Growth-associated molecules and axonal regeneration

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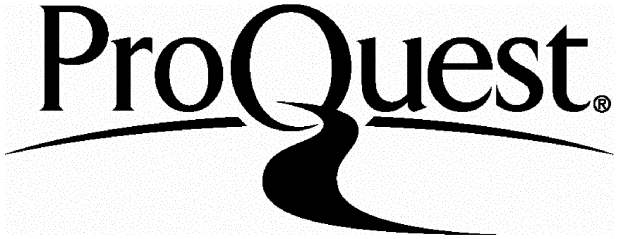

Abstract

An important aspect of successful axonal regeneration is the neuronal response to axotomy. In order to characterise the changes in gene expression associated with axonal regeneration, expression of mRNAs for three candidate regeneration-associated molecules, SCG10, CAP-23 and FKBP12, along with the prototypical growth-associated molecule GAP-43, was examined by in situ hybridisation (ISH). *SCG10* and *CAP-23* were shown to be upregulated in regenerating neurons following sciatic nerve injury. Expression was downregulated by 6 weeks postoperation when regrowing axons have reached their targets, unless this was prevented by ligation of the injured nerve. Dorsal rhizotomy did not provoke any upregulation. All three candidate molecules were upregulated by CNS neurons regenerating axons into peripheral nerve grafts implanted into either the thalamus or the cerebellum, identified by retrograde labelling. These molecules therefore appear to be coregulated with GAP-43. A study was also made of the effects of overexpression of GAP-43 in transgenic mice on regeneration in circumstances in which the axotomy-induced gene upregulation in the injured neuron is minimal and insufficient to promote regeneration. GAP-43 overexpression in primary sensory neurons failed to augment consistently regeneration or sprouting following injury of the ascending dorsal columns in the spinal cord, or injury to the dorsal roots. Compared to wild-type controls, enhanced growth was sometimes seen after lumbar dorsal column injuries, while after thoracic dorsal column injury less growth was observed. Finally, the response to axotomy of neurons projecting down the spinal cord was characterised. Using retrograde labelling and ISH, expression of a panel of axotomy-associated genes was examined in corticospinal neurons following axotomy in the cervical cord or in the cortex, and in rubrospinal neurons following cervical axotomy. Corticospinal neurons showed no response to distal axotomy, but upregulated *GAP-43*, *SCG10*, *c-jun*, *ATF3*, *L1* and *CHL1* following proximal axotomy. Rubrospinal neurons clearly upregulated only *L1*, *c-jun* and *ATF3*.

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Abbreviations and nomenclature

Frequently used abbreviations are listed below:

CNS	Central nervous system	**ISH/CTB**	In situ hybridisation and CTB immunohistochemistry
CRE	Cyclic-AMP response element	**JNK**	Jun N-terminal kinase
CSPG	Chondroitin sulphate proteoglycan	**LGN**	Lateral geniculate nucleus
CT-HRP	Cholera toxin B-subunit - horseradish peroxidase conjugate	**MAG**	Myelin-associated glycoprotein
CTB	Cholera toxin B-subunit	**NCAM**	Neural cell-adhesion molecule
DAB	3-3'-diaminobenzidine tetrahydrochloride	**NGF**	Nerve growth factor
DEPC	Diethylpyrocarbonate	**PB**	Phosphate buffer
DEPC.H_2O	Diethylpyrocarbonate-treated Ultrapure water	**PBS**	Phosphate buffered saline
dH_2O	Distilled water	**PCR**	Polymerase chain reaction
DIG	Digoxigenin	**PKA**	Protein kinase A
DREZ	Dorsal root entry zone	**PKC**	Protein kinase C
DRG	Dorsal root ganglion	**PNS**	Peripheral nervous system
FGF	Fibroblast growth factor	**RT-PCR**	Reverse transcription and polymerase chain reaction
GFAP	Glial fibrillary acidic protein	**RyR**	Ryanodine receptor
IP_3R	Inositol triphosphate receptor	**SNpc**	Substantia nigra pars compacta
ISH	In situ hybridisation	**TRN**	Thalamic reticular nucleus

Nomenclature:

In the main text and the figure legends, the convention has been observed whereby proteins are referred to in non-italicised lettering and with an initial capital letter if appropriate, whereas genes and mRNAs are referred to in italics. The figures themselves, however, are labelled with non-italic names for greater readability.

Some of the molecules investigated in the experiments presented here have several alternative names. For reference, these are listed below:

Molecule name used	**Alternative names**
GAP-43	B-50, F1, pp46, neuromodulin
ATF3	LRF-1
CAP-23	NAP-22, BASP-1
Krox-24	NGFI-A, Egr-1, Zif268, ZENK

Chapter 1

Introduction

1.1 The failure of regeneration in the central nervous system: an overview

In adult mammals, injury to a peripheral nerve results in vigorous regeneration of the damaged axons, often resulting in functional recovery, but in the central nervous system (CNS) regeneration normally fails. The responses to axonal injury in the CNS have been much studied over the last twenty years and the current evidence suggests that the causes of the failure of regeneration can be separated into two major components. The first is the environment within the central nervous system, which is believed to be inhibitory to axon growth for the vast majority of neurons; the second is the nature and magnitude of the neuronal response to axotomy, which is believed in many cases to be inadequate to support vigorous axonal regeneration. The first experiments giving rise to this point of view were performed by Tello (1911). He was the first to find evidence that implantation of a graft of peripheral nerve into the CNS resulted in invasion of the graft by axons, which were assumed to be from CNS neurons. However, because it was known that peripheral axons could also regenerate into nerve grafts, this interpretation remained a doubtful one until the advent of new retrograde and anterograde tracing technology allowed Aguayo and colleagues to demonstrate conclusively that axons from CNS neurons did indeed grow into peripheral nerve grafts implanted into the brain and spinal cord (Richardson et al., 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982; reviewed by Aguayo, 1985). The possibility remained that the growing axons originated only from collateral sprouts of intact axons, and not injured axons, until Berry et al. (1986) used peripheral nerve grafts attached to severed optic nerves to demonstrate conclusively that axotomised retinal ganglion cells could regenerate axons into peripheral nervous tissue.

Once this was established, it became apparent that many neurons of the CNS are capable of regenerating axons over long distances given a suitably supportive environment. This implies that the usual environment encountered by axotomised CNS neurons is at best unsupportive and at worst inhibitory to growth. Since CNS axons do not regenerate into freeze killed nerve grafts (Berry et al., 1988), it appears likely that successful regeneration requires the presence of growth-promoting molecules derived from Schwann cells, or possibly other non-neuronal cells within the grafts, to grow axons in vivo. Many such molecules will be present on the surface of Schwann cells in the grafts, or released by them. Following injury to peripheral nerves, Schwann cells in the denervated portion of

the nerve proliferate and upregulate neurotrophins and cell-adhesion molecules. They therefore not only provide a permissive environment but also actively encourage growth (reviewed by Fawcett and Keynes, 1990; Terenghi, 1999). It seems likely that the failure of growth within the CNS is partly due to the lack of such supportive molecules. However, there is also considerable evidence that the injured CNS contains a number of factors which are actively inhibitory to growth. These may be present in the intact CNS (Caroni and Schwab, 1993) or produced following injury. The astrocytic scar formed at lesion sites appears to be refractory to growth, and the molecular basis of this is a current area of research (see Fawcett and Asher, 1999).

However, some neurons are capable of regenerating within the adult CNS and this suggests that inhibitory influences in the intact CNS are not absolute. For example, transplanted embryonic neurons or neuronal precursor cells (Bjorklund and Stenevi, 1979; Bjorklund et al., 1979; Foster et al., 1985; Wictorin et al., 1990; Wictorin and Bjorklund, 1992; Li and Raisman, 1993; Davies et al., 1993; Davies et al., 1994) and even transplanted adult dorsal root ganglion (DRG) neurons (Davies et al., 1997; Davies et al., 1999) readily grow axons within the adult CNS following injury. In these cases the enabling factors may be the very vigorous axon growth activity of the neurons themselves, which may be sufficient to overcome inhibitory signals, or possibly an absence from the transplanted neurons of receptors for inhibitory molecules. The pattern of gene expression in the injured neuron is, therefore, an important factor in considering the chances of successful regeneration within the CNS, and is also relevant even when neurons are presented with the growth-promoting environment of a peripheral nerve graft. Some classes of neuron, e.g. Purkinje cells, fail completely to regenerate axons into such grafts and generally these neurons show little or no cell body response to axotomy (reviewed by Anderson et al., 1998). In addition, some neurons which can regenerate axons into such a graft will only do so if stimulated strongly enough by an appropriate form of axotomy. For example the ascending dorsal column collaterals of DRG neurons show a much greater ability to invade a graft in the spinal cord if these neurons also receive an injury to the peripheral branches of their axons (e.g. a sciatic nerve crush) (Richardson and Issa, 1984), and axotomised rubrospinal neurons only regenerate axons into peripheral nerve grafts if the axotomy is sufficiently proximal to elicit a strong enough growth response (Richardson et al., 1984; Jenkins et al., 1993a; Fernandes et al., 1999).

In summary, the success of axon growth depends on the balance of inhibitory versus permissive and growth-promoting molecules in the environment, on the responses of individual neurons to such molecules and on the vigour of their axon-growth program. This program consists of changes in neuronal gene expression which occur after axotomy and accompany successful axon regeneration and it is these which are the subject of the present study.

1.2 Peripheral nerve regeneration

It is first worth considering regeneration in the peripheral nervous system (reviewed by Fawcett and Keynes, 1990; Ide, 1996; Terenghi, 1999). Injuries to peripheral nerves result in full-scale regrowth of the injured fibres and reinnervation of target tissues, although the extent of functional recovery varies between species and, for example, is much greater in rats than in humans where only 50% of patients with repaired nerves report a useful recovery of function (Lee and Wolfe, 2000). Peripheral nerve injury in the rat therefore serves as a useful model for the study of successful regeneration.

Axonal regeneration in the peripheral nervous system (PNS) benefits from two factors: the environment of an injured peripheral nerve is highly favourable to axon growth; and injured neurons with axons in peripheral nerves show a very strong response to axotomy and, as a consequence, strongly express a variety of growth-associated molecules. Following injury, the distal part of the axon degenerates, in the process known as Wallerian degeneration (Waller, 1850). At the same time Schwann cells in the distal part of the nerve respond in a number of ways. They endocytose and begin degradation of axonal debris and the myelin sheath before transferring the remaining debris to macrophages. They also proliferate and extend processes along the nerve, to form Schwann cell columns, also known as the bands of Bungner (Salzer and Bunge, 1980). Successful regeneration is dependent on the presence of Schwann cells and on contact between these and the re-growing axons (Anderson et al., 1983; Hall, 1986; Nadim et al., 1990).

Schwann cells facilitate regeneration by providing a guiding structure and by producing various growth-promoting molecules, including surface-bound cell-adhesion molecules and

diffusible trophic factors. The cell-adhesion molecules L1, NCAM, and N-cadherin are all expressed on Schwann cells and are found at points of contact with axons (Martini and Schachner, 1988; Rathjen, 1988; Rutishauser et al., 1988; Shibuya et al., 1995), and all are believed to promote axon growth. Schwann cells in the distal stump also upregulate many molecules with neurotrophic action, such as NGF (Heumann et al., 1987), BDNF (Meyer et al., 1992), bFGF (Grothe et al., 2001), GDNF (Hammarberg et al., 1996), IGF (Pu et al., 1995) and express CNTF (Sendtner et al., 1992). These molecules all exert trophic effects on PNS axons (reviewed by Terenghi, 1999). Schwann cells in peripheral nerves also produce a basal lamina containing extracellular matrix molecules such as laminin, type IV collagen and tenascin-C. Laminin is the archetypal growth-promoting substrate in vitro and may therefore be of importance in peripheral nerve regeneration (reviewed by McKerracher et al., 1996; Luckenbill-Edds, 1997), although its contribution may be limited by the fact that most parts of regenerating axons are separated from the basal lamina by the Schwann cell processes.

PNS neurons and extrinsic CNS neurons also show a vigorous cell body response to axotomy, which includes strong expression of many growth-associated molecules (see Section 1.4 *iv*) and the downregulation of some molecules which are unnecessary for axonal elongation. In contrast, the response of intrinsic CNS neurons to axotomy varies considerably and contributes to the lack of regeneration in the CNS.

1.3 The growth-inhibitory nature of the central nervous system environment

Is the CNS actively inhibitory to axon growth, and if so what is the molecular basis for this inhibition? In the context of regeneration following injury, most of the CNS environment can be divided into three types of tissue: myelinated fibre tracts; grey matter; and the lesion site, which generally contains astrocytic scar tissue and, often, tissue cavities. Of these, the astrocytic scar tissue is probably the most inhibitory to axon growth. White matter also appears to be inhibitory, largely due to factors in CNS myelin. Grey matter may not actively support axon growth over long distance in most circumstances but appears to be less refractory to axonal sprouting and growth than white matter.

i. Myelin-associated growth inhibitors

The hypothesis that CNS myelin, or its breakdown products, could be inhibitory to axonal growth was first proposed by Berry (1982). Although support for the hypothesis was slow to emerge, considerable evidence has been gathered that CNS myelin is inhibitory to axon growth. The earliest in vitro studies to support this view were based on assays of fibroblast spreading and neurite growth (Schwab and Caroni, 1988; Caroni and Schwab, 1988a) and suggested that mature oligodendrocytes and isolated CNS myelin were inhibitory for these processes. Further analysis ascribed this property to two specific fractions of CNS myelin (Caroni et al., 1988; Caroni and Schwab, 1988a; Caroni and Schwab, 1988b). Furthermore, cultured oligodendrocytes caused growth-cone collapse in axons of cultured neonatal rat DRG and other neurons, whereas astrocytes supported growth (Schwab and Caroni, 1988; Fawcett et al., 1989; Bandtlow et al., 1990). Immature oligodendrocytes were also permissive for adhesion and growth (Schwab and Caroni, 1988). Similar results were obtained when frozen sections of adult rat CNS tissue were used as substrates for cultures of embryonic chick peripheral or neocortical neurons, and neonatal rat DRG neurons. Grey matter was consistently permissive for cell adhesion and neurite growth, but white matter was clearly not (Savio and Schwab, 1989; Crutcher, 1989; Watanabe and Murakami, 1989; Watanabe and Murakami, 1990).

There is considerable circumstantial evidence that CNS myelin is inhibitory to axonal growth in vivo. Regeneration of spinal axon tracts occurs in the neonatal rat and neonatal opossum following spinal injury (Xu and Martin, 1991; Saunders et al., 1992; Bates and Stelzner, 1993) but this capacity is soon lost in the postnatal animal (see also Nicholls and Saunders, 1996). In both species, the end of the regeneration-permissive period coincides with the appearance of oligodendrocytes and the onset of myelination (Caroni and Schwab, 1989). Similar findings have been made in the chick embryo (Shimizu et al., 1990; Keirstead et al., 1992). Furthermore, prevention of myelin formation or myelin removal appears to prolong the regeneration-permissive period. In rats in which myelin formation was prevented by X-irradiation of neonates, which kills oligodendrocytes, regeneration of the corticospinal tract was found in animals 2-3 weeks old (Savio and Schwab, 1990). Antibodies against CNS myelin or its components also have dramatic effects on axonal regeneration in the spinal cord (see below).

Experiments with isolated myelin extract from adult rat CNS, as well as providing evidence of the inhibitory nature of myelin, have led to the identification of individual protein constituents of CNS myelin with growth inhibitory properties. Currently, two major inhibitory components of myelin have been identified; myelin-associated glycoprotein (MAG) and Nogo.

MAG was first identified as an inhibitory component of myelin by Mukhopadhyay et al. (1994) and McKerracher et al. (1994). MAG is inhibitory to neurite outgrowth from a wide variety of neurons, including postnatal retinal ganglion cells, hippocampal neurons, superior cervical ganglion neurons and spinal neurons and adult DRG neurons (Mukhopadhyay et al., 1994; DeBellard et al., 1996). Purified MAG, recombinant MAG, and a solubilised form are all inhibitory to neuronal cell lines and primary cultures (McKerracher et al., 1994; Li et al., 1996; Tang et al., 1997). However, although MAG seems to be as powerful an inhibitor of axonal growth in vitro as Nogo, studies of MAG knockout mice found no or only minor improvement in regeneration in vivo or in neurite outgrowth on myelin preparations. (Bartsch et al., 1995; Li et al., 1996).

Nogo was first identified as the two growth-inhibitory components of myelin, NI-35 and NI-250, described by Caroni and Schwab (1988a; 1988b). The monoclonal antibody IN-1 was raised against NI-250 (Caroni and Schwab, 1988b) and has been used in a variety of experiments to promote regeneration, suggesting it neutralises growth-inhibitory properties of these molecules. In vitro, IN-1 treatment allowed neurite growth on CNS myelin and into optic nerve explants (Caroni and Schwab, 1988b). In vivo, administration of IN-1 antibodies, using implanted hybridomas, promoted regeneration of the corticospinal tract (Schnell and Schwab, 1990; Schnell and Schwab, 1993). IN-1 was also shown to promote regeneration of other descending tracts (Bregman et al., 1995) and to promote sprouting of uninjured Purkinje cell axons (Buffo et al., 2000). Recently, the Nogo cDNA was cloned and is thought to represent the gene encoding NI-35 and NI-250. Its protein products have three forms, Nogo A, B and C (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) and growth-inhibitory properties have been described for two separate domains of the three proteins. A receptor for one of these domains, Nogo66, has also been described (Fournier et al., 2001).

