td>
</tr>
<tr>
<td>2 w</td>
<td>VP 196</td>
<td>CHL1, c-jun, GAP-43</td>
<td>DN</td>
<td>2 25 16 0 0</td>
<td>0 5 2 0</td>
<td></td>
</tr>
<tr>
<td>3 w</td>
<td>VP 258</td>
<td>CHL1, c-jun, GAP-43</td>
<td>DN</td>
<td>2 4 6 0 0</td>
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</tr>
<tr>
<td>4 w</td>
<td>VP 210</td>
<td>L1, CHL1, c-jun, GAP-43</td>
<td>DN/BS</td>
<td>3 12 17 10 0</td>
<td>0 0 1 0</td>
<td></td>
</tr>
<tr>
<td>6 w</td>
<td>VP 236</td>
<td>L1, CHL1, c-jun, GAP-43</td>
<td>DN/BS</td>
<td>1 3 4 8 1</td>
<td>0 0 1 2</td>
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</table>

This table summarized expression data for 7 animals (from 3 dpo-6 wpo), in which three or four mRNAs were probed. Colocalization of at least two mRNAs in some individual cells could be identified. Two further animals in which four mRNAs (VP 235; 6 wpo) or three mRNAs (VP 195; 7 wpo) were probed were also studied for retrogradely labelling with CT-HRP applied to the graft, the results are therefore included in Table 8.6 and omitted here to avoid repetition. The animals in which two mRNAs were probed were not included in this Table, although in those cases some corresponding labelled neurons were also found. Labelled neurons presented in this Table were counted as described in Section 1: Summary of methods used.

Abbreviation: BS, brainstem; DN, deep cerebellar neurons
**Figure 8.1** Retrogradely labelled DN neurons in the cerebellum 4 weeks following the implantation of a living PN graft.

8.1a,b) Low power photomicrograph of the cerebellum implanted with a PN graft (G), showing a group of neurons, close to the graft tip, retrogradely labelled with CT-HRP (a, *arrows*) in DN neurons (lateral nucleus). Some of the labelled regenerating neurons are shown at higher magnification in b. Arrowheads in a indicate CT-HRP labelled regenerating axons in the graft. Note the absence of CT-HRP labelled neurons in the cerebellar cortex. Scale bars = 250µm (8.1a); 50µm (8.1b).

Abbreviation: G, PN graft.
Figure 8.2-3 Expression of L1 mRNA in the cerebellum following implantation of a living PN graft.

8.2a,b) Low power photomicrographs of the cerebellum implanted with a PN graft 2 weeks previously, showing distribution and localization of L1 mRNA in the interpositus nucleus of the DN of the ipsilateral cerebellum (a) and the corresponding region of the interpositus nucleus of the contralateral cerebellum (b). A cluster of interpositus neurons near the graft tip (a, arrow) expresses high levels of L1 mRNA compared with the same group of neurons (which show only a moderate level of expression of this gene) on the contralateral side (b, arrow). Scale bar = 200μm (8.2a,b).

8.3a-c) Comparison between L1 mRNA expressing cells (b) and retrogradely labelled neurons (a,c) in three immediately adjacent sections of the cerebellum at 5 wpo. Neurons of the medial nucleus display strong L1 mRNA expression (b) and CT-HRP (a,c). The graft is close to these neurons in adjacent sections. The numbered neurons (1-7) expressing high levels of L1 message in b also contain HRP reaction product in two adjacent sections in a (neurons 1-4) and c (neurons 5-7), confirming that they have regenerating axons into the distal graft. Scale bar = 100μm (8.3a-c).

Abbreviation: G, PN graft
**Figure 8.4** Expression of L1 mRNA (8.4a,b) in identified regenerating DN and brainstem neurons (8.4c) from an animal at 8 wpo with the graft tip embedded in the brainstem.

8.4a,b) Low power photomicrograph of the junction between the cerebellum and brainstem (a) showing upregulation of L1 in brainstem neurons after implantation of a PN graft. Neurons in hypoglossal prepositus nucleus (a, *arrow*) and the medial vestibular nucleus (neurons 1-3) just ventral to the cerebellum and the fourth ventricle, express high levels of L1 mRNA. Part of this figure is shown at higher magnification in b, showing three neurons in the medial vestibular nucleus (1-3) expressing high levels of L1 mRNA. In 8.4a the midline is just beyond the left edge of the field. Scale bar = 200μm (8.4a); 100μm (8.4b).

8.4c) An adjacent section to that shown in 8.4a and b, showing the same three neurons labelled with CT-HRP (neurons 1-3; dark field image), confirming they are regenerating neurons. V indicates the same blood vessel in all 3 panels. Scale bar = 100μm.

**Abbreviations:** G, PN graft; v, small blood vessel
Figure 8.5-7 Expression of CHL1 mRNA in the cerebellum following the implantation of a living PN graft. Fig. 8.5a duplicates Fig. 7.5a, Fig. 8.6a duplicates Fig. 7.5d and Fig. 8.7 duplicates Fig. 7.10. The reason for duplication is explained in the text.