Clearly, there is a large amount of evidence to suggest that CNS myelin is inhibitory to axon regeneration. Against this, the experiments of Davies et al. (1997, 1999) indicate that axon growth from adult neurons is certainly possible within CNS white matter. DRGs from adult transgenic mice expressing green fluorescent protein were transplanted into the spinal cord of adult rats, in such a way as to minimise trauma to the cord and subsequent gliosis and scarring (see Section 1.3 *ii*). The transplanted neurons grew axons long distances within the dorsal funiculus. Axons grew in both otherwise uninjured white matter and in white matter undergoing degeneration as a consequence of an earlier lesion to the dorsal funiculus. In the latter case, the inhibitory components of CNS myelin so far described would be exposed and would be expected to prevent any regeneration. Similarly, the findings of Berry et al. (1996, 1999) and Leon et al. (2000) show that retinal ganglion cells, when suitably stimulated, can regenerate axons within the optic nerve, which is purely white matter. None the less, the weight of evidence still suggests that CNS myelin is inhibitory to axonal regeneration, but it may be that maximally stimulated neurons either downregulate receptors for inhibitory molecules or express growth promoting molecules so strongly that they are able to ignore the presence of myelin or overcome its effects.

ii. Lesion cavities and the glial scar

Injuries to the spinal cord commonly provoke the formation of two types of structure which are likely to represent obstacles to subsequent axon growth: the glial scar and tissue cavities. The former, which is characterised by the presence of glial fibrillary acidic protein (GFAP)-positive astrocytes has been better studied and is described in some detail below. The latter are a major feature of spinal cord injuries in experimental models in which the injury is caused by compression or contusion of the cord (e.g. Noble and Wrathall, 1985; Bresnahan et al., 1991). These result in large cavities being formed, but lesser cavitation is observed even in laceration injury models. Such cavities are likely to be formed by processes similar to those which lead to the formation of large fluid-filled cysts which are a feature of some human spinal injuries (Bunge et al., 1993). Cavity formation is thought to be a consequence of extensive cell death, caused both directly by the lesion and by secondary processes, and occurring by both necrosis and apoptosis (reviewed by Beattie et al., 2000). The cavity itself generally does not contain a large number of GFAP-positive astrocytes which are characteristic of inhibitory scar formation (see below), but these can

be found around the margin of the cavity and in the surrounding parenchyma (Farooque et al., 1995; von Euler et al., 1997). Typically, various cell types are found within the cavity including macrophages/microglia, Schwann cells, cells immunopositive for the chondroitin-sulphate proteoglycan NG2, a few astrocytes and, if the meninges have been breached, fibroblasts and meningeal cells (Bunge et al., 1994; Zhang et al., 1997; P. Anderson, personal communication). While tissue cavities may present an obstacle to regeneration, their properties of inhibition or otherwise of axon growth have not been fully investigated. Axons have frequently been observed within lesion cavities, many of which may arise from the dorsal roots (Bunge et al., 1994; Beattie et al., 1997). Corticospinal and reticulospinal fibres can grow into the lesion cavity (Hill et al., 2001).

The glial scar is one of the main obstacles to regeneration of axons within CNS fibre tracts (Reier et al., 1983; Reier and Houle, 1988; Snow et al., 1990; McKeon et al., 1991; Silver, 1994; Fawcett and Asher, 1999). When examined by electron microscopy, the scar appears to be largely composed of astrocytes (Reier et al., 1983), but it also contains oligodendrocytes, oligodendrocyte precursors and microglia. In response to the lesion, astrocytes around the lesion, and to some extent around newly formed or forming tissue cavities, upregulate the intermediate filament proteins glial acidic fibrillary protein (GFAP) and vimentin, and some astrocyte cell division occurs. There is also evidence that progenitor cells multiply and differentiate into astrocytes but the relative contribution of these cells to scar astrocyte numbers is not established (Frisen et al., 1995; Johansson et al., 1999). Existing and newly formed astrocytes become hypertrophic and produce a dense meshwork of enlarged processes. Where the physical boundary of the CNS tissue has been breached, this structure encapsulates the damaged CNS tissue and forms a reconstituted glia limitans, separating the CNS from surrounding tissue. Meningeal cells also participate in recreating the glia limitans (Kruger et al., 1986). Reformation of the glia limitans also results in the formation of its associated basal lamina (Reier et al., 1983), a boundary layer of ECM molecules, largely composed of laminin and collagen (Timpl, 1996). Astrocytes lining lesion cavities may also produce a basal lamina along the cavity border (Bunge et al., 1994; Zhang et al., 1997). CNS injury also results in the deposition of collagenous scar tissue within the lesion (Tobin et al., 1979).

Axon growth is typically arrested at the scar in vivo (reviewed by Reier et al., 1983; Reier and Houle, 1988). The basis of prevention of axon growth by this scar tissue has yet to be determined. The final form of the scar consists of many tightly interwoven fine astrocyte processes (Reier et al., 1983; Berry et al., 1983; Eng et al., 1987; Reier and Houle, 1988) and this may present a physical barrier to growing axons, although the presence of inhibitory molecules is probably more important. The basal lamina and the associated collagenous ECM deposits in the scar may also represent a physical barrier, and prevention of formation of these with 2,2'- dipyridine results in increased regeneration of the fimbria-fornix (Stichel et al., 1999), but, however, fails to augment regeneration of the corticospinal tract (Weidner et al., 1999).

The importance of the glial scar in preventing regeneration in the spinal cord was clearly demonstrated by Davies et al. (1999). Microtransplantation of DRG neurons from mice into rat spinal cords resulted in long distance growth in both normal and degenerating white matter. The inhibitory nature of the glial scar was confirmed by the observation that axon growth was halted by the glial scar tissue associated with a concomitant lesion of the cord. Growth arrest by the glial scar can be observed in many sorts of lesions of the CNS, including lesions of the ascending dorsal columns of the spinal cord (Bavetta et al., 1999; Neumann and Woolf, 1999) and the corpus callosum (Davies et al., 1994).

Physical obstruction of axon growth, while it may play a role, is probably not the most important mechanism of inhibition of axon growth by the astrocytic scar. Evidence for this comes from observations of regenerative failure at the transition zone between the central and peripheral nervous sytems, the dorsal root entry zone (DREZ). Following an injury to a dorsal root, the incoming axons will regenerate towards the DREZ, where Schwann cells give way to oligodendrocytes and astrocytes. These astrocytes react to such an injury with upregulation of GFAP and by growing processes into the root, and the DREZ becomes similar to other astroglial scars (Reier et al., 1983; Murray et al., 1990). Carlstedt (1985) and Liuzzi and Lasek (1987) observed that axons apparently arrested at the DREZ were similar in appearance to normal synaptic terminals, except that they were apposed to astrocyte processes instead of neurons. This contrasted with the appearance of peripheral axons when presented with a ligated peripheral nerve, which prevents axonal elongation physically and results in a neuroma, with individual axons having large swollen

terminals containing accumulated neurofilaments and organelles. A small number of axons with these characteristics were observed at the dorsal root entry zone, where axons were obstructed by collagenous tissue rather than astrocytic processes. Liuzzi and Lasek (1987) suggested that at the DREZ regenerating axons are arrested by specific stop signals from reactive astrocytes, rather than by a physical barrier. However, many axons do not stop at the DREZ but instead loop back and regenerate retrogradely towards the periphery (Reier et al., 1983; Zhang et al., 2001). Furthermore, even regenerating dorsal root axons in contact with Schwann cells sometimes show the ultrastructural characteristics which Luzzi and Lasek (1987) associate with specific stop signals (Chong et al., 1999).

Molecules which are thought to be inhibitory to axon growth are associated with the glial scar, and may be at least partly responsible for the failure of axon regeneration. The primary candidates for this activity are the chondroitin-sulphate proteoglycans (CSPGs) (reviewed by Hoke and Silver, 1996; Fawcett and Asher, 1999; Bovolenta and Fernaud-Espinosa, 2000). Chondroitin sulphate linear polysaccharides, known as glycosaminoglycans (GAGs), are attached to a number of different core proteins to form CSPGs. CSPGs are expressed at lesion sites in CNS injury and some colocalise with GFAP-positive astrocytes (e.g. McKeon et al., 1991). In the experiments by Davies and co-workers described above, axons regenerating in the dorsal columns of the rat spinal cord stopped growing when they reached CSPG associated with the glial scar adjacent to an injury site (Davies et al., 1997; Davies et al., 1999). Many of the CSPGs have been shown to be inhibitory to growth or to block interactions with growth-promoting molecules. This is often a function of the GAG sidechains but can also be a function of the core protein. For example, neurocan has been found to block the neurite-growth-promoting properties of Ng-CAM and L1 (Friedlander et al., 1994) whereas NG2 appears to block the growth-promoting properties of laminin (Smith-Thomas et al., 1994). NG2 was shown to be largely responsible for growth inhibition by the inhibitory astrocyte cell line Neu7 (Fidler et al., 1999). There is disagreement as to whether inhibition of growth by NG2 is an activity of the GAGs or the core protein. Chondroitinase treatment of NG2, which removes the GAG sidechains from CSPGs was found by Fidler et al. (1999) to abolish the growth-inhibitory effect of NG2 but Dou and Levine (1994) found that chondroitinase had no effect. Recently, Moon et al. (2001) were able to promote regeneration in the nigrostriatal pathway after injury by the infusion of chondroitinase.

iii. Grey matter

The ability of CNS grey matter to inhibit axonal growth has been less well investigated than that of white matter. Little axonal regeneration has been reported in grey matter, although there are many examples where it has been shown to support axonal sprouting. Several experimental treatments have been shown to promote growth of severed corticospinal axons, and often this growth is observed in the uninjured grey matter of the spinal cord (Schnell and Schwab, 1990; Grill et al., 1997; von Meyenburg et al., 1998; Blesch et al., 1999; Blits et al., 2000). Transplanted embryonic adrenergic and serotinergic neurons were also reported to grow axons significant distances through adult CNS grey matter (Bjorklund et al., 1979; Nornes et al., 1983; Foster et al., 1985). In addition, intact grey matter is capable of supporting short-range axon growth in the adult in the context of cortical plasticity in the adult cat (Darian-Smith and Gilbert, 1994) or collateral sprouting in the dorsal horn following peripheral nerve injury in the rat (Woolf et al., 1992a; Woolf et al., 1995). In vitro assays indicated that grey matter was considerably more permissive to cell adhesion and axon growth than white matter (Savio and Schwab, 1989; Crutcher, 1989; Watanabe and Murakami, 1989; Watanabe and Murakami, 1990). Therefore, the general failure of growth in grey matter may be less due to intrinsically inhibitory properties than to a lack of the growth-factors and adhesion molecules found in peripheral nervous tissue. Grey matter is also unlikely to provide a suitable structural framework for significant linear growth, and lastly, in theory at least any growth may also be arrested by encountering neuronal cell bodies and dendrites which constitute valid synaptic targets, so that injured axons stop and form synapses (e.g. Bernstein and Bernstein, 1971). Indeed, in the transplantation experiments of Davies et al. (1997, 1999) it was shown that regenerating axons which entered the grey matter had shorter, more branching courses.

iv. Other molecules

In addition to the growth-promoting and growth-inhibitory molecules already mentioned, a rather large number of molecules have been identified which may be involved in promotion or inhibition of growth, or in guidance of axons, or both. The importance of most of these for inhibiting regeneration in the CNS or of promoting it in peripheral

nervous tissue has yet to be demonstrated. Those molecules not already mentioned which may be of importance include the ephrins (reviewed by Mellitzer et al., 2000), semaphorins (reviewed by Raper, 2000), netrins (reviewed by Culotti and Merz, 1998; Kennedy, 2000) and ECM molecules such as tenascin-R (reviewed by Pesheva and Probstmeier, 2000).

v. Overcoming CNS growth-inhibition

Much work has been devoted to devising strategies to promote regeneration of CNS axons and most studies have focused on providing a more favourable environment for the regrowth of CNS axons. This has been attempted with, amongst other things, treatments to neutralise inhibitory components of myelin (Schnell and Schwab, 1990; Bregman et al., 1995; Tatagiba et al., 1997; Dyer et al., 1998; Huang et al., 1999), degradation of CSPGs in the glial scar (Moon et al., 2001), provision of neurotrophic factors (e.g. Tuszynski et al., 1994; Tuszynski et al., 1996; Grill et al., 1997; Ye and Houle, 1997; Zhang et al., 1998; Bradbury et al., 1999), implantation of Schwann cells or olfactory ensheathing cells which are presumed to provide a growth-promoting environment (Li and Raisman, 1994; Paino et al., 1994; Li et al., 1997; Li et al., 1998; Ramon-Cueto et al., 1998; Ramon-Cueto, 2000; Ramon-Cueto et al., 2000), implantation of embryonic nervous tissue (Tessler et al., 1988; Houle and Reier, 1988; Bregman et al., 1989; Houle and Reier, 1989; Nothias and Peschanski, 1990; Itoh et al., 1992; Iwashita et al., 1994), or a combination of such approaches (Schnell and Schwab, 1993; Schnell et al., 1994; Xu et al., 1995; von Meyenburg et al., 1998; Blits et al., 2000). More recently there have been attempts to block signal transduction within the growth cone from receptors for inhibitory molecules (Lehmann et al., 1999). The focus of this study is, however, on neuron-intrinsic factors, which have been subject to less extensive previous investigation.

1.4 Neuron-intrinsic factors

i. Many CNS neurons can regenerate axons into peripheral nerve grafts

While the environment of the CNS is clearly inhibitory to axon regeneration, many neurons of the CNS retain the capability for regeneration over long distances when presented with a suitable environment. This was first demonstrated convincingly by Agauyo and

colleagues (Richardson et al., 1980; David and Aguayo, 1981; reviewed by Aguayo, 1985) who showed that neurons in various parts of the CNS would grow axons into implanted grafts of peripheral nerve. This was done by retrogradely labelling the regenerating cells from the distal end of the graft, which remained outside the CNS. Retrogradely labelled neurons were found in the spinal cord and brainstem (Richardson et al., 1980; David and Aguayo, 1981) when grafts were used to bridge a spinal cord injury, and were found in the forebrain when a nerve graft was implanted here (Benfey and Aguayo, 1982). Subsequent studies showed most parts of the CNS contained neurons that would regenerate axons into nerve grafts. In the neural retina, the retinal ganglion cells will do so following implantation of a nerve graft into the retina (So and Aguayo, 1985), and, importantly, following anastomosis of a peripheral nerve graft to the severed optic nerve (Berry et al., 1986). In the latter case regenerating axons unequivocally originated from axotomised CNS neurons. The thalamus and cerebellum also contain regeneration competent neurons (Dooley and Aguayo, 1982; Benfey et al., 1985; Morrow et al., 1993; Chaisuksunt et al., 2000a; see part *ii* below). The promotion of axon growth by peripheral nerve grafts is almost certainly due to the properties of the living cells, presumably the Schwann cells, within the grafts as nerve grafts rendered acellular by repeated freezing and thawing are not invaded by regenerating axons when implanted into the CNS (Berry et al., 1988; Smith and Stevenson, 1988). Following injury to a peripheral nerve, Schwann cells in the distal stump provide a highly favourable environment to axon growth, removing myelin debris and expressing a number of trophic factors and cell-adhesion molecules (see Section 1.2).

ii. Variation among CNS neurons and the neuronal response to axotomy

However, the nerve grafting experiments described above, and subsequent similar experiments, also revealed that there is a marked variation in the capacity of CNS neurons to regenerate axons, even given a favourable environment. Peripheral nerve grafts implanted into various sites in the CNS will usually elicit axon regeneration in large numbers only from particular classes of neuron, and not from other types of neuron around the graft, which must also have been axotomised. Many types of neuron will only regenerate into a graft if axotomised close to the cell body, although what constitutes a sufficiently proximal axotomy varies between neuronal populations. It is unclear what

determines whether neurons are capable of regenerating into a peripheral nerve graft as no universal phenotypic correlates of this ability have been identified in uninjured neurons, either in terms of morphology, neurotransmitter content, antigenicity, or gene expression. Much remains to be understood, but what is known suggests that a key determinant of the success of axon regeneration into peripheral nerve grafts is the magnitude and nature of the neuronal response to axotomy. The cell body response to axotomy was originally described as a set of morphological and cytological changes (Lieberman, 1971), and since then it has also been shown to be accompanied by characteristic changes in gene expression. Although the exact set of changes may vary, neurons which demonstrate a regenerative response to axotomy typically upregulate the transcription factor c-Jun, GAP-43, particular tubulin isoforms and other growth-associated molecules such as L1 and CHL1, and down-regulate neurofilament proteins and molecules involved in neurotransmission (Bisby and Tetzlaff, 1992; Anderson et al., 1998). These initial changes coincide with the period of sprouting that occurs immediately after a lesion and if regeneration occurs (supported by a nerve graft) then they are usually prolonged; otherwise gene expression returns to normal within about 2 weeks following injury.

Various models have been used for studying differential powers of axonal regeneration and their correlates in terms of gene expression; these will now be discussed.

When a graft is implanted into the thalamus, retrograde labelling from the distal end of the graft reveals that at least 80% of regenerated axons come from neurons of only one nucleus, the thalamic reticular nucleus (TRN) (Benfey et al., 1985; Morrow et al., 1993). This nucleus forms the rostrolateral boundary of the thalamus, and consists of GABA-ergic neurons which project to nuclei in the remainder of the thalamus (the dorsal thalamus), but not outside it (Jones, 1985). Insertion of a graft into the thalamus must necessarily axotomise, in addition to TRN neurons, large numbers of neurons in the dorsal thalamus, corticothalamic projection neurons and afferents from various brainstem nuclei. Regeneration by neurons in most of these sites is poor in comparison to those of the TRN or non-existent. Neurons in and around the suprageniculate nucleus in the dorsal thalamus are also good at regenerating axons into grafts placed sufficiently caudally, and some regeneration is observed from small numbers of cells in the raphé nucleus, the reticular formation and the hypothalamus (Anderson et al., 1998). Small numbers of neurons in

several other nuclei of the dorsal thalamus are sometimes found to regenerate axons, but these neurons' cell bodies are always very close to the graft (Benfey et al., 1985; Morrow et al., 1993).

Successful regeneration by TRN neurons correlates with a strong response to axotomy in the cell body, manifested as a significant upregulation of growth-associated molecules. Thus, many neurons of the TRN upregulate *GAP-43*, *c-jun*, *L1* and *CHL1* mRNAs following implantation of a graft, and immunoreactivity for c-Jun, GAP-43 and L1 also increases in these cells, in axons within the graft or in the surrounding parenchyma (Campbell et al., 1991; Vaudano et al., 1995; Zhang et al., 1995; Vaudano et al., 1998; Chaisuksunt et al., 2000b). This expression lasts for many weeks in neurons with axons in the grafts. A transient upregulation of *GAP-43* and c-Jun also occurs in the TRN following a stab wound or insertion of a freeze-killed nerve graft. Small numbers of neurons in the dorsal thalamus also upregulate these after a stab wound, but the response is weaker and more short-lived than in the TRN. Therefore, in this model there is a good correlation between those neurons which respond to axotomy, with or without a nerve graft, and those which will successfully regenerate axons into a graft. It is also apparent that nerve grafts can maintain the expression of growth associated genes in neurons for long periods, likely to be well beyond the time required for an axon to regenerate through the complete length of the Schwann cell columns.

A similar situation exists when grafts are inserted into the cerebellum. This results in axon regeneration into the grafts by neurons of the cerebellar deep nuclei (the medial, lateral and interposed cerebellar nuclei) but never from Purkinje cells or other neurons of the cerebellar cortex (Dooley and Aguayo, 1982; Chaisuksunt et al., 2000a). Neurons in the brainstem will also regenerate, in particular those in the inferior olivary nucleus and the vestibular nuclei. In the work from this laboratory, grafts have generally been inserted to terminate in the deep nuclei. These nuclei receive projections from Purkinje cells and so these neurons must be axotomised, but regeneration by these neurons has never been observed (Chaisuksunt et al., 2000a).

Again, successful regeneration by neurons of the deep cerebellar nuclei correlates with the upregulation of growth-associated molecules. These neurons upregulate *GAP-43*, *c-jun*,

L1 and *CHL1* after implantation of a graft and may be contrasted with Purkinje cells, which do not regenerate and of these four molecules show only a moderate upregulation of *c-jun*, and that only when axotomised proximally (Chaisuksunt et al., 2000a).