8.5a,b) The cerebellum 3 days after graft insertion, showing many CHL1 positive cells at the brain/graft interface (a, arrowheads), presumably reactive astrocytes, as well as putative Golgi cells (shown in b at higher magnification in an adjacent section of the boxed area of cortex in a), along the course of the graft. Strong hybridisation signals are also apparent in the interpositus nucleus (a, left open arrow), and in the medial deep nucleus (a, right open arrow) of the DN. Note that Purkinje cells (b, small arrows, pointing downwards from the molecular layer) are CHL1 mRNA negative. Scale bars = 500μm (8.5a) and 300μm (8.5b).

8.6a,b) Neurons of the interpositus nucleus (a, arrow) of the DN labelled with CHL1 cRNA probe are seen in the ipsilateral cerebellum 1 week after graft implantation in a. The same group of interpositus neurons on the contralateral side of the cerebellum in the same section is shown in b where the neurons are CHL1 mRNA negative. Scale bars = 200μm (8.6a,b).

8.7a,b) Comparison between CHL1 mRNA expressing cells (a) and retrogradely labelled neurons in an immediately adjacent section (b) of the cerebellum implanted with a living PN graft 6 weeks previously. Neurons in the interpositus nucleus of the DN expressing high levels of CHL1 mRNA (neurons 1-8 in a) are shown also to be retrogradely labelled with CT-HRP (neurons 1-8 in b) in an adjacent section, confirming that they have regenerating axons in the distal graft. G indicates the position of the graft, which was lost during tissue processing. Scale bar = 100μm (8.7a,b).

Abbreviations: G, PN graft; Gr, granular layer of the cerebellum; Mo, molecular layer of the cerebellum.
Figure 8.8-10 Expression of c-jun mRNA in the cerebellum following the implantation of a living PN graft.

8.8) The cerebellum 3 days following the implantation of a PN graft, showing neurons in the medial deep nucleus (large open arrows) of the DN expressing high levels of c-jun mRNA. Note Purkinje cells (small arrows) are also labelled with the c-jun probe. Scale bar = 200µm.

8.9a,b) The cerebellum 4 weeks following the implantation of a PN graft, showing neurons in the lateral and interpositus nuclei (e.g. at open arrows) of the DN heavily labelled with c-jun probe. Neurons in the same region on the contralateral side of the cerebellum shown in b, express very low levels of c-jun mRNA. Scale bars = 400µm (8.9a); 200µm (8.9b).

8.10a,b) Comparison between c-jun mRNA expressing cells (a) and retrogradely labelled neurons in an immediately adjacent section (b) of the cerebellum 6 weeks following the implantation of a living PN graft. Neurons of the interpositus nucleus are heavily labelled with c-jun mRNA (a) and with CT-HRP (b). Colocalization of c-jun mRNA and CT-HRP product appear in neurons 1-7 confirming that they have axons regenerating into the distal graft. G indicates the position of the graft. Scale bar = 100µm (8.10a,b).

Abbreviation: G, PN graft.
**Figure 8.11-13** Expression of GAP-43 mRNA in the cerebellum following the implantation of a living PN graft

8.11) The cerebellum at 3 days following the implantation of a PN graft, showing neurons heavily labelled with GAP-43 probe in the interpositus nucleus (e.g. at open arrows). Note the absence of Purkinje cell labelling in the portion of cortex present at top right. Scale bar = 200μm.

8.12a,b) The cerebellum at 4 weeks following the implantation of a living PN graft, showing numerous neurons in the medial nucleus (a, left arrow) and in the lateral nucleus (a, right arrow) of the DN expressing high levels of GAP-43 mRNA adjacent to a graft. The corresponding region of DN on the contralateral side of the cerebellum in the same section are shown in b where only very low levels of GAP-43 mRNA expression are apparent. The faint diagonal streak at top left of b is the granular layer of an area of cortex. Scale bars = 400μm (8.12a); 200μm (8.12b).

8.13a,b) Comparison between GAP-43 mRNA expressing cells (a) and retrogradely labelled neurons in an immediately adjacent section (b) of the cerebellum 6 weeks following the implantation of a living PN graft. Neurons in the lateral nucleus expressing high levels of GAP-43 mRNA (1-9 in a) are also labelled with CT-HRP in an adjacent section (1-9 in 13b), confirming they are neurons with regenerating axons. G indicates the position of the graft. Scale bar = 100μm (8.3a,b).

Abbreviation: G, PN graft
Figure 8.14  Comparison of the expression of c-jun and GAP-43 mRNAs in Purkinje cells in the cerebellum 3 days following a living PN graft implantation.

8.14a,b) A pair of immediately adjacent sections, probed for c-jun mRNA (a) and GAP-43 mRNA (b), showing a part of the granular layer close to a graft (G). Some Purkinje cells are labelled with c-jun mRNA probe (a), but none of the cells containing c-jun mRNA is labelled with the GAP-43 probe (b). Scale bar = 100μm (8.14a,b).