Graft implantation into the striatum provides another model where axotomised neurons show differential powers of regeneration into a nerve graft, although in this case the correlates in terms of gene expression are less consistent with those generally observed elsewhere. The neostriatum receives projections from the cortex and the substantia nigra pars compacta (SNpc), and graft insertion must also axotomise neurons in the neostriatum itself, including projection neurons, cholinergic interneurons and other interneurons. In a study by Woolhead et al. (1998), retrograde labelling revealed that the majority of retrogradely labelled neurons were found in the SNpc or in the globus pallidus, with a minority also found in the neostriatum. Striatal projection neurons and corticostriatal projection neurons therefore appear to be poor at regeneration into a graft. However, in this model, most regenerating neurons of the SNpc fail to upregulate *GAP-43* or *L1*, and only small numbers upregulate *c-jun* (Anderson et al., 1998; Woolhead et al., 1998; Chaisuksunt, 1999). Some striatal neurons, probably the small population of cholinergic interneurons, upregulate *GAP-43* and *c-jun*.

While many CNS neurons will regenerate axons into peripheral nerve grafts, in the forebrain at least they are probably outnumbered by those which will not. Some types of neurons are particularly recalcitrant in this respect, most notably hippocampal neurons and corticospinal neurons. Although there exist reports from one group of regeneration into predegenerated grafts by hippocampal neurons (Lewin-Kowalik et al., 1992; Lewin-Kowalik et al., 1997), it has never been observed in our laboratory or reported elsewhere. Corticospinal neurons are of course particularly relevant to work on spinal cord injuries and again these generally do not regenerate into peripheral nerve grafts in the spinal cord (Richardson et al., 1982a; Ye and Houle, 1997; Blits et al., 2000) although they have been reported to do so once (Cheng et al., 1996).

iii. Proximal versus distal axotomy

In addition to the variation between neurons discussed above, differences in the success of axon regeneration are observed in some types of neuron according to the distance of the axotomy from the cell body. This is also a well-established factor affecting the strength of the cell body response to axotomy (Lieberman, 2001). This phenomenon is clearly demonstrated by the finding that following graft insertion into the spinal cord, intrinsic spinal neurons which had grown axons into the grafts were generally located close to the graft tip, within 3-4mm (David and Aguayo, 1981). Most of the axons which form the long fibre tracts in the spinal cord appear to respond to axotomy in a distance-dependent manner (Richardson et al., 1984). Segments of nerve were inserted into the cord at different levels and neurons which had grown axons into the grafts were identified by retrograde labelling. Following insertion of the nerve graft at low cervical level, labelled neurons were found in brainstem nuclei, in particular the red nucleus, and in the thoracolumbar spinal cord. Grafts inserted at the high cervical level induced larger numbers of neurons in the brainstem to regenerate but virtually no neurons in the thoracolumbar cord. Conversely, grafts inserted at midthoracic or lumbar level resulted in very few or no brainstem neurons being retrogradely labelled from the graft. Thus for both rubrospinal neurons and spinal projection neurons, the most proximal injury is the most effective at eliciting regeneration.

Retinal ganglion cells also show similar behaviour. Grafting of a peripheral nerve to a severed optic nerve 2mm from the eye resulted in successful regeneration by retinal ganglion cell axons, but at a distance of 7mm the response was poor (Richardson et al., 1982b; Berry et al., 1986). It seems that many neurons respond strongly enough to regenerate only if axotomised within a critical distance of the cell body, however it should be noted that this distance varies widely between different classes of neurons.

For several of these results, changes in gene expression can be correlated with successful regeneration. Thus rubrospinal neurons have been reported to upregulate c-Jun and the mRNAs for GAP-43 and tubulin, and to downregulate neurofilament mRNAs following a lesion to the lateral spinal cord at cervical level, but not at thoracic level (Tetzlaff et al., 1991; Jenkins et al., 1993a; Fernandes et al., 1999). Retinal ganglion cells upregulate c-Jun

and GAP-43 after optic nerve injury if the injury is 2-3mm from the eye, but not at 6-7 mm (Doster et al., 1991; Hull and Bahr, 1994).

iv. The response of neurons of the peripheral nervous system to axotomy

Following peripheral nerve injury, there is extensive regrowth of axons and reinnervation of target tissues may occur. While the success of such regeneration is partly due to the trophic and structural support of Schwann cells in the distal stump, PNS neurons also show a strong cell body response to axotomy, which includes a manifold increase in the production of c-Jun (Jenkins and Hunt, 1991) and GAP-43 protein and message (van der Zee et al., 1989; Woolf et al., 1990; Chong et al., 1992; Schreyer and Skene, 1993).

The changes in production of various cytoskeletal components which take place in peripheral neurons after axotomy have also been well described. Thus α- and β-tubulins genes and proteins are upregulated in neurons of the superior cervical ganglion, the facial nucleus and lumbar motor and sensory neurons following axotomy (Quesada et al., 1986; Hoffman et al., 1987; Hoffman and Cleveland, 1988; Tetzlaff et al., 1988; Miller et al., 1989; Muma et al., 1990). In particular, the Tα1 and TβII isoforms of tubulin are upregulated (Hoffman and Cleveland, 1988; Miller et al., 1989). Neurofilaments are down-regulated following axotomy (Hoffman et al., 1987; Wong and Oblinger, 1987; Tetzlaff et al., 1988; Greenberg and Lasek, 1988; Oblinger and Lasek, 1988; Muma et al., 1990). These changes persist until regeneration is complete and target tissues are reinnervated.

The importance of the neuronal response to axotomy in determining the degree of regeneration which may result is most clearly observed in experiments on the central branches of dorsal root ganglion neurons. Curiously, injury of the dorsal roots elicits a very small response in the cell body, reflected by a smaller and more transient upregulation of c-Jun and essentially no increase in GAP-43 (Schreyer and Skene, 1993; Jenkins et al., 1993b; Chong et al., 1994a; Kenney and Kocsis, 1997). Downregulation of neurofilament is also less than after peripheral axotomy (Greenberg and Lasek, 1988; Oblinger and Lasek, 1988). Central axotomy also results in a relatively poor response in terms of regeneration but a concomitant injury of the peripheral branch of the appropriate nerve,

which of course induces greater changes in gene expression, results in much more vigorous regeneration of the central axon. Several experiments have illustrated this point.

Richardson and Verge (1987) showed that peripheral axotomy accelerates axonal regeneration in the injured dorsal root. Animals were given bilateral crush injuries to the L5 dorsal root, and a sciatic nerve crush on one side. Contralateral to the sciatic nerve injury, axons in the dorsal root regenerated at a rate of 1 mm per day, but on the ipsilateral side growth was accelerated approximately 3-fold. Similarly, when the L4 dorsal root was transected and a peripheral nerve segment anastomosed, many more dorsal root axons regenerated through the graft when the sciatic nerve was injured (Chong et al., 1996). The central processes of primary sensory afferents which ascend in the dorsal columns of the spinal cord will also regenerate into a peripheral nerve graft inserted into the cord. However, such regeneration is achieved by only a small proportion of injured sensory neurons unless the peripheral processes of these neurons are also injured. This procedure increased the numbers of axons invading the graft by up to a hundred fold (Richardson and Issa, 1984; Oudega et al., 1994). Lastly, following a transection of the dorsal columns but in the absence of a peripheral injury, axons show minimal sprouting and invasion of the lesion and stop at the injury site. A sciatic nerve injury at the time of, or prior to, the spinal cord lesion provoked considerable growth into and in some cases beyond the lesion, as well as through grey matter around the lesion (Bavetta et al., 1999; Neumann and Woolf, 1999).

v. Cell death

Another aspect of the neuronal response to axotomy which deserves mention is the propensity towards cell death which it also includes. Axotomy of some populations of CNS neurons results in death of a significant proportion of the axotomised cells and in these neurons, cell death and the vigour of the regenerative response are both correlated with the distance of the axotomy from the cell body. For example, at least 90% of retinal ganglion cells die following intraorbital axotomy (Sievers et al., 1987; Sievers et al., 1989) but after intracranial axotomy the survival rate is significantly higher, at least initially (Misantone et al., 1984; Aguayo, 1985; Hull and Bahr, 1994) but the vigour of regeneration is reduced (Richardson et al., 1982b). In the spinal cord, 30% of neurons in

Clarke's nucleus die after axotomy at L1, but the death rate falls to 15% when the axotomy is performed at C5 (Loewy and Schader, 1977). Death rates of septal cholinergic neurons after transection of the fimbria-fornix also vary with the distance of the cells from the lesion (Sofroniew and Isacson, 1988). It appears that in many cases the same stimulus which is required for successful regeneration of the axon also increases the likelihood that the neuron will die. This may be a consequence of the increased expression of c-Jun in injured neurons, as this molecule has also been implicated in both regeneration and apoptosis (Ham et al., 1995; Herdegen et al., 1997).

vi. Summary

Broadly speaking it appears that those neurons which are capable of regenerating axons into nerve grafts respond to axotomy with changes in gene expression, although there are variations and exceptions among different classes of neurons. Those which are not capable generally show little or no response to axotomy. In those neurons in which successful regeneration into a graft requires a sufficiently proximal axotomy, the magnitude of the cell body response and the associated changes in expression of these genes also varies with the proximity of the axotomy to the cell body. It appears that in order to regenerate an axon into a peripheral nerve graft, a neuron must be stimulated strongly enough by the axotomy to initiate a program of axon regeneration. The neuronal cell-types which fail to regenerate into peripheral nerve grafts also fail to upregulate any of the growth-associated molecules mentioned above, although it should be noted that in many cases they express them at a low level in the uninjured animal. Clearly the nature and strength of the neuronal response to axotomy is a major factor in determining whether any attempt at regeneration is made and whether it is successful. Some of the molecules expressed as part of this response, such as GAP-43 and L1, are expected to play a direct role in axon elongation or pathfinding processes in the growth cone. It would therefore be useful to establish which genes are upregulated by neurons regenerating their axons. Characterisation of the changes in gene expression following axotomy and during regeneration would aid our understanding of what molecules may be necessary for a neuron to successfully regenerate an axon, allow studies into how the expression of these molecules is controlled and perhaps lead to approaches where regeneration may be enhanced by the manipulation of gene expression. Therefore, the experiments described in Chapters 3-6 were designed to

establish whether three candidate molecules, CAP-23, SCG10 and FKBP12 are upregulated by neurons in response to axotomy and during axonal regeneration.

While the properties of the CNS environment which may make it unfavourable to regeneration have been thoroughly investigated, and several strategies have been developed to try to overcome these, there have been surprisingly few studies where regeneration has been promoted by enhancing the expression of genes for growth-associated molecules. Studies of transgenic mice which overexpressed GAP-43 in neurons of the dorsal thalamus or in Purkinje cells failed to reveal greater regeneration of these cells' axons into peripheral nerve grafts or Schwann cell implants (Buffo et al., 1997; Mason et al., 2000). Very recently, Bomze et al. (2001) reported overexpression of GAP-43 and CAP-23 in primary sensory neurons enhanced regeneration of dorsal column axons into peripheral nerve grafts. The potential for such an approach has been clear since the effects of conditioning peripheral lesions on regeneration of DRG central axons was established (Richardson and Issa, 1984; Richardson and Verge, 1986; Richardson and Verge, 1987). The more recent experiments by Neumann and Woolf (1999), which resulted in significant dorsal column fibre growth through a spinal cord lesion if the primary sensory neurons were stimulated by a conditioning peripheral lesion, further demonstrate this. Therefore, the experiments described in Chapter 7 were designed to evaluate the extent to which overexpression of GAP-43 in primary sensory neurons of transgenic mice might compensate for the lack of a cell body response following injury to the central axon branches of these neurons.

1.5 Molecules investigated

A number of molecules have been examined in the studies presented here. In Chapters 3-6, SCG10, CAP-23 and FKBP12 have been studied as candidates for regeneration-associated molecules. The well established growth-related molecule GAP-43 has been examined as a control for these experiments.

1. GAP-43

i, GAP-43, an established axon growth associated molecule

GAP-43 was one of the first growth-associated molecules to be identified in regenerating axons (Skene and Willard, 1981; reviewed by Skene, 1989; Benowitz and Routtenberg, 1997), although it was first identified as a presynaptic phosphoprotein (Ehrlich and Routtenberg, 1974; Zwiers et al., 1976). It is a major component of growth cones in developing and regenerating neurons, where it comprises about 1% of total protein. Its expression correlates well with axon growth and plasticity and while its function has remained somewhat unclear there is accumulating evidence that GAP-43 lies on the pathway between the receptors for extracellular guidance cues and regulation of the cytoskeleton in the growth cone. It also has apparent roles in such processes as long-term potentiation and synaptic vesicle release (see Oestreicher et al., 1997, for a review of all aspects of GAP-43). Other names which have been used for GAP-43 are B-50, neuromodulin, pp46 and F1.

ii. Expression by developing and regenerating neurons

GAP-43 is highly expressed by neurons in the developing mammalian nervous system during the period of axogenesis. In the developing rat brain, GAP-43 expression is very low while neuronal precursors divide and migrate, but at the onset of neurite outgrowth high-levels of GAP-43 protein become detectable along the developing axons. Thereafter, GAP-43 expression remains strong in the neuropil until synaptogenesis is completed (Neve et al., 1987; Dani et al., 1991; Chong et al., 1992). GAP-43 is then down-regulated in nearly all neurons, although low levels persist in most neurons in the adult, with higher

levels in restricted sets of cells, particularly in areas believed to show high levels of plasticity, such as hippocampus and the association areas of human neocortex (Neve et al., 1988; McGuire et al., 1988; Meberg and Routtenberg, 1991; reviewed by Benowitz and Routtenberg, 1997). Similarly, during peripheral nerve development, DRG neurons express GAP-43 while they are growing their axons, beginning soon after the cells become post-mitotic and continuing until postnatal day 4 (Biffo et al., 1990; Chong et al., 1992). A subset of DRG neurons, predominantly smaller cells, retains moderate GAP-43 expression in the adult.

GAP-43 expression in neurons of adult animals correlates very well with axon regeneration. Neurons with axons in peripheral nerves upregulate GAP-43 soon after axonal injury and expression continues throughout the period of axon regeneration and the re-formation of synaptic contacts. Following a sciatic nerve crush, GAP-43 expression in DRG neurons is up-regulated 8-16 hours after the crush, and in motor neurons is detectable by 24 hours (van der Zee et al., 1989; Woolf et al., 1990; Chong et al., 1992). Expression persists for at least 2 weeks, but if reconnection with targets is prevented by cutting and ligating the sciatic nerve, expression is maintained for much longer periods (Chong et al., 1994b).

GAP-43 expression also correlates with CNS regeneration in lower vertebrates. In the toad and goldfish, regenerating retinal ganglion cells begin to express GAP-43 4 days after axotomy (Benowitz et al., 1981; Skene and Willard, 1981), which is shortly before the onset of axon regeneration (Landreth and Agranoff, 1976).

GAP-43 is also upregulated by nearly all CNS neurons regenerating axons into peripheral nerve grafts. Neurons of the thalamic reticular nucleus, and the substantia nigra pars compacta and deep cerebellar nuclei all express high levels of *GAP-43* mRNA when regenerating axons into a peripheral nerve graft inserted into the thalamus, cerebellum or striatum respectively (Vaudano et al., 1995; Woolhead et al., 1998; Chaisuksunt et al., 2000a). The mRNA is upregulated in regenerating neurons in each case, with the exception of neurons in the substantia nigra pars compacta where *GAP-43* is expressed at a high constitutive level but there is no detectable upregulation. GAP-43 protein is also detectable in axons within the grafts (Campbell et al., 1991). *GAP-43* upregulation lasts

at least 6 weeks, probably reflecting the fact that no synaptic targets are available in the grafts for the regenerating axons to contact. Neurons such as Purkinje cells, which do not regenerate axons into grafts, do not upregulate *GAP-43*. Insertion of freeze-killed grafts into the brain does not elicit a long-lasting upregulation of *GAP-43* and axons do not regenerate into these grafts (Chaisuksunt et al., 2000a).

Thus, no neurons have been shown to regenerate axons in vivo without expressing GAP-43 and, although the characteristics of the cell body response to axotomy varies between neurons (Anderson et al., 1998), the upregulation of GAP-43 expression is one of the most constant markers of successfully regenerating cells. The high degree of correlation between GAP-43 expression and axon growth and regeneration suggests a role for GAP-43 in axon elongation towards targets.

iii. In vitro studies

In vitro studies on the effect of GAP-43 also suggest it has a direct role in neurite outgrowth. Antisense oligonucleotides to *GAP-43* mRNA inhibit neurite outgrowth by PC12 cells (Jap Tjoen et al., 1992), block outgrowth by embryonic chick sensory neurons on poly-L-lysine, and result in atrophied growth-cones on more permissive substrates (Aigner and Caroni, 1993; Aigner and Caroni, 1995). Similarly, axon growth by neuroblastoma cells is prevented or attenuated by transfection with anti-GAP-43 antibodies (Shea et al., 1991). Depletion of GAP-43 also leads to a deficiency in growth-factor induced spreading and branching of the growth cone (Aigner and Caroni, 1995). However, cultured DRG neurons from GAP-43 knockout mice (see part *v*) grow axons at the same speed as wild-type neurons.

Conversely, overexpression of GAP-43 in non-neuronal cells can be enough to induce the formation of neurite-like processes and growth-cone-like structures (Zuber et al., 1989; Widmer and Caroni, 1993; Nielander et al., 1993; Verhaagen et al., 1994). GAP-43 overexpression also potentiates NGF-induced neurite outgrowth in PC12 cells (Yankner et al., 1990).

iv. In vivo studies with transgenic mice

The most convincing evidence that GAP-43 is not essential for axon growth comes from studies of GAP-43 knockout mice (Strittmatter et al., 1995). These animals develop a nervous system described as grossly normal, although some serious misrouting of axons was discovered. The effect of the loss of GAP-43 on pathfinding was demonstrated at the optic chiasm, a well-defined decision point where growing axons must choose between entering the ipsilateral or contralateral optic tracts. At P1 most axons in the GAP-43-deficient animals had failed to cross the optic chiasm, in contrast to wild-type animals. Most axons continued to grow within the chiasm in apparently random directions. Those axons which had crossed the chiasm were randomly distributed between the two tracts, and showed no sign of the normal correlation between path choice and the position of the retinal ganglion cell perikarya in the retina (Sretavan and Kruger, 1998). By P15, however, many retinal ganglion cell axons had grown into the optic tracts, although there was still an abnormally large proportion growing ipsilaterally, and many axons growing aberrantly into the contralateral optic nerve.

Studies on a second strain of GAP-43 (-/-) mice revealed that retinal ganglion cell axons also failed to form normal terminal fields in the lateral geniculate nuclei, suggesting the GAP-43 deficient axons had failed to invade these regions (Zhu and Julien, 1999). It has also been found that the anterior and hippocampal commisures and the corpus callosum do not form in GAP-43 knockout mice (Shen et al., 2000) and these animals also exhibit a disrupted cortical somatotopic map (Maier et al., 1999).

These data all suggest that GAP-43 is not essential for axonal growth and, in combination with the results of in vitro studies, lead to the alternative idea that it may play a major role in axonal pathfinding. This is consistent with data linking GAP-43 to cell recognition molecules, second-messenger systems and actin cytoskeleton dynamics in the growth-cone (see parts *vii-ix* of this section).

Transgenic mice which overexpress GAP-43 in neurons in the adult have also been generated (Aigner et al., 1995). Several strains of animals were generated expressing either mouse or chick GAP-43 under the control of the modified Thy-1 promoter, which

drives expression in a neuron-specific manner, commencing postnatally. Spontaneous sprouting of motor axons was found at the neuromuscular junctions of unoperated animals and axonal sprouting in response to botulinum-toxin induced paralysis was enhanced. Hippocampal mossy fibres also showed aberrant sprouting in the transgenic animals. The authors also reported that the speed of peripheral nerve regeneration was increased. Again, this evidence supports the view that GAP-43 promotes axonal growth.

v. GAP-43 expression without regeneration

While GAP-43 is almost certainly important for axon growth and accurate pathfinding, it is not necessarily sufficient on its own to promote regeneration into peripheral nerve grafts or Schwann cell implants in neurons which will not normally do so. Many CNS neurons which do not regenerate into peripheral nerve grafts express GAP-43 at a low or moderate level in the intact animal, and experimentally induced expression of GAP-43 is insufficient to induce regeneration.

In the thalamus, neurons of the dorsal thalamus are poor at regenerating into nerve grafts, and those in some nuclei will almost never do so (Vaudano et al., 1995). These neurons constitutively express moderate levels of GAP-43, although they fail to upregulate it following axotomy. Overexpression of GAP-43 in these neurons in transgenic mice does not enable them to regenerate axons into peripheral nerve grafts (Mason et al., 2000).

Pyramidal cells of the neocortex fail to regenerate into peripheral nerve grafts in the spinal cord (Richardson et al., 1982a; Richardson et al., 1984; Blits et al., 2000) although they constitutively express GAP-43. Purkinje cells, on the other hand, do not regenerate axons into nerve grafts and neither express GAP-43 normally nor upregulate it following injury (Vaudano et al., 1998; Chaisuksunt et al., 2000a), so it was thought this might account for their failure to regenerate. However, Purkinje cells of transgenic mice which express GAP-43 also fail to regenerate axons even into an apparently favourable environment provided by Schwann cell grafts (Buffo et al., 1997). Clearly, GAP-43 has additional functions in adult neurons, most probably related to synaptic activity.

vi. Interactions with second messenger systems and the cytoskeleton

The precise mechanism of action of GAP-43 in the complex pathways controlling growth cone behaviour is unclear, but it has several properties which might allow it to intervene between the cell membrane and the cytoskeleton. GAP-43 interacts with several second-messenger systems in the growth cone, including protein kinase C (PKC), the G-protein G_0, and the calcium-binding-protein calmodulin (Benowitz and Routtenberg, 1997). An important feature of GAP-43 is its association with the cytoskeletal elements actin, α-actinin, talin and fodrin (Moss et al., 1990; Meiri and Gordon-Weeks, 1990). Expression of GAP-43 in COS cells causes the formation of filopodia, and in these circumstances GAP-43 associates with filamentous actin (Widmer and Caroni, 1993). Interaction between GAP-43 and actin has also been demonstrated in cell-free systems (Hens et al., 1993; He et al., 1997).

vii. Phosphorylation and membrane linkage

GAP-43 activity appears to be regulated at transcriptional and post-transcriptional levels. Its activity is influenced by phosphorylation (it is the major neuronal substrate for protein kinase C) and it is also possible that there is some control of its membrane association. GAP-43 contains a short amino-acid sequence which is subject to phosphorylation by PKC at serine 41, and also binds calmodulin. These two functions are mutually exclusive, i.e. phosphorylated GAP-43 cannot bind calmodulin and calmodulin-bound-GAP-43 cannot be phosphorylated (Alexander et al., 1987). This property suggests the domain functions as a two-way switch, such that the phosphorylation/calmodulin-binding status of GAP-43 regulates its activity.

Several lines of evidence indicate that the phosphorylation status of GAP-43 affects its activity. Mutation of the phosphorylation site of GAP-43 such that it can no longer be phosphorylated reduced its association with the membrane cytoskeleton (Meiri et al., 1996) and overexpression of this form of GAP-43 in transgenic mice induced less sprouting at the neuromuscular junction than did wild-type GAP-43 (Aigner et al., 1995). The properties of each form with respect to actin binding were investigated by He et al. (1997). Both forms of GAP-43 bind F-actin, but phosphorylated GAP-43 stabilised long

actin filaments, whereas calmodulin-bound dephosphorylated GAP-43 inhibited actin polymerisation. The phosphorylation state of GAP-43 in growth cones and its relationship with growth-cone behaviour was investigated by the same group (Dent and Meiri, 1998), using double-labelling immunofluorescence with one antibody which was specific for phosphorylated GAP-43 and one which detected GAP-43 regardless of its phosphorylation state. They found that while GAP-43 was distributed fairly uniformly throughout growth-cones, phosphorylated GAP-43 was often found in localised areas, particularly in the shaft of the neurite proximal to the growth-cone and in the lamellae, or distinct areas of them. High levels of GAP-43 phosphorylation were associated with lamellar expansion and with stability of both the neurite shaft and the central region of the growth cone, while low levels were found during retraction of lamellae, and during retraction of the neurite shaft and the central region of the growth cone. This supports the idea that GAP-43 is involved in the regulation of such cytoskeletal activity in the growth cone as is necessary for motility and adhesion, in a manner determined by its phosphorylation status. However, phosphorylation status has been found to be unimportant in at least one model: Aarts et al. (1998) demonstrated that GAP-43 promoted the formation of filopodia in fibroblasts but found that the effects of phosphorylation site mutants of GAP-43 were indistinguishable from those of the wild-type protein.

Membrane attachment of GAP-43 is mediated by the N-terminal domain (Liu et al., 1991; Liu et al., 1994) which contains two cysteine residues (Cys3 and Cys4). These are reversibly modified by the addition of palmitic acid (Skene and Virag, 1989) and palmitoylation is required for membrane targeting (Liu et al., 1993; Aarts et al., 1995) and association with the cortical cytoskeleton (Wiederkehr et al., 1997). GAP-43 is specifically localised to 'raft' membrane microdomains (Maekawa et al., 1997; Aarts et al., 1999; Laux et al., 2000), cholesterol rich regions which are resistant to detergent extraction and contain high concentrations of receptors and signalling molecules (reviewed by Simons and Toomre, 2000). Targetting of GAP-43 to rafts requires other parts of the N-terminal domain in addition to the palmitoylated cysteine residues (Wiederkehr et al., 1997). There is some evidence that GAP-43 activity may also be controlled by regulation of its attachment to the plasma membrane. Activation of L-type voltage-sensitive calcium-channels in growth cones in vitro results in phosphorylation of GAP-43 and the release of GAP-43 from the membrane (Fukura et al., 1996; Ohbayashi et al., 1998). However,

phosphorylation of GAP-43 in itself does not lead to dissociation from the membrane (Wiederkehr et al., 1997; Laux et al., 2000). Both membrane-bound and cytosolic GAP-43 co-exist in the growth cone in vivo (Skene and Virag, 1989). That palmitoylation-mediated membrane attachment may be of importance for GAP-43 activity was shown by Aarts et al. (1998) who found that GAP-43-induced formation of filopodia was abolished by mutation of the Cys3 and Cys4, which prevents acylation. Palmitoylation status has been proposed to regulate GAP-43 membrane binding (Sudo et al., 1992) but there is little evidence for active regulation of palmitoylation in vivo.

viii. Mechanism of action of GAP-43

Evidence has been found for at least 5 mechanisms by which GAP-43 might act in the growth-cone:

1. Regulation of calmodulin availability. Because only non-phosphorylated GAP-43 binds calmodulin, it has been proposed that GAP-43 acts by regulating calmodulin availability (Liu and Storm, 1990; Estep et al., 1990). In support of this idea, Slemmon and Martzen (1994) demonstrated that in low physiological concentrations of Ca^{2+} GAP-43 inhibited nitric oxide synthase (NOS), a calmodulin target. NOS inhibition was abolished by increased Ca^{2+} concentration or phosphorylation of GAP-43, both of which cause calmodulin to dissociate from GAP-43 (Andreasen et al., 1983; Cimler et al., 1985; Alexander et al., 1987).

2. Regulation of G-protein activity. Strittmater et al. 1990 showed that GAP-43 stimulates the heterotrimeric G-protein G_0. However, activated G-proteins such as G_0 generally inhibit growth-cone motility (Igarashi et al., 1993), whereas GAP-43 generally promotes it. This contradiction may be explained by the finding that palmitoylation prevents the action of GAP-43 on G_0 (Sudo et al., 1992); whether non-palmitoylated GAP-43 (which may be free of the membrane) may have a distinct role in the growth-cone is unclear.

3. Control of actin polymerisation via direct interactions. The association of GAP-43 with actin in vivo and its binding of actin in vitro have been well-demonstrated (Moss et al., 1990; Meiri and Gordon-Weeks, 1990; Strittmatter et al., 1992; Hens et al., 1993; He et

al., 1997). As already mentioned, He et al. (1997) also showed that GAP-43 affects actin polymerisation in different ways depending on its phosphorylation/calmodulin binding status. Phospho-GAP-43 favoured stabilisation of long actin filaments while dephospho-GAP-43 inhibited extension of short filaments. The authors went on to suggest that GAP-43 functions as a membrane-bound actin barbed-end capping protein, which regulates actin polymerisation, and consequently controls actin filament extension away from the membrane.

4. Control of cytoskeletal dynamics via Rho-type GTPases. These are a group of molecules involved in a very wide range of cellular processes (see Hall and Nobes, 2000 and Luo, 2000 for review), and the exact function of each one depends on the context in which they are active. Commonly, Rho-type GTPases regulate assembly and disassembly of cytoskeletal components. Aarts et al. (1998) demonstrated that vector driven expression of GAP-43 in the Rat1 fibroblast cell line caused a significant increase in filopodia formation, and showed not only that this was dependent on palmitoylation (see part *viii* above), but that in this model GAP-43 acts through the small GTPase Rho. A dominant negative mutant of Rho prevented the morphological actions of GAP-43, while a constitutively active mutant of Rho mimicked them.

5. Control of actin dynamics by regulating the availability of the lipid second messenger $PI(4,5)P_2$. This mechanism was proposed by Laux et al. (2000) for both GAP-43 and CAP-23 and is discussed in the section on CAP-23, below.

Each of these mechanisms may be important for GAP-43 function, but the relative importance of each one remains to discovered.

ix. Other information

A dominant-negative form of GAP-43 was recently generated (Laux et al., 2000). This protein lacks the domain which contains the calmodulin-binding activity and the protein kinase C phosphorylation sites. Expression of dominant-negative GAP-43 in PC12 cells prevented neurite outgrowth in response to NGF. Transgenic mice expressing this protein in neurons showed a reduction in the speed of peripheral nerve regeneraton following a

crush injury, although there was no difference in the final extent of reinnervation of the muscles. In addition, the dominant negative GAP-43 greatly inhibited botulinum-toxin induced sprouting at the neuromuscular junction of the soleus muscle. Surprisingly, however, sprouting was induced at synapses in the medial gastrocnemius muscle. These synapses do not sprout greatly in wild-type animals.

Meiri et al. (1998) found convincing evidence that GAP-43 acts on a pathway between cell-adhesion molecules and growth cone motility. The promotion of neurite outgrowth by the NCAM was abolished in neurons from GAP-43 knockout mice, while integrin-mediated neurite outgrowth was normal. Outgrowth on NCAM induced phosphorylation of GAP-43, as did the presence of solubilised forms of both NCAM and L1. Activation of the FGF receptor or pharmacological activation of the arachidonic acid signalling cascade, both of which are thought to mediate signalling by these cell-adhesion molecules, also led to GAP-43 phosphorylation.

Additional data on GAP-43 will be discussed in the following section on CAP-23.

2. CAP-23

i. CAP-23: a molecule with similar properties to GAP-43

CAP-23, also known as NAP-22 or BASP-1, was first identified in the chick by Widmer and Caroni (1990). Subsequently, the rat, bovine and human forms were also identified independently (Maekawa et al., 1993; Mosevitsky et al., 1994; Mosevitsky et al., 1997). The similarities between CAP-23 and GAP-43, described below, suggest that the two molecules may have similar functions and that CAP-23 may also have an important role in axon growth during development, regeneration and in circumstances of synaptic plasticity. It was first isolated from a protein fraction enriched in elements of the cortical cytoskeletal (which underlies the plasma membrane) and was identified by its anamolous behaviour on polyacrylamide gels, a property it shares with GAP-43 (Widmer and Caroni, 1990). CAP-23 is expressed by all tissues during development but declines in most tissues, until at birth it persists only in the nervous system and, to a lesser degree, in the intestines (Widmer and Caroni, 1990). In 8-week old rats its expression was brain-specific

(Maekawa et al., 1993). CAP-23, GAP-43 and a third molecule, MARCKS (myristoylated alanine-rich protein kinase C substrate), share a number of biochemical properties (Maekawa et al., 1993), and have been regarded as a distinct functional group of membrane-associated cytoskeleton-modulating molecules (Wiederkehr et al., 1997; Frey et al., 2000; Laux et al., 2000). These shared properties are: an unusual amino acid composition; they are highly hydrophilic; they are protein kinase C substrates; they are bound by calmodulin; and they are membrane-linked via acylation. Some sequence similarity was detected between CAP-23 and GAP-43 (Widmer and Caroni, 1990; Maekawa et al., 1993) but was ascribed by the former to the similar unusual amino acid composition.

ii. Biochemical properties

GAP-43 is membrane-linked via palmitoylated cysteine residues, and the analogous modification of CAP-23 is myristoylation at the N-terminal glycine following cleavage of the initial methionine (Maekawa et al., 1994a; Takasaki et al., 1999). As there are no hydrophobic domains in the amino acid sequence, the hydrophobic myristate group and the N-terminus of the protein are thought to be responsible for the membrane localisation of CAP-23 (Maekawa et al., 1994a; Mosevitsky et al., 1997). Unlike GAP-43, which exists in both acylated and non-acylated forms in the growth cone (Skene and Virag, 1989), all or almost all CAP-23 is apparently myristoylated (Mosevitsky et al., 1997).

Like GAP-43, CAP-23 also localises to raft fractions of neuronal membrane (Maekawa et al., 1997; Maekawa et al., 1999; Laux et al., 2000). These two proteins were found in the same detergent-resistant fraction, along with subunits of G-proteins and the tyrosine kinases src and fyn (Maekawa et al., 1997). In fact, GAP-43, CAP-23 and MARCKS are all associated with plasmalemmal rafts and colocalise at the plasma membrane (Laux et al., 2000) and may be involved in raft assembly (Maekawa et al., 1999; Laux et al., 2000). Most of the CAP-23 protein was associated with rafts, whereas a significant proportion of GAP-43 was not.

The myristoylated N-terminal region also contains the calmodulin binding site and the PKC phosphorylation site on Ser6 (Maekawa et al., 1994b; Takasaki et al., 1999). Consistent

with this, myristoylation appears to be essential for calmodulin-binding and enhances phosphorylation by protein-kinase C (Takasaki et al., 1999). As with GAP-43, phosphorylation at this site and calmodulin-binding are mutually exclusive (Maekawa et al., 1994b). However, both calmodulin binding and phosphorylation have been reported to interfere with membrane binding. Calmodulin binding caused dissociation of CAP-23 from liposomes and from brain raft fraction (Maekawa et al., 1999), and Laux et al. (2000) reported that, unlike GAP-43, CAP-23 dissociates from the membrane in response to phoshorylation. Somewhat inconsistent with this is the observation that in vivo, most CAP-23 is membrane associated (Maekawa et al., 1997). While it is tempting to assume that phosphorylation of CAP-23 is dynamically regulated as is that of GAP-43, it should be noted that physiological phosphorylation of CAP-23 has not been demonstrated in vivo, and analysis of extracted brain protein indicated that no or almost no CAP-23 was phosphorylated by PKC at Ser5 (Mosevitsky et al., 1997). However, in cultured embryonic chick spinal cord neurons and in cultured brain slices, CAP-23 was phosphorylated by pharmacological activation of PKC (Widmer and Caroni, 1990; Maekawa et al., 1994b).

Further biochemical differences have been identified between GAP-43 and CAP-23: GAP-43 binds calmodulin at lower Ca^{2+} concentration (Andreasen et al., 1983; Cimler et al., 1985), whereas CAP-23 binding of calmodulin requires higher Ca^{2+} concentration (Maekawa et al., 1993); a co-sedimentation assay demonstrated little association of CAP-23 with actin (Maekawa et al., 1993), unlike GAP-43 (Hens et al., 1993; He et al., 1997); lastly, CAP-23 contains no domain equivalent to the G_0-interacting domain of GAP-43 (Maekawa et al., 1993), and no such interaction has been reported. CAP -23 also shows different subcellular localisation to GAP-43 in primary neuronal cultures, being found in dendrites and the cell-body as well as the axon (Widmer and Caroni, 1990; Maekawa et al., 1993).

iii. Function

Functional similarity between GAP-43, CAP-23 and MARCKS was demonstrated in vitro. In the growth cones of cultured neurons and in transfected non-neuronal cells, GAP-43, CAP-23 and MARCKS showed a high degree of colocalisation, and are distributed in a

characteristic punctate pattern (Wiederkehr et al., 1997). Vector-driven expression of these three proteins in non-neuronal cells all caused similar changes at the cell surface, including the formation of blebs, filopodia and long processes. CAP-23 and MARCKS potentiated NGF-induced neurite-outgrowth in PC12 cells to the same extent as GAP-43 (Yankner et al., 1990; Laux et al., 2000).

Transgenic mice which overexpress CAP-23 have been generated (Caroni et al., 1997). These animals express CAP-23 in the spinal motor neurons of the adult, and showed a similar phenotype to mice overexpressing GAP-43, namely spontaneous sprouting at the neuromuscular junction and a potentiation of sprouting caused by botulinum-toxin induced paralysis. Sprouts induced by CAP-23 were longer but less numerous than those induced by GAP-43, and fewer growth-cone-like structures were detected. Double transgenic animals expressing GAP-43 and CAP-23 showed much more sprouting than either of the individual lines, suggesting the effects of the two proteins are additive in these circumstances. As had been shown for GAP-43, CAP-23 expression also enhanced the immediate regenerative sprouting response to sciatic nerve injury (before the arrival of lesion-induced proteins transported from the cell body). An increase in synaptic density at the neuromuscular junction was found in both lines of single transgenic mice. However, in contrast to GAP-43 (Aigner et al., 1995), CAP-23 failed to induce sprouting of hippocampal mossy fibres, despite strong expression in the dentate gyrus.