Abbreviation: G, PN graft
Figure 8.15-18 Co-expression of L1 (8.15a) and CHL1 mRNAs (8.15b); L1 (8.16a) and c-jun mRNAs (8.16b); c-jun (8.17a) and CHL1 mRNAs (8.17b); GAP-43 (8.18a) and c-jun mRNAs (8.18b) in the DN neurons following the implantation of a living PN graft into the cerebellum.

8.15a,b) DN regions from a pair of adjacent sections, probed for L1 mRNA in one section and for CHL1 mRNA in the other from the cerebellum 4 weeks after graft insertion, showing co-expression of both genes in the interpositus nucleus neurons (neurons 1-5). The graft tip is close to this area but is not visible in these sections. V indicates the same blood vessel in both panels. Scale bar = 100μm (8.15a,b).

8.16a,b) DN regions from a pair of adjacent sections, probed for L1 mRNA in one section and for c-jun mRNA in the others, of the cerebellum 2 weeks after graft insertion, showing co-expression of both genes in neurons in the medial nucleus (neurons 1-6). V indicates the same blood vessel in both panels. Note that many more neurons expressing L1 mRNA are present in a than neurons expressing c-jun in b. Scale bar = 100μm (8.16a,b).

8.17a,b) DN regions from a pair of adjacent sections, probed for c-jun mRNA in one section (a) and for CHL1 mRNA in the others (b), of the cerebellum 1 weeks after graft insertion, showing co-expression of both genes in neurons in medial nucleus (neurons 1-5). Scale bar = 50μm (8.17a,b).

8.18a,b) DN regions from a pair of adjacent sections, probed for GAP-43 mRNA in one section (a) and for c-jun mRNA in the others (b), of the cerebellum 1 weeks after graft insertion, showing co-expression of both genes in neurons in the interpositus nucleus (neurons 1-11). Note that a few neurons with strong c-jun expression in b are not present or do not express GAP-43 in a. Scale bar = 100μm (8.18a,b).

Abbreviations: G, PN graft, V, small blood vessel
Figure 8.19 Co-expression of GAP-43 mRNA (8.19a,c) and CHL1 mRNA (8.19b)

8.19a-c) Three immediately adjacent cerebellar sections, probed for GAP-43 mRNA (a,c) and CHL1 mRNA (b), from an animal at 6 wpo, showing expression of CHL1 and GAP-43 mRNAs in individual neurons in the interpositus nucleus of the DN. Note that all 7 of the numbered CHL1-expressing neurons in b show GAP-43 mRNA signal (neurons 1-3 in a and neurons 4-7 in c) but that there are also a few GAP-43-expressing neurons which do not show detectable CHL1 signal. V indicates the same blood vessel in all 3 sections. Scale bar = 50μm for all panels.
Figure 8.20 Camera lucida drawings to show the distribution of neurons (black dots) displaying strong signals for L1 (a), CHL1 (b), c-jun (c) and GAP-43 mRNAs (d) in the DN, brainstem nuclei and - in the case of c-jun only - in the cerebellar cortex, in representative sections through the cerebellum 3 days after implantation of a peripheral nerve graft. The in situ positive neurons are shown as black dots which, in the interests of clarity, are larger than to scale. The graft is hatched but in a (L1) and c (c-jun), the outline of the lesion track is shown by a continuous outline extending to the medial nucleus in a, and through the lateral vestibular nucleus (LVe) into the spinal vestibular nucleus (SpVe) in c. Note that more neurons express c-jun and GAP-43 mRNA than L1 and CHL1 mRNA and that Purkinje cells in the cerebellar cortex close to the graft express c-jun mRNA. Approximate nuclear boundaries are indicated by dashed lines. The CHL1, c-jun and GAP-43 sections are taken from the same animal (VP224; 3 dpo) and the L1 section is from animal YZ202 (3 dpo). Scale bar = 1mm for all panels.

Abbreviations: DC, dorsal cochlear nucleus; icp, inferior cerebellar peduncle; I, interpositus nucleus; L, lateral nucleus; M, medial nucleus; LVe, lateral vestibular nucleus; Spve, spinal vestibular nucleus.
Figure 8.21 Comparison of the expression pattern of mRNAs for L1 (8.21b), CHL1 (8.21c), c-jun (8.21d), and GAP-43 (8.21e) and of the distribution of CT-HRP retrogradely labelled regenerating neurons (8.21f) in the DN of the cerebellum in a series of adjacent sections taken from a single rat 6 weeks after implantation of the graft.

8.21a) Camera lucida drawing from a section, hybridised with c-jun mRNA probe, adjacent to the section from which 8.21d is taken, and shows the termination of the graft in the interpositus nucleus. Many labelled neurons in the interpositus nucleus of the DN are situated near the graft tip and a few labelled Purkinje cells are also seen close to the graft and close the lateral nucleus of the DN (large black dots). This drawing provides an orientation guide to panels b-f. The graft is indicated by the hatched outline.

8.21b-e) L1 (b), CHL1 (c), c-jun (d), and GAP-43 (e) mRNAs are strongly upregulated in the same group of neurons (open arrows), confirmed as regenerating neurons by retrograde labelling (open arrows in f). V indicates the fourth ventricle in panels b-f. Fig. 8.21c and f are immediately adjacent sections, and neurons labelled with CHL1 probe (1-8 in c) are also shown in f to contain CT-HRP reaction product (1-8) and are thus identified as neurons with axons regenerating in the distal graft. These neurons are illustrated at higher magnification in Figs. 7.9c, 7.10a,c and 8.7a (CHL1 probe) and Figs. 7.9d, 7.10 b,d and 8.7b (CT-HRP labelling). The neurons in d and e are shown at higher magnification and rotated through 90° in Figs. 8.10a and 8.13a, respectively. Scale bar = 200μm (8.21b - f).

Abbreviations: BS, brainstem; G, PN graft; M, medial nucleus of the DN; I, interpositus nucleus of the DN; L, lateral nucleus of the DN.
Figure 8.22 Expression of mRNAs for L1 (8.22a), CHL1 (8.22b), c-jun (8.22c) and GAP-43 (8.22d) in the cerebellum of a single rat, 2 weeks following the implantation of a freeze-killed graft.

8.22a-d) Four sections hybridized with 4 different probes: L1 (a), CHL1 (b), c-jun (c) and GAP-43 (d), showing DN regions containing labelled neurons in the interpositus and lateral nuclei. Note that there is very little upregulation of L1 or CHL1 mRNAs in those DN neurons. However, c-jun and GAP-43 mRNAs are strongly expressed in many of these DN neurons. Scale bar = 200μm for all panels.

Abbreviation: G, (freeze-killed) PN graft.
CHAPTER 9

General discussion

The work reported in this thesis is concerned with the patterns of expression of several regeneration-relevant molecules in neurons of the striatum, thalamus and cerebellum of adult rats after implantation of a PN graft, and the correlation of these pattern of expression with differences in the regenerative capacity of the neurons. The present results, as well as previous results from this laboratory, have shown that many different populations of intrinsic neurons in these three regions of the brain and some of the neurons projecting to these regions, namely striatal cholinergic interneurons, neurons of the SNpc, TRN, DN and brainstem, express the same set of mRNAs, i.e., L1, CHL1, c-jun and GAP-43 during axon regeneration. In contrast, neurons which failed to regenerate axon into the graft, such as projection neurons of the striatum and Purkinje cells, failed to maintain or express mRNAs of these molecules. These findings suggest that the expression of these four mRNAs is involved in promoting axon regeneration in these CNS neurons. Importantly, induction and maintenance of expression of the growth-related molecules in regenerating CNS neurons certainly requires signal(s) from PN grafts containing living Schwann cells, since freeze-killed grafts failed to promote such expression.

However, the patterns of expression of these growth-related molecules among CNS neurons displaying high regenerative capacity are not identical but differ from one another in interesting ways. For example, following the implantation of a living graft, strong upregulation of expression of L1 (Zhang et al., 1995), CHL1 (Chapter 6), c-jun (Vaudano et al., 1998) and GAP-43 mRNAs (Vaudano et al., 1995) was seen in TRN neurons, up to the longest survival times examined (over 6 wpo), whereas such expression declined after 6 wpo in DN and brainstem neurons (Chapter 8). In the case of cholinergic striatal interneurons, expression of CHL1, c-jun and GAP-43 mRNA declined after 4 wpo, but upregulation of L1 from the moderate level of constitutive expression that characterizes normal adult cholinergic striatal interneurons, was not
detectable. In contrast to other CNS neurons, axotomized SNpc neurons apparently upregulated only c-jun mRNA expression (Chapter 5), but the levels of constitutive expression of L1 (Woolhead et al., 1998), CHL1 (Chapter 4) and GAP-43 mRNAs (Woolhead et al., 1998) in these neurons were high under normal conditions.

In this Chapter, the expression of growth-related molecules in injured CNS neurons reported in this thesis are summarized below and in Table 9.1. In order to provide complete data, the results from previous studies from this laboratory (Zhang et al., 1995; Woolhead et al., 1998; Vaudano et al., 1998) are included both in this general discussion and in the Table. Finally, the possible roles of four growth-related molecules, L1, CHL1, c-jun and GAP-43 in promoting axonal regeneration of CNS neurons are considered.

9.1) **Expression of growth-related molecules in regenerating CNS neurons following implantation of a living PN graft** (see Table 9.1)

A) In the striatum and substantia nigra

Expression of CHL1 and c-jun mRNA was upregulated by many cell types in the striatum, including GFAP-positive reactive astrocytes, very early after axotomy. The implantation of a living graft could maintain expression of CHL1 and c-jun mRNAs only in a small number of presumptive cholinergic interneurons near the graft tip, the group of neurons which could be retrogradely labelled from the graft (Chapter 3 and see Woolhead et al., 1998). These neurons also upregulate GAP-43 mRNA following living graft insertion (Woolhead et al., 1998). There was no evidence of upregulation of L1 mRNA in axotomized cholinergic interneurons under the same conditions; however the constitutive expression of this gene in intact neurons is rather high. Moreover, after PN grafting, presumptive cholinergic interneurons also upregulated trkA and p75 proteins and such cells could be retrogradely labelled from the distal end of the graft. Upregulation of trkA and p75 in these injured cholinergic interneurons may be considered as responses to a trophic factor, possibly NGF, from the graft (as discussed in Chapter 3). It is uncertain if the same factor induces and maintains expression of CHL1, c-jun and GAP-43 mRNAs in the same neuronal group. However, the evidence that expression of c-jun in damaged neurons is not regulated by
NGF or BDNF (De Felipe and Hunt, 1994), suggests that other factors released from living Schwann cells may control expression of this gene in cholinergic interneurons and also in other CNS neurons.