The possibility of functional similarity between CAP-23 and GAP-43 was explored by Frey et al. (2000). The authors generated mice with null mutations at the CAP-23 allele, and further strains in which the GAP-43 gene was knocked in to the CAP-23 locus ($CAP23^{GAP-43/GAP-43}$), or in which CAP-23 expression was driven by the adult and neuron specific Thy1 promoter. CAP-23 knockout animals showed increased neonatal mortality. No analysis was made of axonal projections and possible misrouting in these animals, but they exhibited enlarged ventricles and axonal and synaptic ultrastructural abnormalites in the CNS, and morphological abnormalities at nerve terminals. The authors also reported a deficit in sprouting at the neuromuscular junction in response to botulinum-toxin induced paralysis. (A similar phenotype was reported for GAP-43 knockout mice.) This phenotype was rescued by Thy1-driven expression of either CAP-23 or GAP-43, suggesting a high degree of functional redundancy between the two molecules. Animals which were

homozygous for the CAP-23 null mutation and the GAP-43 knock-in at the CAP-23 locus were essentially rescued with respect to all aspects of the phenotype described above.

Cultured neurons from the CAP-23 knockout animals showed complex deficits in neurite outgrowth, including reduced growth-cone stability, thinner and more winding processes, or shorter processes. Some of these deficits were restored in neurons taken from the $CAP23^{GAP\text{-}43/GAP\text{-}43}$ animals. In addition, some aspects of this phenotype were induced in wild-type neurons by cytochalasin D, which inhibits actin polymerisation, implicating CAP-23 (and GAP-43) in control of actin dynamics.

One observation that has been made by Frey et al. (2000) is that although GAP-43 appears able to substitute for CAP-23 functionally, it tends to promote neurite growth with a higher frequency of branching than does CAP-23. This was based on observations of the $CAP23^{GAP\text{-}43/GAP\text{-}43}$ animals as well as a comparison of the animals which overexpress either GAP-43 or CAP-23.

Less data is available for CAP-23 than for GAP-43 (for example, at present, there is no data on possible effects of CAP-23 on actin polymerisation) but the functional redundancy between these two molecules supports the notion that they act via similar mechanisms. It seems reasonable, therefore, to suppose that some of the information gathered about GAP-43 may also be broadly applicable to CAP-23.

iv. Proposed mechanism of action

Laux et al. (2000) presented evidence that GAP-43, CAP-23 and MARCKS may act through the lipid second messenger phosphatidyl inositol (4,5) bisphosphate (PI $(4,5)P_2$). Overexpression of any of these 3 proteins promoted PI $(4,5)P_2$ retention at plasmalemmal rafts, a property which was dependent on the presence of a full effector domain (the calmodulin-binding domain/PKC phosphorylation site). The authors suggest that these proteins act by binding PI $(4,5)P_2$ and regulating its availability, and that PI $(4,5)P_2$ in turn regulates other molecules which control the actin cytoskeleton. Agents which lower plasmalemmal PI $(4,5)P_2$ levels blocked neurite outgrowth by PC12 cells following NGF treatment and process formation induced by GAP-43, CAP-23 or MARCKS. The PI

(4,5)P_2 sequestering agent neomycin had complex effects on PC12 cells and simulated some of the activities of the three proteins with respect to the cytoskeleton, but inhibited others.

v. CAP-23 protein distribution in the nervous system

The distribution of CAP-23 protein has been examined in the CNS of young rats (Iino and Maekawa, 1999; Iino et al., 1999). It was found to be widespread with dense immunoreactivity in the neuropil, while most neuronal cell bodies and most nerve fibre bundles were CAP-23 negative, although the pyramidal tract in the spinal cord was heavily immunopositive. Ultrastructural localisation revealed that most CAP-23 immunoreactivity is confined to axon terminals, where it is found in association with synaptic vesicles, and dendritic spines.

vi. Summary

CAP-23 shares a number of biochemical properties with GAP-43, and in addition, these two molecules appear to have partially overlapping activities in vivo and in vitro. Given the considerable evidence that GAP-43 has an important role in axon growth, it seems likely that CAP-23 may be an important molecule for axon regeneration. Caroni et al. (1997) reported that intramuscular nerves, which were previously devoid of CAP-23 immunoreactivity, became immunopositive after nerve injury. However, no study has been made of CAP-23 expression during axon regeneration.

3. SCG10

i. SCG10, a developmentally regulated neuronal growth-cone protein

The *SCG10* cDNA was originally cloned from adult rat superior cervical ganglion neurons (Anderson and Axel, 1985). Much less data is available for this molecule as compared to GAP-43 and CAP-23, but there is evidence that it is important for axon growth and participates in the regulation of microtubule dynamics. It appears to be functionally similar to stathmin (also known as Op18) to which it is related (Schubart et al., 1989; Okazaki

et al., 1993). SCG10 is neuron specific and is expressed by neurons of the PNS and CNS during development, with expression beginning around the time of differentiation of neuronal precursor cells into neurons (Anderson and Axel, 1985). In the embryonic rat brain *SCG10* shows a high level of co-expression with *GAP-43*, although this diminishes during post-natal development (Sugiura and Mori, 1995). *SCG10* mRNA is upregulated by PC12 cells which are induced to differentiate by NGF (Anderson and Axel, 1985; Stein et al., 1988a). This upregulation is observable from 6 hours after NGF is applied and persists for at least 2 weeks. Immunohistochemistry for SCG10 protein on embryonic nervous system tissue and cell cultures reveals staining on axons and the presence of protein in growth cones, where it appears to accumulate (Stein et al., 1988b). Also seen is a punctate staining pattern in the perinuclear cytoplasm, which corresponds to localisation of SCG10 to the Golgi apparatus (Di Paolo et al., 1997a; Lutjens et al., 2000). Some SCG10 staining has also been observed in dendrites in vitro (Di Paolo et al., 1997b).

ii. Membrane association of SCG10

While it lacks a membrane-embedding signal peptide, a significant proportion of SCG10 protein appears to be tightly membrane-associated, specifically with the smooth microsomal membrane fraction, which represents organelles and vesicles (Stein et al., 1988b). In fact, SCG10 associates with growth-cone vesicles, but not with the plasma membrane or organelles other than the Golgi apparatus (Lutjens et al., 2000). Membrane attachment is effected by 34 amino acids at the N-terminal of the protein. This domain targets the protein to the Golgi apparatus and to the growth cone. Removal of this region resulted in most protein becoming cytosolic in transfected COS-7 cells and PC12 cells (Di Paolo et al., 1997a; Lutjens et al., 2000). Interestingly, the membrane attachment appears to be via palmitic acid covalently linked to two cysteine residues within this region, a mechanism also used by GAP-43 (Skene and Virag, 1989; see Section 1.5 *viii*). Both palmitoylated cysteine residues are required for correct growth-cone targetting (Lutjens et al., 2000).

iii. The stathmin family

SCG10 belongs to a family of which the most studied member is the ubiquitous cytosolic protein stathmin (Schubart et al., 1989; Okazaki et al., 1993), and which also includes the neuron-specific protein SCLIP (Ozon et al., 1998) and three splice variants of the *RB3* gene (RB, RB' and RB") which are expressed in neurons and glial cells (Ozon et al., 1997). Stathmin is involved in the regulation of microtubule dynamics (see McNally, 1999; Walczak, 2000 for review) and promotes microtubule depolymerisation. In the context of growth cone motility and navigation, microtubule dynamics play an important role. The two ends of a microtubule (plus and minus) have different properties. The minus end is generally stable while the plus end switches between phases of elongation and rapid depolymerisation (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997). This dynamic instability is necessary for both forward movement and turing of growth cones (Letourneau and Ressler, 1984; Bamburg et al., 1986; Williamson et al., 1996; Challacombe et al., 1997). Its exact role in neurite extension is not clear although, interestingly, microtubule depolymerisation can directly produce a motor force (Lombillo et al., 1995).

iv. Functions of stathmin

As stathmin has been studied in more detail than has SCG10, it is worth summarising briefly some of what is known about this molecule and its role in microtubule dynamics (McNally, 1999; Walczak, 2000). Stathmin acts to destabilise microtubules, primarily by increasing the rate of microtubule catastrophe (catastrophe is the process whereby the microtubule switches from elongation to rapid depolymerisation) (Belmont and Mitchison, 1996; Marklund et al., 1996). Two modes of action have been proposed: firstly, that it sequesters free tubulin dimers, thus reducing their availability for polymerisation; secondly that it acts by directly inducing catastrophe at the plus end. Stathmin and tubulin form complexes in vitro (Belmont and Mitchison, 1996; Curmi et al., 1997; Jourdain et al., 1997), which is consistent with the first idea. However, Howell et al. (1999) demonstrated that stathmin employs both modes of action, and that each activity could be ascribed to separate parts of the protein. Consistent with this, in tissue culture, mutated stathmin which has greatly diminished activity on microtubule dynamics still binds tubulin

effectively (Larsson et al., 1999a), and conversely a C-terminal-deleted protein showed much reduced tubulin-binding activity but retained most of its ability to induce microtubule depolymerisation (Larsson et al., 1999b).

The probable mechanism by which stathmin directly promotes catastrophe has also become clear (McNally, 1999). Microtubules are assembled from heterodimers of α- and β-tubulin. Each monomer of free tubulin dimers binds a molecule of GTP. In microtublules, the β-tubulin-bound GTP may be hydrolysed to GDP, and this causes the conformation of the tubulin dimer to become favourable to dissociation from the microtubule. In fact, on addition to an existing microtubule the 'incoming' tubulin dimer hydrolyses the GTP associated with the tubulin previously at this end. Consequently, on each microtubule only this last tubulin dimer to be added at the plus end does not have its associated GTP molecule hydrolysed to GDP. The microtubule is maintained in the polymerised state by the single subunit at the plus end which still retains its GTP. (It appears that rapid depolymerisation can only occur in the plus to minus direction. For example, cleavage of a microtubule results in catastrophe of the exposed plus end, but not of the exposed minus end (Tran et al., 1997).) Stathmin acts by promoting hydrolysis of GTP bound to this capping tubulin, so the cap effectively ceases to exist and depolymerisation results (Howell et al., 1999). This may be partly by stimulating the GTPase activity of tubulin (Larsson et al., 1999b).

Stathmin activity is regulated by multiple kinases. It is phosphorylated in vivo by protein kinase A (PKA), MAP kinase and several other kinases, and phosphorylation inhibits the activity of stathmin in promoting microtubule disassembly (Melander et al., 1997; Larsson et al., 1997; Di Paolo et al., 1997c; Gavet et al., 1998; Gradin et al., 1998). Phosphorylation also reduced the formation of stathmin-tubulin complexes.

As a ubiquitous protein cytosolic protein stathmin is expected to be important for diverse cellular processes, but has been studied most in the context of chromosome segregation during mitosis. A role in neurite outgrowth may perhaps be implied by the finding that PC12 cells show a requirement for stathmin expression in order to differentiate morphologically (Di Paolo et al., 1996).

v. Function of SCG10

SCG10 appears to have a similar function to stathmin, presumably modified to be useful in a neuron-specific context. It co-purifies with microtubules, and in vitro it inhibits microtubule polymerisation and promotes disassembly of existing microtubules (Riederer et al., 1997). It also counteracts the effects of microtubule-associated proteins in promoting microtubule assembly, and can induce partial depolymerisation of microtubules stabilised by taxol. Microtubule depolymerising activity is common to all members of the stathmin family (Gavet et al., 1998), making it highly likely that the stathmin homology region in these proteins is functionally conserved, and that SCG10 and its other relatives act in a similar way to stathmin.

SCG10 is phosphorylated at serines 50 and 97 by protein kinase A, serines 62 and 73 by MAP kinase and serine 73 by cyclin-dependent kinase (CDK5/p25)(Antonsson et al., 1998). Two phospho-isoforms and the non-phosphorylated form of SCG10 were found to exist in vivo in postnatal rat brain. As with stathmin, phosphorylation reduces the microtubule-destabilising activity of SCG10. Various phosphorylation site mutants of SCG10 were expressed in cultured COS-7 cells and the microtubule-destabilising activity of each was analysed. SCG10 with non-phosphorylatable residues at all four sites showed a significant increase in microtubule depolymerisation activity, while pseudophosphorylated mutants showed decreased activity (Antonsson et al., 1998). SCG10 was also more active in soluble form than in membrane-bound form.

vi. SCG10 and neurite outgrowth

PC12 cell lines engineered to overexpress SCG10 showed increases in both the frequency and speed of neurite outgrowth in the presence of NGF, suggesting a significant role for SCG10 in neurite outgrowth, although no neurite outgrowth was observed in these lines in the absence of NGF (Riederer et al., 1997).

vii. Expression of SCG10 in the adult

The expression pattern of the *SCG10* mRNA, and mRNAs for its family members, has been examined in the adult rat brain (Himi et al., 1994; Ozon et al., 1999). *SCG10* is expressed in most areas of the brain, with particularly strong signals reported in hippocampus and cerebellar cortex. Moderately strong expression was found in neocortex, and the deep cerebellar nuclei, while lower levels were found in the thalamus. Himi et al. (1994) suggested that SCG10 expression correlates with long axons or extensive dendritic arborization, and that it may be involved in synaptic plasticity in the adult, as has been suggested for GAP-43. This possibility was supported by the finding that after unilateral partial ablation of the cerebral cortex, SCG10 was upregulated in the part of the contralateral cortex which projects across the midline to the deafferented striatum, coincident with the formation of new synapses by these neurons (McNeill et al., 1999).

viii. Summary

SCG10 expression has not been examined in regenerating neurons, but its developmentally regulated expression, its location in the growth cone, and its possible role mediating signalling to the microtubule cytoskeleton make it a suitable candidate for a regeneration-associated molecule.

4. FKBP12

i. FKBP12

FKBP12 was first identified as a target of the immunosuppressant drugs FK506 and rapamycin and is one of a class of such molecules known as immunophilins, which comprises other targets of FK506 (FK-binding proteins) and the cyclophilin family. Interest subsequently developed in the roles of immunophilins in the nervous system when it became known that FK506 and related compounds (immunophilin ligands) showed neurotrophic activity in the contexts of axon regeneration and neuroprotection (reviewed by Gold, 1997; Hamilton and Steiner, 1998). A number of experiments have subsequently provided further evidence that FK506 and related compounds promote neurite growth and

axon regeneration, but the relative importance of FKBP12, as opposed to the other FK-binding proteins, for regeneration and its possible role in this processs are not clear.

ii. Enzymatic activity

When it was first identified, FKBP12 was shown to have peptidylprolyl cis-trans isomerase, also known as rotamase, activity (Siekierka et al., 1989; Harding et al., 1989). The amide bonds of the amino acids which make up proteins can exist chemically in two isomeric forms, *cis* and *trans*. The normal form of these bonds is the *trans* form but in the case of proline, a relatively high proportion of its associated amide bonds are found in the *cis* form. The enzymatic activity of FKBP12 converts this bond back into the *trans* form. It shares this activity with other immunophilins (Hamilton and Steiner, 1998).

iii. FKBP12 as a target of immunosuppressant drugs

FKBP12 mediates the immunosuppressive actions of the drugs FK506 and rapamycin. Both drugs bind the rotamase active site of FKBP12 and inhibit this enzyme activity and both molecules also contain additional domains which extend over other parts of FKBP12. Each drug, however, acts through a different pathway. The immunosuppressant actions of FKBP12-FK506 complexes (as well as cyclosporin A acting on cyclophilin), are thought to be due to inhibition of calcineurin, resulting in increased phosphorylation of the transcription factor NF-AT (Armistead and Harding, 1993).

Rapamycin acts on a different pathway, inhibiting T-cell proliferation in response to IL-2, via the kinase known as FRAP (Brown et al., 1994) or RAFT1 (Sabatini et al., 1994); see also Brown and Schreiber (1996). Because inhibition of rotamase activity does not appear to be important for the immunosuppressive actions of FK506 and rapamycin (Bierer et al., 1990; Dumont et al., 1992; Ocain et al., 1993) these two molecules have been thought of as each containing a rotamase-inhibition domain and an 'effector' domain which may confer a gain-of-function to FKBP12 (in the case of FK506 this is a calcineurin-binding domain; Schreiber, 1991).

iv. Cellular roles of FKBP12

FKBP12 interacts with several proteins involved in calcium signalling, including the intracellular calcium channels, the ryanodine receptor (RyR) and the inositol-1,4,5-triphosphate receptor (IP_3R) and also interacts with transmembrane receptor for TGFβ family ligands. FKBP12 forms an integral part of the RyR and IP_3R (Collins, 1991; Cameron et al., 1995). In both cases, without FKBP12 the calcium flux properties of the channel are altered such that it is less effective at both retaining calcium when closed and releasing it when open (Jayaraman et al., 1992; Brillantes et al., 1994; Cameron et al., 1995). FKBP12 serves a related role for the TGFβ receptor and appears to inhibit spontaneous self-activation of this receptor and so reduce the basal level of signalling activity (Wang et al., 1996; Chen et al., 1997).

Both RyR and IP_3R are regulated by phosphorylation and are dephosphorylated by calcineurin. Calcineurin associates with FKBP12 in the context of immunosuppression and and with the FKBP12-IP_3R complex, raising the possibility that in the IP_3R and RyR receptor complexes, and possibly in the TGFβ receptor, FKBP12 may be functioning as a docking protein for this phosphatase (Cameron et al., 1997; Hamilton and Steiner, 1998).

v. FKBP12, immunophilin ligands and regeneration

The first evidence that FKBP12 may be involved in axon regeneration was the observation that FK506 and rapamycin increased the rate of neurite outgrowth in vitro (Lyons et al., 1994). Neurite outgrowth from PC12 cells was measured by counting the number of cells with processes over 5μm in length. PC12 cells showed an increase in this measure of neurite outgrowth in the presence of either drug. In cultured embryonic rat dorsal root ganglia, FK506 also enhanced neurite outgrowth, increasing the number and length of processes. Anti-NGF antibodies reduced the effect of FK506 suggesting the drug was increasing the sensitivity of DRG cells to NGF produced by Schwann cells in the culture.

FK506 was shown to improve regeneration of the injured sciatic nerve in vivo in the rat (Gold et al., 1994), as assessed both behaviourally and morphologically. Animals were

given a sciatic nerve crush, and speed of recovery was assessed by measuring the time until the subjects could move the toes and the time until they could walk on the foot. FK506 significantly decreased the time required for regeneration to restore these functions. Drug treatment also led to improved toe-spreading. Morphological assessment of regeneration indicated an increased density of axonal profiles in nerve cross-sections. FK506 also caused an increase in size of axons in the distal stump (Gold et al., 1995).