Intact neurons of SNpc express rather high levels of CHL1 (Chapter 4), L1 and GAP-43 (Woolhead et al., 1998), but low levels of mRNA for c-jun (Chapter 5). After axotomy, SNpc neurons did not show any detectable changes in the levels of L1 and GAP-43, but transiently upregulated c-jun expression and such expression was prolonged in the presence of a living graft.

B) In the thalamus

Neurons of the TRN, a discrete population of thalamic neurons showing a strong regenerative response into living PN grafts (Campbell et al., 1991; Morrow et al., 1992), express undetectable levels of mRNAs for CHL1 (Chapter 6), L1 (Zhang et al., 1995), c-jun (Vaudano et al., 1998), and GAP-43 (Vaudano et al., 1995). These neurons upregulated expression of all four molecules after axotomy and the expression was maintained at very high levels up to the longest survival times investigated. In some cases, such expression was found in TRN neurons whose cell bodies were located up to 1.5mm away from the graft tip. In contrast, only a few injured neurons of dorsal thalamic nuclei, located close to the graft tip, upregulated three of the mRNAs but not L1.

C) In the cerebellum and brainstem

Following the implantation of living grafts, DN neurons upregulated expression of CHL1, c-jun and GAP-43 mRNAs from undetectable levels and L1 mRNA from moderate levels and coexpression of at least 2 mRNAs in individual neurons was demonstrated. Similarly, neurons of the brainstem, which are located close to the cerebellum and have axon connections with cerebellar neurons (i.e., neurons of the vestibular nuclear complex, cochlear nuclei and hypoglossal prepositus nucleus), also upregulated expression of the four mRNAs. These DN and brainstem neurons were also shown to have axons which regenerated into the graft. Surprisingly, upregulation of expression of GAP-43 and c-jun mRNAs was also detected in a small number of DN
Table 9.1 Summary of expression patterns of CHL1, L1, c-jun and GAP-43 mRNAs in the striatum, substantia nigra (pars compacta), thalamus, cerebellum and brainstem following the implantation of freeze-killed and living PN grafts

<table>
<thead>
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<th>Grafted site</th>
<th>Striatum</th>
<th>Thalamus</th>
<th>Cerebellum &amp; Brainstem</th>
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</thead>
<tbody>
<tr>
<td>Neuronal population</td>
<td>Cholinergic interneurons</td>
<td>Other striatal neurons</td>
<td>SNpc neurons</td>
</tr>
<tr>
<td>Type of graft</td>
<td>FK</td>
<td>L</td>
<td>FK</td>
</tr>
<tr>
<td>CHL1</td>
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</tr>
<tr>
<td>L1</td>
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<tr>
<td>c-jun</td>
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</tr>
<tr>
<td>GAP-43</td>
<td>*</td>
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</tr>
</tbody>
</table>

Symbols:
- upregulated from very low level; strong expression still observed after 4 wpo.
- upregulated from very low level; strong expression still observed after 4 wpo, but number of labelled neurons reduced.
- upregulated from very low level, but expression is downregulated after 4 wpo.
- upregulated from moderate level of constitutive expression; strong expression still observed after 4 wpo.
- upregulated from moderate level of constitutive expression but only a few positive cells found.
- upregulated from very low level with return to background level by 2 wpo.
- upregulated from moderate level with return to background level by 2 wpo.
- strong constitutive expression; no upregulation apparent.
- moderate constitutive expression; no upregulation apparent.
- undetectable data ; * data not available

Abbreviations: BS, brainstem; DN, deep cerebellar nuclei; FK, freeze-killed PN graft; L, living PN graft; SNpc, substantia nigra pars compacta; TRN, thalamic reticular nucleus

Note: Some data presented in this table are taken from: Woolhead et al., 1998 (1); Zhang et al., 1995 (2); Vaudano et al., 1995 (4), 1998 (3). In cases of the expression of c-jun and GAP-43 mRNAs in the thalamus, the data apparent in column FK derived from stab wounds instead of freeze-killed graft implantation.
and brainstem neurons in animals implanted with freeze-killed grafts even after long survival times, whereas expression of CHL1 and L1 under the same condition was transient. Generally, neurons of the cerebellar cortex showed no response after axotomy or implantation of PN grafts, with the exception of some neurons close to the graft which transiently upregulated CHL1 and c-jun mRNAs. In contrast, some axotomized Purkinje cells upregulated c-jun expression irrespective of the types of graft implanted.

The results from the different regions of the brain described above are in keeping with the modern theme that expression of CHL1, L1, c-jun and GAP-43 and regenerative propensity of CNS neurons are correlated. It is clear that CNS neurons which were able to successfully regenerate axons into PN grafts expressed high levels of all growth-related genes. Although in some regenerating neurons, some mRNAs were not upregulated, constitutive expression of those mRNAs in the neurons was always present, as in the case of SNpc neurons. These data suggest that the four growth-related molecules may have functional links in relation to the promotion of axon regeneration of CNS neurons.

9.2) Growth-related molecules: their roles in promoting axonal regeneration in injured CNS neurons

A) Cell recognition molecules: CHL1 and L1

L1 is a transmembrane cell recognition molecule of the immunoglobulin superfamily. L1 appears to be particularly important for axonal growth on Schwann cells (Seilheimer and Schachner, 1988) and plays a role in axonal pathfinding during development (Dahme et al. 1997). Ample evidence indicates that expression of L1 promotes neurite outgrowth (Poltorak et al., 1993, 1997; Doherty et al., 1995; Walsh et al., 1997) and neuronal plasticity (Poltorak et al., 1993; Gopinath et al., 1996; Schmidt and Schachner, 1998) and its expression is upregulated in regenerating axons during axonal elongation and downregulated upon target innervation (Aubert et al., 1998). Zhang et al. (1995) also correlated the regeneration of axonal profiles into PN grafts implanted into the thalamus with upregulation of L1 mRNA in TRN neurons, which regenerate axons vigorously into PN grafts. In keeping with these data, the present study provides additional strong evidence that L1 mRNA was expressed by neuronal populations which regenerated axon into grafts but not by neurons which failed to do so.
(Chapter 8). In some CNS neurons expressing moderate to high levels of L1 mRNA (i.e., striatal cholinergic interneurons and SNpc neurons), there was no sign of enhancement of expression of this gene detectable after implantation of living PN grafts. Nevertheless, those neurons regenerated axons very well into the graft. High constitutive expression of L1 mRNA in CNS neurons, capable of regeneration, may be considered as a pre-adaptation of these neurons for axonal regeneration into PN grafts.

Expression of L1 mRNA was upregulated more slowly than other growth-related genes, CHL1, c-jun and GAP-43 (Chapter 8), even in neurons which expressed undetectable level of this gene such as TRN neurons (Zhang et al., 1995). It has been clearly shown in the present study that upregulation of L1 in CNS neurons was induced by living PN grafts. The delayed expression of L1, therefore, suggest that exogenous factor(s) from the graft which regulate expression of L1 on one hand and those that regulate CHL1, GAP-43 and c-jun expression on the other hand, may differ from one another.

L1 is capable of homophilic binding to L1 on the surfaces of other cells and heterophilic binding to other recognition molecules. Expression of L1 on Schwann cell surfaces and on regenerating axons, may help to support axons growing into the graft by way of axon-axon interactions and interactions between axons and Schwann cells in the grafts. Additionally, in recent years it has become clear that L1 is able to stimulate neurite outgrowth via second messenger systems (Doherty et al., 1996a,b; Burden et al., 1997; Heiland et al., 1998). It has been suggested that L1 and N-CAM activate signal transduction cascades in neurons via the fibroblast growth factor (FGF) receptor, containing a domain homologous to one found on L1 (William et al., 1994a,b; Doherty et al., 1995; Walsh et al., 1997). In view of these results, not only would these axons benefit from enriched cell-substrate adhesion but also receive a stimulus which would enhance neurite outgrowth via signal transduced through FGF receptors. Significantly, SNpc neurons are known to express mRNA for the FGF-1 receptor (Wanaka et al., 1990); this pre-adaptation may contribute to the regenerative ability of these neurons (Woolhead et al., 1998).
CHL1, a recently identified recognition molecule (Holm et al., 1996), is one of the genes, including c-jun and GAP-43, which injured CNS neurons upregulate soon after injury. Similar to L1 and the other two molecules, there is no doubt that CHL1 is expressed by regenerating CNS neurons, as demonstrated in TRN neurons (Chapter 6) and in DN and brainstem neurons (Chapter 8). It is not clear what the role of neuronal CHL1 in axonal regeneration may be, since up to now there have been only a few studies investigating the function of CHL1 (Holm et al., 1996; Hillenbrand et al., 1999).

CHL1 does not bind homophilically or by heterophilic interactions with L1 or N-CAM (Hillenbrand et al., 1999) and its ligands are unknown. Consequently, the role of neuronal CHL1 expression in the regeneration of axons is more a matter of speculation than that of neuronal L1. If CHL1 is expressed on the shafts of the pioneering axons during regeneration, it could promote the growth of later regenerating axons, even if those neurons do not express CHL1. Alternatively, if it is secreted by the growing axons, CHL1 could act as an autocrine or paracrine factor promoting regeneration; CHL1 promotes neurite growth even when in a soluble form (Hillenbrand et al., 1999) and apparently more powerfully than does L1. Finally, CHL1 could act as a receptor for its ligand (presumably on the surface of Schwann cells) transducing a growth-promoting signal into the axon. However, more studies are needed to elucidate the role of CHL1 in the nervous system and in particular, its role in promoting regeneration.