Following these reports it was found that after a peripheral nerve injury, FKBP12 is upregulated by the axotomised neurons (Lyons et al., 1995). *FKBP12* mRNA was upregulated in axotomised neurons supplying the sciatic nerve and the facial nerve following injury, with a time course similar to that of the upregulation of GAP-43. The authors also showed that FK506-binding activity is axonally transported and is found in neonatal rat forebrain growth cone fraction, although at a lower level than in brain as a whole.

It was also found that FK506 treatment caused a greater upregulation of *GAP-43* mRNA and c-Jun protein in axotomised DRG neurons (Gold et al., 1998a; Gold et al., 1999). Beneficial effects of FK506 on axon regeneration have been reported in several other models. Following a photothrombotic lesion of the spinal cord, FK506 treatment resulted in greater upregulation of GAP-43 in injured neurons near the lesion (Madsen et al., 1998). The authors also reported an improvement in behavioural scores following drug treatment. FK506 caused an increase in size of axons regenerating into a predegenerate peripheral nerve graft implanted into the injured spinal cord (Wang and Gold, 1999) and unexpectedly promoted regeneration by rubrospinal neurons into the grafts.

The non-immunosuppressive FK506 analogues GPI-1046, L-685,818 and V-10,367 have also been reported to have equivalent neurotrophic activity to FK506 (Gold et al., 1997; Steiner et al., 1997a; Steiner et al., 1997b).

vi. Further data concerning neurotrophic actions of immunophilin ligands and the role of FBKP12

More recently findings have emerged which raise doubts about both the neurotrophic activities of these molecules and the role of FKBP12 in mediating them. Harper et al. (1999) found that GPI-1046, which inhibits the rotamase activity of FKBP12 but does not cause calcineurin inhibition or immunosuppression, has minimal effects on neurite outgrowth in chick sensory neuronal cultures, and Parker et al. (2000) found that FK506 is also ineffective on PC12 cells. FK506 fails to promote regeneraton in the injured optic nerve (Campbell et al., 1999).

Against this, the effects of FK506 of promoting neurite outgrowth from DRG neurons and of accelerating regeneration and functional recovery following peripheral nerve injury were confirmed (Carreau et al., 1997; Doolabh and Mackinnon, 1999; Jost et al., 2000; Lee et al., 2000) although the analogue V-10,367 was not shown to be effective (Becker et al., 2000).

There is also some uncertainty about what is the important target of these drugs for neurotrophic activity. Gold et al. (1999) reported that in fact, FKBP12 is not required for this activity, and instead the actions of FK506 and its analogues on neurite growth are mediated by FKBP52. FK506 caused a similar increase in speed of neurite outgrowth in cultures from both FKBP12 knockout and wild-type mice. The authors went on to show that an antibody to another FK506-binding protein, FKBP52, blocked the effect of FK506 in promoting neurite outgrowth, and suggested that FK506 acts by dissociating FKBP52 from steroid-receptor complexes.

vii. Summary

Overall, the importance of FKBP12 in axonal regeneration remains unclear, and some disagreement exists over whether FK506 or its analogues have genuine neurotrophic effects, and whether any such effects are mediated by FKBP12. However, the reported upregulation of FKBP12 by regenerating neurons supplying injured peripheral nerves suggests that it does have a role in axon regeneration. Extending such findings to other systems in which axonal regeneration can be induced would add weight to the idea that FKBP12 is involved in this process.

1.6 The response of spinal axons to injury

i. Overview

As discussed above, neurons of the CNS show variable abilities to regenerate into peripheral nerve grafts, and their ability to regenerate correlates with changes in gene expression in the neuron. Of particular relevance to research on spinal cord injury are neurons whose axons form the long fibre tracts of the spinal cord. Two important descending tracts are the corticospinal and rubrospinal tracts. Together these illustrate the differences between CNS neurons discussed earlier: rubrospinal axons will regenerate into a peripheral nerve graft if axotomised sufficiently proximally, but corticospinal neurons will generally not do so (Richardson et al., 1982a; Ye and Houle, 1997; Blits et al., 2000). The final experiments presented in this study, described in Chapter 8, are concerned with characterising gene expression in these two sets of neurons following axotomy.

ii. Corticospinal axons

Corticospinal axons have seldom been reported to regenerate into peripheral nerve grafts. The only published example where this has been observed is a study by Cheng et al. (1996), in which multiple transplants of intercostal nerve were used to bridge a complete transection of the spinal cord, in combination with αFGF administered in fibrin glue. In this study, the grafts were aligned to lead axons from the rostral white matter of the cord to the caudal grey matter. The authors observed some regeneration of corticospinal fibres through the grafts and into the distal grey matter. While this result remains unreplicated, several other groups have attained some regeneration of corticospinal fibres by introducing neurotrophic factors with or without peripheral nerve grafts or Schwann cell transplants. A common feature of these experiments is that corticospinal axons often appear to show a marked preference for the intact grey matter of the spinal cord over other tissue, including Schwann cells (Schnell and Schwab, 1990; Grill et al., 1997; von Meyenburg et al., 1998; Blesch et al., 1999; Blits et al., 2000; Ferguson et al., 2001). The most striking example is the recent work by Blits et al. (2000). In this study intercostal nerves were transduced with an adenoviral vector such that Schwann cells in the nerves would express NT-3, which is one of the few neurotrophins not usually made in injured peripheral nerve.

The transduced nerves were then implanted into the spinal cord below a dorsal hemisection, which injured the component of the corticospinal tract found in the dorsal funiculus. Curiously, the corticospinal axons grew greater distances if the nerves were made to express NT-3, but did so in the grey matter underlying the nerve graft and no corticospinal axons entered the nerve itself. This would suggest that the combination of neurotrophins released by NT-3 producing Schwann cells is sufficient to drive growth in these axons, but that they find grey matter a more attractive substrate than peripheral nerve tissue. Ferguson et al. (2001) also found enhanced corticospinal axon growth following implantation of predegenerated peripheral nerve tissue into a chronic spinal cord lesion, combined with the provision of additonal neurotrophic factors, including NT-3. Again, axon regeneration was confined to the grey matter around the lesion. Corticospinal axons therefore have an unusual combination of properties, namely, that they will grow axons in the presence of suitable trophic factors, or if certain inhibitory molecules are neutralised, but are apparently do not find peripheral nervous tissue a favourable substrate for growth. This aspect of corticospinal axon regeneration is little understood.

It is possible that it may reflect a lack of expression or upregulation of growth-associated molecules, including those such as L1 and CHL1 which are involved in axon-Schwann cell interactions, but gene expression in injured corticospinal neurons has been little studied. Tetzlaff and co-workers reported that they upregulate GAP-43 following a proximal (subcortical) lesion, but not following axotomy at the level of the pyramids (Tetzlaff and Giehl, 1991; Tetzlaff et al., 1994), but studies of transcallosal neurons, another population of pyramidal cortical projection neurons, found that a proximal lesion did not cause upregulation of GAP-43 or of alpha tubulin (Elliott et al., 1997; Elliott et al., 1999). More distal lesions of corticospinal neurons were found to result in a general down-regulation of GAP-43 and cytoskeletal components (Mikucki and Oblinger, 1991; Tetzlaff et al., 1994) attributed by the latter group to a general down-regulation of cell metabolism. However, there have been no reports on expression by corticospinal neuron of other molecules associated with regeneration and axotomy. Therefore it would be useful to know what, if any, changes in gene expression occur in injured corticospinal neurons.

iii. Rubrospinal neurons

As already discussed, gene expression in axotomised rubrospinal neurons has been examined after thoracic and cervical lesions to the spinal cord. Thoracic lesions produce little change in immediate-early gene expression in the red nucleus, but cervical lesions produce upregulation of c-Jun in identified rubrospinal projection neurons (Jenkins et al., 1993a), beginning at 12 hours post-injury and increasing until 10 days. Cervical lesions were also reported to cause upregulation of GAP-43 and down-regulation of neurofilament (Tetzlaff et al., 1991; Fernandes et al., 1999). Tubulins and actin were initially upregulated but then declined after the first week to levels below those seen in the contralateral control nucleus (Tetzlaff et al., 1991; Fernandes et al., 1999), but stronger expression of c-Jun, GAP-43 and the Tα1 tubulin subtype persisted in some neurons for up to 7 weeks (Tetzlaff et al., 1991; Jenkins et al., 1993a). The expression of these growth-associated molecules in cervically injured rubrospinal neurons is reflected by the fact that some of them are able to regenerate axons into a suitably placed peripheral nerve graft after such an injury. However, the correlation in this case is minor: the number of rubrospinal neurons which regenerate is very low, being under 2% (Richardson et al., 1982a; Fernandes et al., 1999) whereas c-Jun and GAP-43 upregulation was found in a majority of cells (Jenkins et al., 1993a; Fernandes et al., 1999). Nonetheless, the responses found so far suggest that they may also upregulate other molecules but to date there is no information on this.

1.7 Molecules studied by in situ hybridisation following spinal cord lesions

The expression of mRNAs for GAP-43, CAP-23 and SCG10 were all examined in axotomised corticospinal and rubrospinal neurons, and these molecules have been discussed extensively earlier. In addition, expression of the mRNAs for the axotomy-associated transcription factors, c-Jun, ATF3 and Krox-24, and the cell-adhesion molecules L1 and CHL1, was studied.

A. Transcription factors

Of the large number of constitutive and inducible transcription factors found in neurons only c-Jun, JunD, Krox-24 (reviewed by Herdegen and Leah, 1998) and recently ATF3 have been found to be upregulated in axotomised neurons for more than brief periods. C-Jun was long considered to be the only one consistently upregulated for the duration of the regeneration process and was therefore thought to be a good candidate for a transcriptional regulator involved in the initiation of the axon-growth program, which includes upregulation of growth-associated molecules and the production of the general machinery of axon growth. Direct evidence for this was generally lacking, however, and the recent discovery that ATF3 is also induced by axotomy may require reassessment of this model. Krox-24 is also upregulated in some axotomised CNS neurons but is not associated with regeneration. JunD expression was not examined in this study.

1. C-Jun

C-Jun is a transcription factor and immediate early gene, and can be expressed by all cell types. C-Jun dimerizes with itself and with other proteins, such as JunD, JunB, Fos family members, and ATF/CREB family members to form the AP-1 transcription factor complex. C-Jun's transcriptional activity is regulated by phosphorylation at two serine residues (ser63 and ser73) by the jun-N-terminal kinase (JNK) family. In most cell types, the AP-1 complex is involved in the response to various stimuli such as UV-irradiation induced stress and exposure to pro-inflammatory cytokines or growth factors, and is also involved in the control of cell proliferation and apoptosis (reviewed by Karin et al., 1997). The apparent role of c-Jun in neurons is at least in part rather different, in that its expression is associated with axotomy, axon regeneration and apoptosis (reviewed by Herdegen et al., 1997). Upregulation of c-Jun has been correlated well with axotomy and subsequent regeneration in several models, and with the strength of the cell body response, and therefore it may play a pivotal role in the neuronal response to axotomy.

C-Jun is upregulated by motor and sensory neurons supplying peripheral nerves following nerve injury or blockade of axon transport (Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1992; Jenkins et al., 1993b). Upregulation occurs within 24 hours and

continues until around the time of target reinnervation. There have been many subsequent reports, too numerous to list here, of c-Jun protein or mRNA upregulation in PNS neurons and extrinsic CNS neurons regenerating axons after nerve injury, and in intrinsic CNS neurons following axon injury (these are listed in Herdegen and Leah, 1998). In addition, enhanced phosphorylation of c-Jun and activation of JNK has been demonstrated in axotomised neurons after sciatic nerve injury (Kenney and Kocsis, 1998). Neuronal c-Jun expression is a constant correlate of peripheral nerve regeneration, and the intrinsic CNS neurons which regenerate into peripheral nerve grafts also upregulate it. Retinal ganglion cells and neurons of the TRN and deep cerebellar nuclei which are axotomised and then successfully regenerate into a peripheral nerve graft have all been shown to upregulate c-Jun or its mRNA for the duration of this regeneration (Hüll and Bähr, 1994; Vaudano et al., 1998; Chaisuksunt et al., 2000a).

A good correlation is also found between c-Jun expression and the strength of the subsequent regenerative response by the neuron. While peripheral nerve inury results in massive c-Jun upregulation and vigorous regeneration, dorsal root injury, which results in a much more limited regenerative response, leads to a more limited and less widespread upregulation in the affected primary sensory neurons (Jenkins et al., 1993b; Kenney and Kocsis, 1997). Similarly, while proximally axotomised retinal ganglion cells upregulate c-Jun and regenerate axons into a peripheral nerve graft, distal (intracranial) axotomy of retinal ganglion cells results in little or no c-Jun induction and poor regeneration into a peripheral nerve graft (Hull and Bahr, 1994; Robinson, 1995). Rubrospinal neurons behave similarly, in that cervical axotomy induces c-Jun and some ability to grow axons into grafts, whereas thoracic axotomy induces neither (Richardson et al., 1984; Jenkins et al., 1993a; Tetzlaff et al., 1994).

The correlation of c-Jun expression with regenerative axon growth would suggest that it may be a transcriptional control point for regeneration-associated genes. However, the correlation is imperfect: for example, insertion of a peripheral nerve graft into the striatum elicits regeneration from nigrostriatal projections neurons, but upregulation of c-Jun is observed in only a few SNpc neurons (Chaisuksunt, 1999). Generally, however, there is good evidence that the increase in c-Jun during regeneration closely parallels the upregulation of other growth-associated genes, such as GAP-43. However, little data is

available on the in vivo importance of AP-1 activity in initiating or regulating transcription of regeneration associated genes, or on its importance for successful regeneration. Only two studies have provided information on the former point: analysis of the GAP-43 promoter revealed that it contains an AP-1 binding site which regulates its transcription (Weber and Skene, 1998) and the gene for vasoactive intestinal peptide was shown to be regulated by AP-1 activity acting via a cAMP-response element motif (CRE site) (Mulderry and Dobson, 1996). Nonetheless, its co-expression with other regeneration-associated molecules suggests it may lie upstream of these as well.

The picture is made more unclear by a second function ascribed to c-Jun in neurons, namely that of control of apoptosis (Ham et al., 1995; Herdegen et al., 1997). C-Jun is associated with neuronal apoptosis following cerebral ischaemia and is required for programmed cell death in sympathetic neurons (Dragunow et al., 1993; Ham et al., 1995). This activity is also associated with the c-Jun upregulation seen following axotomy as the strong cell body response to axotomy which is associated with a vigorous regenerative response is also associated with increased likelihood of cell death. It is not clear how the opposing functions c-Jun is believed to have of regulating axon growth and apoptosis would be separately regulated. A further indication that c-Jun has additional neuronal functions is the fact that it is expressed at a moderate basal level in many neurons, including some primary sensory neurons, and parts of the hippocampus and neocortex, although given the variety of partners with which c-Jun can dimerize to form active transcription factors it is unsurprising that it may have multiple functions.

2. ATF3

ATF3 (also known as LRF-1) is also a transcription factor and a product of an immediate early gene. It may dimerize with itself to form a transcriptional repressor, or with multiple other partners, including c-Jun, to form a transcriptional activator (Hai et al., 1999). ATF3/c-Jun dimers recognise the CRE/ATF motif, as well as the AP-1 motif normally associated with c-Jun (Hai and Curran, 1991).

Evidence has recently emerged that ATF3 is also expressed following axotomy, and may be a more specific marker for axotomised neurons than c-Jun (Tsujino et al., 2000).

Following sciatic nerve cut, *ATF3* mRNA was induced in ipsilateral DRG neurons within 12 hours and in motor neurons within 24 hours. In addition, a near perfect correlation was found between retrograde labelling of axotomised neurons and *ATF3* expression. While, as would be expected, a high degree of colocalisation of ATF3 and c-Jun was found, there were some differences in expression of these two molecules. In the DRGs almost half the neurons expressing ATF3 did not express c-Jun, but c-Jun expression was not found without ATF3. In motor neurons nearly 100% colocalisation was found. Additionally, ATF3 was not expressed at all in these neurons in the untreated animal, in contrast to c-Jun which is expressed at a low level in motor and primary sensory neurons.

ATF3 is also expressed following axotomy of retinal ganglion cells (Takeda et al., 2000). Its expression was found to be more transient than c-Jun, and begins to decline before the onset of cell death. It was also expressed in fewer cells than c-Jun at any given time.

Although the formation of ATF3/c-Jun heterodimers has yet to be demonstrated in these models, it is likely to occur. The mechanisms by which ATF3 expression is regulated have not yet been thoroughly investigated, but initial evidence points to regulation by c-Jun, possibly in conjunction with the constitutively expressed transcription factor ATF-2. Activation of the jun N-terminal kinase pathway in HeLa cells results in ATF3 induction, as does vector driven expression of c-Jun and ATF-2 (Hai et al., 1999). Little is known about transcriptional targets of ATF3 homodimers, and there is no published data on targets of ATF3/c-Jun dimers. It remains to be seen whether other regenerating or axotomised neurons also express ATF3.

3. Krox-24

Krox-24, also known as NGFI-A, Zif/268, Zenk and Egr-1, is a transcription factor and immediate early gene, but does not interact with AP-1 proteins although it is often co-expressed with them (Herdegen and Leah, 1998). It is upregulated by some CNS neurons following axotomy. Following transection of the medial forebrain bundle and the mammillothalamic tract, Krox-24 expression was induced in the nucleus mammillaris, the parafascicular thalamic nucleus, the ventral tegmental area and the substantia nigra pars compacta (Leah et al., 1993; Herdegen et al., 1993a). Upregulation began between 1.5-3

days after injury and persisted at least 10 days. Krox-24 expression has also been reported in axotomised retinal ganglion cells of the rat (Herdegen et al., 1993b; Robinson, 1994), but is not expressed in these cells if they are regenerating axons, either in the goldfish optic nerve, or in the rat when regenerating into a peripheral nerve graft (Herdegen et al., 1993b; Robinson, 1995). Krox-24 is not expressed by regenerating PNS neurons following sciatic nerve crush (Herdegen et al., 1992). These data suggest that Krox-24 expression is associated with axotomy in some neurons, but is not associated with regeneration. This therefore qualifies it as a candidate for expression in corticospinal and rubruspinal neurons following axotomy.

Krox-24 may be of particular interest in corticospinal neurons as its expression is also associated with plasticity and learning in several contexts (Dragunow, 1996). It is expressed in neurons during consolidation of long-term potentiation in the rat (Abraham et al., 1991; Abraham et al., 1993), during behavioural learning tasks in primates (e.g. Okuno and Miyashita, 1996), and during reorganisation of cat visual cortex after retinal lesions (Obata et al., 1999). This last example involves growth of axon collaterals of cortical pyramidal neurons within grey matter (Darian-Smith and Gilbert, 1994; Das and Gilbert, 1995). Given the marked preference for grey matter shown by corticospinal axons described in Section 1.6 *ii* this raises the interesting possibility that a Krox-24 response in axotomised corticospinal neurons might direct axon growth in the grey matter of the spinal cord.