B) c-jun and GAP-43

The inducible transcription factor c-jun increases in neurons in response to axotomy, and it has been postulated that c-jun may regulate genes involved in promoting either degeneration or regeneration of axotomized neurons (for review, see Herdegen et al., 1997b). The results of the present study showed that expression of c-jun uniquely among the molecules studied, was upregulated by injured CNS neurons, both regenerating neurons (e.g., DN and brainstem neurons) and non-regenerating neurons (e.g., Purkinje cells), either after implantation of a living or freeze-killed graft. Expression of c-jun in both regenerating and non-regenerating injured neurons may reflect the pivotal role of this gene in more than one signalling pathway. Upregulation
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of expression of c-jun mRNA in regenerating CNS neurons found in this study indicates a role of c-jun in regulating a signalling pathway or pathways, leading to cell survival and regeneration. Although the precise role of c-jun in the regenerative response, is unclear, it has been hypothesised that c-jun activation following axon damage leads to an increased expression of particular genes which are essential for the regenerative response (e.g., Herdegen et al., 1997b; Anderson et al., 1998; Vaudano et al., 1998). Coexpression of c-jun and other growth-related molecules: L1, CHL1 and GAP-43, in individual neurons in neuronal populations capable of regenerating axons into PN grafts (Chapter 8), suggests that c-jun may regulate the expression of these other genes. The similar patterns of expression of c-jun and GAP-43 mRNAs found in the cerebellum (Chapter 8) and in the thalamus (Vaudano et al., 1995, 1998) following the implantation of PN grafts has given a clue as to the close relationship between two mRNAs.

Most types of neuron, in both the PNS and CNS, upregulate c-jun and GAP-43 expression when they regenerate their axons, and the extent of that upregulation can be broadly correlated with the vigour of their regenerative response. Injury to the central axons of DRG cells, either in the dorsal roots or in the dorsal columns of the spinal cord, produces a much less vigorous regenerative response than injury to their peripheral axons (Richardson and Issa, 1984; Richardson and Verge, 1987; Chong et al., 1996) and little or no upregulation of c-jun or GAP-43 (Jenkins et al., 1993a; Schreyer and Skene, 1993; Chong et al., 1994a, 1996). When CNS neurons regenerate their axons, they usually upregulate both c-jun and GAP-43. For example, in addition to neurons in the DN and the TRN, retinal ganglion cells (RGCs) also increase their expression of GAP-43 mRNA and protein, and c-jun mRNA, when they regenerate into grafts attached to the severed optic nerves (Hüll and Bähr, 1994b; Schaden et al., 1994; Ng et al., 1995; Jung et al., 1997). However, a massive upregulation of GAP-43 cannot be essential for axonal regeneration, since neurons in the SNpc regenerate axons into nerve grafts in the neostriatum but do not appear to upregulate GAP-43 (Woolhead et al., 1998). On the other hand, SNpc neurons express GAP-43 mRNA even in unoperated animals (Kruger et al., 1993; Woolhead et al., 1998). The precise role of GAP-43 in axonal regeneration remains uncertain but it may function by controlling
growth cone activity (Aigner and Caroni, 1993), perhaps through interacting with the
growth cone membrane and actin cytoskeleton (Wiederkehr et al., 1997). The nervous
system develops in animals carrying a null mutation of the GAP-43 gene (Strittmatter
et al., 1995), but there are axonal pathfinding abnormalities in such mice. Furthermore,
it is not necessarily the case that the growth of axons during development is identical to
regeneration in the adult: the distances axons regenerate in the adult are often much
greater than the distance over which they grow towards their targets during
development and the cellular substratum used by the regenerating axons is different. No
neurons have been shown to be capable of regenerating their axons over long distances
without expressing GAP-43.

9.3) Coordinated expression of growth-associated molecules?

Increased binding of c-Jun to AP-1 sites, and elevated c-Jun protein levels have
been described in DRG neurons after axotomy (Kenney and Kocsis 1998). GAP-43 is a
prominent growth-cone component widely correlated with competence for axon
regeneration (Aigner and Caroni, 1993; Aigner, 1995; Basi et al., 1987) and its
promotor contains a highly conserved AP-1 binding sites (Eggen et al., 1994). Several
reports provide evidence that c-jun and GAP-43 are regulated in parallel following
neuronal damage (Bisby et al., 1995; Tetzlaff et al., 1994; Schreyer and Skene, 1993;
Schaden et al., 1994). In the present study, c-jun and GAP-43 showed very similar
patterns of expression in the cerebellum following PN graft insertion. Moreover, it has
been found that expression of c-Jun and GAP-43 is upregulated by DRG neurons
within hours after sciatic nerve crush, and GAP-43 mRNA level drop as regeneration is
completed (Van der Zee et al., 1989). Taken together, these findings strongly support
the possibility that c-jun may directly regulate expression of the GAP-43 gene via the
AP-1 binding site in its regulatory region.