B. Other growth-associated molecules: L1 and CHL1

L1 and CHL1 (close homologue of L1) are cell-adhesion molecules which are thought to be important for the promotion of axon growth and correct guidance in vivo. These two molecules belong to a family of four close relatives in the L1 gene family in vertebrates. In the rat, mouse and humans the other family members are NrCAM and neurofascin. L1 family members are well-conserved across species. This family itself belongs to the larger group of immunoglobulin-domain cell-adhesion molecules (Ig-CAMs) which includes the NCAM and TAG-1 families, all belonging to the Ig superfamily (reviewed by Walsh and Doherty, 1997; Hortsch, 2000). L1 and CHL1 are both thought to be growth-associated and growth-promoting molecules.

1. L1

During development, L1 is expressed by CNS neurons and by peripheral neurons and Schwann cells. It is found on developing peripheral axons until myelination begins (Martini and Schachner, 1986; Moscoso and Sanes, 1995). Observations made of axon growth in vitro on L1 and laminin substrates suggest it probably promotes the fasciculation of axons. Axon growth on laminin is normally fasciculated, but the introduction of anti-L1 antibodies prevents fasciculation, suggesting fasciculation is mediated by an interaction between L1 molecules on different axons (Stallcup and Beasley, 1985; Drazba and Lemmon, 1990). Axon growth on L1 is defasciculated. In humans, mutations in the L1 gene result in developmental defects of the nervous system, particularly malformation of the corticospinal tract and corpus callosum (Wong et al., 1995) and similar defects were observed in L1 knockout mice (reviewed by Kamiguchi et al., 1998). Expression of L1 is maintained in the peripheral nervous system in the adult (Mirsky et al., 1986; Zhang et al., 2000).

L1 is thought to be important for axon-Schwann cell interactions during peripheral nerve regeneration. Although *L1* is not upregulated by axotomised neurons, it is expressed normally by motor and some sensory neurons (Zhang et al., 2000). During peripheral nerve regeneration L1 is upregulated by Schwann cells in the degenerating nerve and is found on regrowing axons and Schwann cells at the points of contact between them, until the onset of myelination (Martini and Schachner, 1988; reviewed by Martini, 1994). It is also upregulated by CNS neurons regenerating axons into peripheral nerve grafts and so may be important generally for axon growth in peripheral nerves. Neurons in the thalamic reticular nucleus (Zhang et al., 1995) and deep cerebellar nuclei (Chaisuksunt et al., 2000a) upregulate L1 when regenerating into peripheral nerve grafts. Expression of L1 in retinal ganglion cells, neurons of the substantia nigra pars compacta and cholinergic striatal interneurons does not increase but is normally at a moderate to high level, which is maintained during axon regeneration (Jung et al., 1997; Woolhead et al., 1998).

That L1 may promote axon growth in peripheral nerve tissue is supported by evidence that it promotes neurite growth in vitro. As a growth substrate in vitro it promotes neurite extension (Lemmon et al., 1989). Axon growth by cultured motor neurons and neonatal

DRG neurons growing axons on Schwann cell monolayers was greatly inhibited by anti-L1 antibodies (Bixby et al., 1988; Seilheimer and Schachner, 1988). L1 expressed from a vector in cultured 3T3 fibroblasts, which do not normally express L1, promoted neurite outgrowth from PC12 cells and cerebellar neurons (Williams et al., 1992; Williams et al., 1994a).

L1 promotes growth by homophilic interactions i.e. by interaction of axonal and glial L1, and by heterophilic interactions of axonal L1 with other Ig CAMs receptors on glial cells. Axon growth on L1as a substrate coated onto culture dishes and on L1 expressed by cultured cells is via homophilic interaction, shown by the use of antibodies which bound specifically to the axonal L1, which blocked neurite growth (Lemmon et al., 1989; Williams et al., 1992). L1 binds heterophilically to several molecules including the cell adhesion molecules TAG1/axonin1 (Kuhn et al., 1991; Felsenfeld et al., 1994), and DM-GRASP (DeBernardo and Chang, 1996) 96). TAG1/axonin1 as a substrate promotes neurite outgrowth in an L1 dependent manner, and conversely anti-DM-GRASP antibodies prevent neurite growth on L1. L1 also interacts with F3/F11 (Brummendorf et al., 1993) the chondroitin sulphate proteoglycans neurocan and phosphacan (Friedlander et al., 1994; Milev et al., 1994), laminin (Grumet et al., 1993) and a_5b_1 and a_vb_3 integrins (Ruppert et al., 1995; Montgomery et al., 1996), although the functions of these interactions are not clear. Heterophilic interactions may be required for adhesion of unmyelinated sensory axons to Schwann cells (Haney et al., 1999).
L1 mediated growth-promotion may be effected by several mechanisms (reviewed by (Burden-Gulley et al., 1997), including cis-activation of the FGF receptor leading to signalling via phospholipase Cγ (Williams et al., 1994b; Doherty and Walsh, 1996; Saffell et al., 1997), direct interaction with cytoskeleton-linked protein ankyrin (Davis and Bennett, 1994) and phosphorylation of L1 by the kinase $p90^{rsk}$ (Wong et al., 1996).

2. CHL1

CHL1, like L1, is expressed by neurons and glia (Hillenbrand et al., 1999). Being closely homologous to L1 (Holm et al., 1996) it may be expected to have related functions in axon growth and axon-Schwann cell interactions. Immunoblotting of primary cultures taken from embryonic or postnatal animals revealed CHL1 protein expression in

astrocytes, Schwann cells, and most neuronal cell types and *CHL1* expression is found in many brain neurons in postnatal mice (Hillenbrand et al., 1999). Similarly to L1, CHL1 promotes neurite outgrowth when expressed by vector in fibroblasts or the Drosophila S2 cell line, and in these experiments was more effective than L1 in this respect. It is not known which molecules CHL1 interacts with to promote growth. Cell aggregation experiments indicate that CHL1 does not promote adhesion by homophilic interactions, and nor does it interact heterophilically with L1 (Hillenbrand et al., 1999).

CHL1 may be considered a growth-associated molecule as it is consistently upregulated by neurons regenerating their axons. Motor and sensory neurons upregulate *CHL1* during peripheral nerve regeneration (Zhang et al., 2000) and neurons of the TRN and deep cerebellar nuclei upregulate *CHL1* when regenerating axons into peripheral nerve grafts (Chaisuksunt et al., 2000a; Chaisuksunt et al., 2000b).

Chapter 2

Materials and Methods

2.1 Animals and anaesthesia

Animals were anaesthetised with a mixture of halothane, oxygen and nitrous oxide for all surgical procedures, which were approved by the local ethical committee and by the Home Office under UK animal experimentation legislation. Unless otherwise stated, all operated animals were adult female Sprague-Dawley rats between 180g and 280g in body weight.

All surgery was performed by the author except for some procedures on wt3 transgenic mice, and dorsal root injuries on rats, performed by Prof. Patrick Anderson (detailed in Sections 2.3 *iii* and 2.9B).

2.2 Transganglionic and retrograde labelling

i. Injection device for transganglionic and retrograde labelling

A 50µl glass capillary tube (Sigma) was pulled in a gas flame to create a needle with a tip 100µm or less in diameter. This was attached to a 5µl Hamilton syringe via polyethylene tubing, internal diameter 0.28 mm (Jencon's Scientific 686-099), and superglue was used to seal the interface between the micropipette and the tubing. Prior to use the device was filled with sterile water, by injecting it into the bore of the Hamilton syringe. 1µl of air was taken up followed by the tracer.

ii. Tracers

Cholera toxin B-subunit (CTB; List Biological Labs, Campbell, CA, USA) was used for retrograde labelling. This was dissolved at 10mg/ml in sterile H_2O. Transganglionic labelling was performed using cholera-toxin B-subunit conjugated to horseradish peroxidase (CT-HRP; List Biological Labs, Campbell, CA, USA), dissolved at 14mg/ml in sterile H_2O.

2.3 Surgical procedures used in studies of gene expression in axotomised neurons after injury to the sciatic nerve or lumbar dorsal roots (Chapter 3).

i. Sciatic nerve injury

A total of 22 animals received a sciatic nerve crush or cut and ligation. Animals were anaesthetised and an incision was made over and parallel to the left tibia and the underlying muscles were separated to expose the sciatic nerve. In sixteen animals the nerve was crushed twice for 10-15 seconds with a pair of watchmakers' forceps. In another 6 animals the sciatic nerve was cut at the same level and the proximal stump ligated with 5/O sutures to prevent axonal regeneration. Animals survived for between 3 days and 6 weeks (see Table 2.1), after which time animals were anaesthetised and decapitated, and the 4th and 5th lumbar spinal cord segments and the L4 and L5 DRGs on both sides were removed and prepared for in situ hybridisation (ISH).

ii. Dorsal root injury

All dorsal root injuries of rats were performed by Prof. Patrick Anderson. The left lumbar dorsal roots were exposed by a hemilaminectomy in 3 animals. The left L4 and L5 dorsal roots were located under an operating microscope and completely transected with microscissors (dorsal rhizotomy). The cut ends were reanastomosed with 10/O sutures. Animals survived for between 3 and 24 days (see Table 2.1) after which animals were anaesthetised and decapitated, and the L4 and L5 DRGs on both sides were removed and prepared for in situ hybridisation.

2.4 Surgical procedures used in studies of gene expression in regenerating neurons after tibial nerve autograft insertion into the thalamus or cerebellum (Chapters 4-6).

i. Nerve grafts in the thalamus

A total of 18 animals received living tibial nerve autografts in the thalamus. Animals were anaesthetised and an incision was made over and parallel to the left tibia and the

Surgical procedure	Survival time	Sections processed for					*n*
		SCG10 *ISH*	CAP-23 *ISH*	GAP-43 *ISH*	FKBP12 *ISH*	CTB *IHC*	
Sciatic nerve crush	1 day	✓	✓	✓			3
	3 days	✓	✓	✓			3
	7 days	✓	✓	✓			3
	14 days	✓	✓	✓			3
	5 weeks	✓	✓	✓			1
	6-7 weeks	✓	✓	✓			3
Sciatic nerve cut and ligation	14 days	✓	✓	✓			1
	5 weeks	✓	✓	✓			2
	6-7 weeks	✓	✓	✓			3
Dorsal root injury	3 days	✓	✓	✓			1
	7 days	✓	✓	✓			1
	24 days	✓	✓	✓			1

Table 2.1. Survival times, processing and animal numbers used in studies of gene expression in axotomised neurons after injury to the sciatic nerve or lumbar dorsal roots (Chapter 3).

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underlying muscles were separated to expose the sciatic nerve. The epineurium was opened and the tibial nerve separated from the other branches. A segment approximately 15mm long was removed and stored temporarily in sterile Hank's Balanced Salt Solution (HBSS). The incision was closed, and the scalp was opened with a parasagittal incision and a 2mm-diameter hole drilled in the skull, 2.5 mm caudal to bregma and 3.0 mm to the left of the midline. An incision was made in the dura mater, and the segment of tibial nerve was inserted proximal end first into the brain using a glass needle, to a depth of 7mm measured from the dura. The distal part of the graft remained outside the skull. These co-ordinates were chosen so that the grafts would terminate in the dorsal thalamus near the TRN. The distal part of the nerve graft was glued to the skull and the scalp incision closed. Some animals received injections of CTB tracer into the graft under anaesthesia, before sacrifice (see below). Animals survived for between 3 days and 6 weeks (see Table 2.2), after which the animals were re-anaesthetised and decapitated, and the forebrain was removed, taking great care not to disturb the graft, and prepared for ISH or ISH and CTB immunohistochemistry (ISH/CTB).

Surgical procedure	Survival time	Sections processed for					*n*
		SCG10 *ISH*	CAP-23 *ISH*	GAP-43 *ISH*	FKBP12 *ISH*	CTB *IHC*	
Tibial nerve autograft in thalamus	3 days	✓	✓	✓	✓		3
	2 weeks	✓	✓	✓	✓		5
	4 weeks	✓	✓	✓	✓		3
	6 weeks	✓	✓	✓	✓		3
Tibial nerve autograft in thalamus, followed by CTB injection into the distal graft	4 weeks	✓	✓	✓	✓	✓	3
	6 weeks	✓	✓	✓	✓	✓	1
Freeze-killed tibial nerve autograft in thalamus	2 weeks	✓	✓	✓	✓		3
Tibial nerve autograft in cerebellum	3 days	✓	✓	✓	✓		3
	2 weeks	✓	✓	✓			4
	4 weeks	✓	✓	✓	✓		2
	6 weeks	✓	✓	✓			3
	6 weeks	✓	✓	✓	✓		2
Tibial nerve autograft in cerebellum, followed by CTB injection into the distal graft	4 weeks	✓	✓			✓	2
	4 weeks	✓	✓		✓	✓	2
	4 weeks				✓	✓	1
Freeze-killed tibial nerve autograft in cerebellum	2 weeks	✓	✓		✓		3
CTB injections into cortex	7 days					✓	2

Table 2.2. Survival times, processing and animal numbers used in studies of gene expression in regenerating neurons after tibial nerve autografting into the thalamus or cerebellum (Chapters 4-6).

N.B. While in many cases the animals used to investigate expression of SCG10, CAP23, GAP-43 after graft implantation were also used to study expression of FKBP12, results for FKBP12 are presented separately.

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ii. Nerve grafts in the cerebellum

A total of 19 animals received living tibial nerve autografts in the cerebellum. A segment of tibial nerve was removed and the scalp opened as described for nerve grafts in the

thalamus, above. A 2mm-diameter hole was drilled in the occipital bone, centred approximately 2-2.5mm to the left of the midline and the same distance rostral to the caudal margin of the bone. An incision was made in the dura, and the graft was inserted, proximal end first, to a depth of 7 mm. These coordinates were chosen so that the graft would terminate in the cerebellar deep nuclei. The distal part of the nerve was glued to the skull and the scalp incision closed. Some animals received injections of CTB tracer into the graft before sacrifice (see below). Animals survived for between 3 days and 6 weeks (see Table 2.2), following which the animals were re-anaesthetised and decapitated, and the cerebellum and brainstem were removed, taking great care was taken not to disturb the graft, and prepared for ISH or ISH/CTB.

iii. Freeze-killed grafts

Six additional animals received tibial nerve autografts in the thalamus or cerebellum in which the constituent cells were killed by repeated freeze-thaw cycles, rendering them non-permissive for axonal regeneration. Tibial nerve segments were removed as described above, and went through 7-10 cycles of freezing in liquid nitrogen or on dry ice and thawing in HBSS. The remainder of the grafting procedures were identical to those used for living grafts. Freeze-killed nerve autografts were implanted into the thalamus in three animals and into the cerebellum in another three animals as described for living grafts.

iv. Retrograde labelling of neurons that have regenerating axons in grafts, with cholera-toxin B-subunit

In order to identify CNS neurons which had regenerated axons into the grafts, 4 animals with grafts in the thalamus and 3 animals with grafts in the cerebellum (see Table 2.2) received injections of CTB retrograde tracer into the distal end of the graft. Animals were re-anaesthetised with halothane and the scalp reopened. Connective and scar tissue which had formed under the scalp was carefully removed to expose the distal graft underneath. The most distal part of the nerve graft was removed and a syringe needle was pushed into the cut end 2-3 times, to disrupt the endoneurium. The injection device (described in Section 2.2 *i*) was loaded with 0.2-0.4µl of CTB and the injection needle was inserted into the cut end of the graft. A small piece of Gelfoam was placed around the needle and over

the end of the graft. The CTB was slowly injected into the graft about 4-5 mm from the craniotomy and the needle carefully removed, leaving the gelfoam in place. Animals survived a further 24 hours before being re-anaesthetised and decapitated.

v. Retrograde labelling with CTB of thalamocortical projection neurons by injection into neocortex

In order to check that thalamocortical projection cells can be successfully labelled with CTB, two additional animals were anaesthetised, the skull was opened as described before and the dura was opened 2-3mm lateral to bregma. Three injections were made into the underlying neocortex as follows: 1-1.5µl CTB was taken up into the injection device and the needle inserted into rostral, caudal and lateral regions of neocortex. The needle was slowly withdrawn as the tracer was injected, to release the tracer along the needle injury tract. Animals were killed 24 hours later, the brains removed and frozen as for ISH/CTB, and sections through the thalamus were cut and processed for CTB immunohistochemistry as described below.

2.5 Surgical procedures used in studies of gene expression in axotomised corticospinal and rubrospinal neurons

Animals were divided into five groups. Animals in the first group (n=2) were untreated. Animals in the second and third groups received a transection of the corticospinal tract at lower C3 and an injection of CTB into the dorsal funiculus immediately above this (upper C3 level) and survived for 1 and 7 days respectively. Animals in the fourth group received an injection of CTB into the cervical spinal cord, and 24 hours later received a slice lesion of the left neocortex to proximally axotomise corticospinal neurons. Animals in the final group received a transection of the left rubrospinal tract at lower C3 and an injection of CTB immediately above this (upper C3 level) in the dorsal lateral funiculus. Experimental groups and survival times are given in Table 2.3. All tissue was processed for ISH using probes for *SCG10*, *CAP-23*, *GAP-43*, *L1*, *CHL1*, *c-jun*, *ATF3* and *krox-24* and for CTB immunohistochemistry.

i. Intracortical axotomy of corticospinal neurons

To produce a proximal axotomy of corticospinal axons, three animals received an intracortical lesion, parallel to the plane of the cortical layers and at the bottom of layer V or within layer VI.

To create these lesions, a device was constructed loosely based on the 'brain scythe' of Dale et al. (1995). This consisted of an eye blade, the end of which was bent to form an acute angle of 75^0 with the shaft of the blade. The tip projected 6mm from the shaft of the blade at this angle. This was attached using Superglue to a glass capillary tube. A 5 cm segment of a larger glass capillary tube was placed over this, and the protruding end of the inner glass tube bent over in a gas flame to form a handle (see Fig. 2.1). The device was designed to be inserted into the lateral neocortex and to make a lesion in a semicircular arc medial to the insertion point and parallel to the cortical layers. A mark was made on the blade which when level with the dura would indicate that the device was in the correct position to make a lesion 1.5mm below the surface of the brain when the blade was turned (see below). The blade and inner tube rotate freely within the outer tube.