In contrast to its effects on GAP-43 expression, there has been no suggestion
that c-jun directly controls the expression of L1 or CHL1. Although the same neurons
in the DN, vestibular nuclei and TRN, which upregulate c-jun when regenerating axons
also upregulate L1 and CHL1, the patterns of expression of L1 and CHL1 differ from
those of c-jun and GAP-43. Expression of L1 and CHL1 mRNAs in the cerebellum
after PN grafting declined sooner than that of c-jun and GAP43. Moreover, in the cases of freeze-killed graft insertion, L1 and CHL1 were not upregulated by DN and brainstem neurons at survival times longer than 1 wpo, whereas, expression of GAP-43 and c-jun mRNAs was detected in those neurons in the same animals for up to 6 weeks postgrafting. There are also marked differences between c-jun expression and the expression of LI and CHL1 following peripheral nerve injury: c-jun is upregulated by regenerating DRG and motor neurons but the expression of mRNA for L1 does not appear to change in either group of neurons and CHL1 mRNA is upregulated by motor neurons but not by large DRG neurons (Roslan, et al., 1998; Zhang et al., submitted for publication). The differences between expression of GAP-43 and c-jun on one hand, and CHL1 and L1 on the other hand, suggest that the two pairs of mRNAs are independently regulated. Finally, it is not yet clear if CHL1 and L1 possess functional AP-1 binding sites, although it is thought that such a site is present in the regulatory region of the L1 gene (Dr. M. Schachner, personal communication). In spite of these uncertainties concerning the regulation and functional coordination of their activities the evidence in the expression of the four mRNAs in regenerating CNS neurons presented in this thesis, indicates that all of them are involved some ways in promoting axon regeneration in the CNS neurons I have studied.

Concluding remarks

The present study has shown that expression of the mRNAs for four molecules: the cell recognition molecules, L1 and CHL1, the immediate early gene, c-jun and the growth associated protein, GAP-43, by injured CNS neurons can be correlated with the regeneration of their axons into a PN graft. Neurons which regenerated axons poorly into the graft (e.g., projection neurons of the striatum, neurons of the dorsal thalamus and neurons of the cerebellar cortex) transiently upregulated only some or none of mRNAs for these molecules. In contrast, neurons which regenerated axons vigorously into the grafts, e.g., neurons of the TRN, DN and brainstem, upregulate gene expression of all examined mRNAs after injury and such expression was prolonged when a piece of living PN graft was inserted. Moreover, the patterns of expression of the four mRNAs in each type of CNS neurons (—investigated in the present study), capable of
axonal regeneration, also differ; these differences presumably reflect the distinct regenerative potential among these neurons. For example, TRN and SNpc neurons, expressed high levels of L1, CHL1, c-jun and GAP-43 mRNAs at all post-injury intervals, including the longest survival times examined. These neurons regenerated axons very well into grafts, even those neurons whose cell bodies were situated far away from the graft. Unlike TRN and SNpc neurons, expression of all mRNAs in DN neurons as well as in cholinergic interneurons of the striatum declined sooner, particularly expression of L1 and CHL1 mRNAs; only neurons located near the graft tip appeared to have regenerated their axons.

The present work provides clear evidence that expression of L1, CHL1, c-jun and GAP-43 mRNAs is involved in the promotion of axon growth in axotomized CNS neurons. However, the roles that these molecules play in axon regeneration and the extent to which these molecules are necessary for such processes are still a subject of speculation. To elucidate these matters, a different type of study is required. The use of transgenic animals, in which expression of these molecules has been selectively altered, either in such a way that the expression of particular genes is exaggerated or by deleting these genes (knocked out), may be useful to investigate the crucial role of each molecule in axon regeneration in injured neurons. The results from recent studies have shown that axotomized Purkinje cells in mice, in which GAP-43 (Buffo et al., 1997) or L1 (recent results from this laboratory) is overexpressed, exhibit profuse sprouting, but do not regenerate axons; these results suggest that the use of transgenic animals in which two (or more) genes are selectively attached would be more advantageous. Furthermore, LM and EM immunocytochemical investigations of expression of these growth-promoting molecules by regenerating neurons, would provide more complete information and also strengthen this correlation. Particularly, in the case of CHL1 for which detailed data on expression patterns such as whether or not it is expressed on axons, such studies would provide useful clues.

It is very likely that the intrinsic regenerative response of adult CNS neurons involves the coordinated expression of a set of growth-related molecules, L1, CHL1, c-jun and GAP-43. However, it is necessary to bear in mind that the basic requirements of neurons for accomplishing a full program of axon regeneration almost certain vary
from one type of neurons to another. For example, in striatal cholinergic interneurons, the expression of trkA and p75 on their cell surface seems to be involved in the regeneration of their axons which is not unexpected in the context of the fact that they are NGF-sensitive, but is very different from what happens in other CNS neurons, most of which do not express these surface molecules. Investigations of the growth-related molecules expressed by regenerating neurons, using an exogenous source of trophic factors such as PN grafts, in wider populations of CNS neurons would be necessary to learn and understand the specific molecular requirements of different neuronal subtypes in the context of axon regeneration after injury. Such information would be valuable in attempts to develop therapeutic strategies for the repair of damaged pathways in the brain and spinal cord.
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