Before performing the intracortical lesions, it was necessary to retrogradely label the corticospinal neurons from the spinal cord. To this end, a method was used also based on that of Dale et al. (1995). Animals were anaesthetised and a parasagittal incision made at the midline over the cervical spinal cord. A second incision was made in the most superficial muscle layers, and the underlying muscles were separated at the midline by blunt dissection. The C3 vertebra was exposed and a bilateral laminectomy performed at this level. An incision was made in the dura and a small area over the midline folded back. 3µl of CTB was taken up into the injection device, and the needle attached to the arm of a stereotaxic frame to provide fine control of the needle position. Using a 27gauge needle a small puncture hole was made in the spinal cord at the midline, to a depth of 2 mm. The injection needle was then inserted into the puncture hole perpendicular to the surface of the spinal cord and to a depth of 2 mm, and then withdrawn to a depth of 1.5mm, ensuring the needle terminated in the dorsal funiculus, and in or near the corticospinal tract, and creating an injury tract through the corticospinal tract to hold the injected tracer. After a delay of five minutes to allow the spinal cord tissue to seal around the needle, the CTB

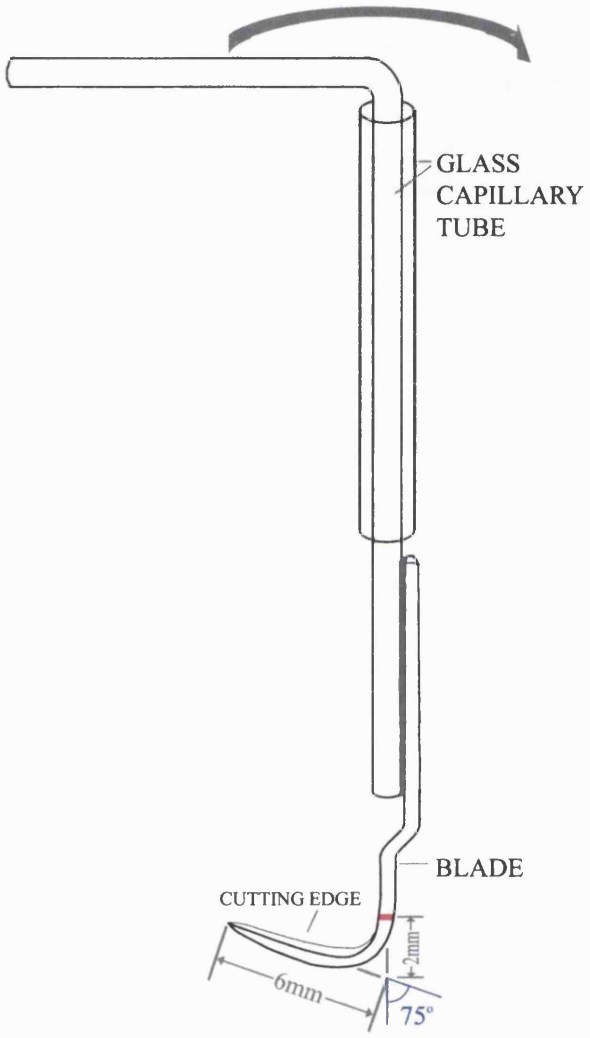

Fig. 2.1. Device used to create an intracortical lesion running parallel to the neocortical layers. The device shown in the diagram was constructed from an eye blade, the tip of which was bent to an angle of 75°, attached to a glass capillary tube. A second capillary tube served as a casing to allow free rotation of the shaft and blade. The red mark is placed such that when level with the dura the device is inserted to the required depth to create a lesion immediately below layer V. See text for details of surgical procedure. Not to scale.

solution was injected slowly, at a rate of 0.2µl every minute. When completed, a further five minutes was allowed to pass before the needle was removed to allow absorption of the tracer into the surrounding tissue. Following removal of the needle, the superficial muscle layers were sutured together and the wound closed.

Intracortical lesions were performed 24 hours later, to allow sufficient time for take-up and retrograde transport of the CTB from the injection site to the cell bodies of the corticospinal neurons. Animals were re-anaesthetised, the scalp was opened and a hole drilled into the skull at the left lateral margin, level with bregma. The device was inserted into the brain such that the blade was pointing in the rostral direction, and the positioning mark was level with the dura. Holding the outer tube of the device and keeping the shaft vertical, the blade was turned through 180° and back again, the device removed and the scalp closed.

Animals survived a further 7 days before being anaesthetised and decapitated. The forebrain was removed and prepared for ISH/CTB.

ii. Cervical corticospinal tract lesions

Animals were anaesthetised, and the cervical spinal cord exposed as described above. In the caudal part of the exposed C3 spinal cord, two puncture holes were made either side of the mid-line to a depth of 2mm, using a 27 gauge needle. Microscissor points were inserted vertically into the needle holes, and the scissors closed. Approximately 1mm above the lesion, an injection of CTB at the midline was made as described above, to retrogradely label corticospinal neurons. Animals survived a further 7 days before being anaesthetised and decapitated. The forebrain was removed and prepared for ISH/CTB.

iii. Rubrospinal tract lesions

Animals were anaesthetised and the cervical spinal column exposed as described above. The left C3 vertebral lamina was removed and a needle inserted approximately midway between the midline and the lateral edge of the cord. Microscissors were used to sever the lateral funiculus, using this needle hole to insert one microscissor blade into the cord.

Rubrospinal neurons were retrogradely labelled using a procedure similar to that described for corticospinal lesions, except that the injection needle was inserted into the lateral funiculus, approximately 1mm above the lesion, first to a depth of 1 mm and then withdrawn to a depth of 0.5 mm, so that the needle terminated in the dorsal lateral funiculus, and creating a short injury tract below to hold injected tracer. The tracer injection was carried out as described above. Animals survived a further 7 days before being anaesthetised and decapitated. The midbrain was removed and prepared for ISH/CTB.

Surgical procedure	Survival time	*n*
CTB injection into medial cervical spinal cord followed by intracortical lesion 24 hours later	8 days	3
Cervical corticospinal tract lesion and CTB injection into medial cervical spinal cord	1 day	3
	7 days	3
Cervical rubrospinal tract lesion and CTB injection into lateral cervical spinal cord	7 days	3

Table 2.3. Survival times and animal numbers used in studies of gene expression in axotomised corticospinal and rubrospinal neurons.

2.6 RT-PCR amplification of *ATF3* and *krox-24* cDNA

In this and the following sections all solvents were obtained from BDH and all other materials used were obtained from Sigma unless stated otherwise.

RNA was extracted from rat brain as follows: tissue was vortexed in 12ml guanidium thiocyanate solution (500mg/ml guanidium thiocyanate, 0.26M sodium citrate pH7, 0.5% Sarcosyl) per gram of tissue, until homogenous. With this was mixed 1 volume of phenol, 0.1 volumes 0.2M sodium acetate pH4 and 0.2 volumes chloroform. This mixture was centrifuged at 12000 rpm at 4°C for 15 minutes. The upper (aqueous) phase was removed and the RNA precipitated by adding 1 volume of isopropanol, and incubating on ice for 15 minutes, followed by centrifugation. The pellet was washed with 75% ethanol, air dried

and resuspended in diethylpyrocarbonate-treated Ultrapure water (DEPC.H_2O) (200µl DEPC was added to 1 litre of H_2O, which was left overnight and then autoclaved).

cDNA was generated as follows: 1-2µg of RNA extract and 1µg oligo-dT (Promega) in 12µl DEPC.H_2O was incubated at 65°C for 10 minutes, and then allowed to cool at room temperature for 10 minutes before being placed on ice. 4µl of 5x reverse transcriptase buffer, 1µl 25mM mixed dNTPs (Promega), 1µl RNase inhibitor (Roche) and 200 units MMLV reverse transcriptase (Promega) were added, and the mixture incubated at 37°C for 1 hour. To stop the reaction the mixture was heated to 90°C for 3 minutes. 0.5-1µl of the resulting mixture was used as the template for PCR.

Primers used for amplification of *ATF3* and *krox-24* cDNA were as follows:

ATF3:	sense:	TAG AAT TC*G GAA CAT TGC AGA GCT AAG C
	antisense:	ATC GAA ATT AAC CCT CAC TAA AGG G*AC GCC AAT GTT GTG CAA GAC
krox-24:	sense:	TAG AAT TC*A GTT TGC CAG GAG TGA TGA AC
	antisense:	ATC GAA ATT AAC CCT CAC TAA AGG G*CT ACT GAC TAG AAG GAC TTG G

Primers for *ATF3* were chosen to amplify bases 691-1252 of Genbank sequence M63282 (Hsu et al., 1991). Primers for *krox-24* were chosen to amplify bases 1482-2011 of Genbank sequence M18416 (Milbrandt, 1987). Both antisense primers also contain the T3 polymerase promoter at the 5' end, to allow generation of probes from PCR product. Both sense primers contain a restriction enzyme site in the first 8 bases. The asterisks mark the beginning of the target gene sequences.

PCR reaction mixture was made up as follows: 2µl each dNTP (10mM), 6µl 25mM Mg^{2+}, 10µl Mg^{2+}-free Taq buffer, 0.5µg each primer, 2U Taq polymerase (Promega), in a total of 100µl H_2O overlaid with light mineral oil (Sigma). Reaction temperature cycles were as follows:

ATF3:	94°C 3' x1; 58°C 1', 72°C 1', 94°C 20" x36; 58°C 1', 72°C 10' x1.
krox-24:	94°C 3' x1; 60°C 1', 72°C 1', 94°C 20" x36; 60°C 1', 72°C 10' x1

PCR reaction product was purified by agarose gel electrophoresis followed by excision of the product band from the gel and extraction of the DNA with Qiagen Gel Extraction Kit. 0.5-1µg of the extracted DNA was used as template for in vitro transcription for the generation of cRNA probes.

The identity of the PCR products was confirmed by restriction enzyme digestion and by comparing *ATF3* and *krox-24* probe ISH signals with the findings of Tsujino et al. (2000) and Schlingensiepen et al. (1991) on the expression of *ATF3* in lumbar dorsal root ganglia after sciatic nerve crush (data not shown) and the expression of *krox-24* in neocortex and hippocampus.

2.7 Digoxigenin-labelled cRNA probes

i. Probe synthesis

cRNA probes were generated from cDNA containing plasmids or from RT-PCR product generated from rat brain RNA (see above), as detailed in Table 2.4. Sense sequence probes are often used as a control in ISH, as they reveal artefacts from the procedure and possible non-specific binding of the probe itself, if it has an unusual base composition. Therefore, both antisense and sense probes were generated for each mRNA, except *ATF3* and *krox-24*, for which only antisense probes were made. However, other sense probes of similar length and base composition served as suitable controls for these two antisense probes.

Minipreps of plasmids containing cDNA sequences for each probe were prepared using Qiagen Spin Miniprep Kits and then linearised using the appropriate restriction enzyme. 1µg of the resultant linear DNA or 0.5-1µg of RT-PCR product was used as template for the generation of cRNA probes labelled with digoxigenin. These were generated by in vitro transcription using digoxigenin (DIG)-labelled ribonucleotides, according to the manufacturer's recommendations (Boehringer Mannheim, Germany) using T3, T7 or Sp6 RNA polymerase as appropriate. Following ethanol precipitation of the labelled RNA, the pellet was resuspended in de-ionised formamide, which prolongs the storage life of RNA molecules (Chomczynski, 1992), or in DEPC.H_2O in the case of L1 and CHL1 (to

Probe	Plasmid	Part of cDNA	Species	Size of template	Hybridisation temperature /°C	Source
CAP-23	pcDNA3	5' end of ORF	Mouse	190 bp	65-68	P. Caroni
SCG10	pcDNA3	Entire ORF	Rat	500bp	62-64	G. Grenningloh
GAP-43	pcDNA3	Entire ORF	Rat	681 bp	62-64	
FKBP12	pBluescript	Entire ORF	Rat	390bp	62-64	S. Dawson
L1	pBlue	Region coding for extra-cellular domain	Mouse	3.4 kb *	62-64	M. Schachner
CHL1	pBlue	Region coding for extra-cellular domain	Mouse	3.4 kb *	62-64	M. Schachner
c-jun	pBlue	Entire ORF	Mouse	1127 bp	62-64	
ATF3	n/a	Mostly 3'UTR	Rat	562 bp	62-64	RT-PCR product
krox-24	n/a	ORF/ 3'UTR	Rat	530 bp	62-64	RT-PCR product

Table 2.4. Details of cDNA templates for cRNA probes used for ISH.

* These probes were reduced to an average size of approximately 600 bases by alkaline hydrolysis prior to use.

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facilitate alkaline hydrolysis). Formamide (Fluka) was de-ionised by treatment with 0.1mg/ml BioRad Mixed Bed Resin (AG501-x8).

Following preparation of the probes, probe quality was assessed by agarose gel electrophoresis followed by blotting of the gel onto Hybond-N+ nylon membrane, and visualisation of the probe using a modified version of the chromogenic reaction used for ISH (for details of these reagents see *In situ hybridisation* below). In the case of probes for L1 and CHL1, this was carried out prior to alkaline hydrolysis. Membrane was incubated in modified blocking medium for 15 minutes, and anti-DIG antibody fragments

were added at a dilution of 1:5000. The membrane was washed for 15 minutes in buffer 1, followed by 15 minutes in ISH buffer 3 and finally 1-24 hours in colour developer. Probe strength was evaluated by dot-blots of neat probe and serial 1:3 dilutions of probe. An established working probe was also examined for comparison. 2µl of each sample was spotted onto Hybond-N+ membrane and the probes visualised on the membane as described above, with colour development proceeding for 1 hour.

ii. Alkaline hydrolysis of L1 and CHL1 probes

To improve tissue penetration, L1 and CHL1 probes were reduced in size after synthesis to an average of 600 bases, by alkaline hydrolysis. The aqueous probe solution was mixed with an equal volume of 80mM $NaHCO_3$, 120mM Na_2CO_3 and incubated at 60°C for 12.5 minutes, according to the formula Time=(initial length - final length)/(0.11x initial length x final length). The reaction was halted by adding 0.03 volumes of 3M sodium acetate (pH 6.0) and 0.05 volumes of 10% glacial acetic acid. Probes were then ethanol precipitated and the pellet resuspended in de-ionised formamide.

2.8 Histochemical procedures: In situ hybridisation (ISH) and CTB immunohistochemistry

i. Preparation of microscope slides for ISH

Silanised slides for ISH were prepared by Julia Winterbottom as follows: glass slides were washed in a 10% solution of Decon detergent for 2 hours, followed by running tap water for 20 minutes and distilled water (dH_2O) for 2-3 minutes. Slides were then immersed in 0.5% HCl 95% ethanol for 15 minutes and washed for 2 minutes in dH_2O. The slides were then autoclaved and allowed to dry completely at room temperature, following which they were dipped in 6% 3-aminopropyltriethoxy-silane in acetone for 5 minutes. They were then rinsed in acetone twice for 2 minutes and in dH_2O twice for 2 minutes and allowed to dry.

ii. Preparation and sectioning of tissue for in situ hybridisation (ISH) or ISH and CTB immunohistochemistry

All tissue dissected for in situ hybridisation was kept moist during dissection, and was immediately placed into OCT (TissueTek) in plastic moulds before being frozen on powdered dry ice.

Cryostat sections of forebrain, cerebellum, spinal cord and DRG were cut at a nominal thickness of 10-12μm and thaw-mounted onto slides coated with 3-aminopropyltriethoxy-silane. Consecutive sections of each spinal cord and DRG (from animals listed in Table 2.1) or grafted forebrain or cerebellum (from animals listed in Table 2.2) were placed in turn onto three or four slides, each slide to be hybridised with a different riboprobe. This allowed comparison of adjacent sections with hybridisation signal for different molecules. Blocks of thalamus or cerebellum containing nerve grafts to be processed for ISH and CTB immunohistochemistry were sectioned in series of 4 consecutive sections mounted alternately onto two slides, one for ISH and one for CTB, ensuring that each of the middle two sections was flanked by sections reacted for the complementary protocol. Forebrain and midbrain from animals which had received corticospinal tract or rubrospinal tract lesions and spinal CTB injections were cut in series of 9 consecutive sections placed onto 9 slides. Odd numbered slides were then reacted for CTB immunohistochemistry while even numbered slides were processed for ISH for one of the mRNAs of interest. This ensured that for every section processed for ISH, both flanking sections had been processed for CTB.

Sections were fixed with 4% paraformaldehyde in phosphate buffer (PB) made in DEPC.H_2O overnight at 4^0 C. Sections were given three washes in 0.1M phosphate-buffered saline (PBS), and were either dehydrated in ascending ethanols for storage at 4°C or -20°C or reacted immediately.

iii. In situ hybridisation (ISH)

In situ hybridisation was carried out as described by Bartsch et al. (1992) and Zhang et al. (1995). All aqueous solutions used up to post-hybridisation washes were made with

DEPC.H_2O RNase-free molecular biology reagents were used until the post-hybridisation washes; these were obtained from Sigma unless otherwise stated.

Sections were rehydrated in descending alcohols if necessary and washed twice in DEPC.H_2O for 5 minutes. To permeabilise the tissue, sections were treated with 0.1 M HCl for 5-10 minutes. Following a brief wash in PBS sections were incubated in 0.1M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 20 minutes. This neutralises positively charged amino-acids which would otherwise cause the negatively-charged RNA probes to bind in a non-specific manner. Sections were washed again with PBS, dehydrated in an ascending ethanol series and air dried. The area of the slide containing sections was delineated with grease pen.

Prehybridisation was carried out at 37^0 C for 3 hours with a mixture of prehybridisation buffer/de-ionised formamide 1:1 (containing 50% formamide, 25 mM ethylenediaminetetra-acetic acid (EDTA), 50mM pH 7.6 Tris-HCl, 2.5x Denhardt's solution, 0.25mg/ml tRNA (Roche), and 20 mM NaCl. Sections to be incubated with this solution were placed in slide incubation chambers containing 20-30ml 0.05M PBS/50% formamide soaked in filter paper, to ensure the interior of the chamber retained sufficient humidity to prevent the sections from drying out.

The digoxigenin labelled **sense and antisense probes for all mRNAs** were prepared at a concentration of 3 µl/ml with hybridisation buffer containing 50% formamide, 20 mM Tris-HCl (pH 7.50), 1 mM EDTA, 1x Denhardt's solution, 0.5 mg/ml tRNA, 0.1mg/ml poly-A RNA, 0.1 M dithiothreitol (DTT), and 10% dextran sulphate. Hybridisation was performed overnight in the incubation chambers (see Table 2.4 for hybridisation temperatures). Further 0.05M PBS/50% formamide was added to the chambers and these were sealed with adhesive tape before being placed at the hybridisation temperature.

After hybridisation, washes were performed at the hybridisation temperature to remove excess probe. Sections were given two five-minute washes in 0.2x standard saline citrate (0.2xSSC; containing 30 mM NaCl and 3mM Na-citrate, pH 7.0) and then in three 30 minute washes in 0.1x SSC/ 50% formamide. Sections were equilibrated with buffer 1

(100mM Tris-HCl, 150 mM NaCl, pH 7.5), then incubated in modified blocking medium (1% blocking reagent (Roche), 0.5% BSA fraction in buffer 1) and incubated with alkaline phosphatase-coupled antibodies to digoxigenin (Roche) at a dilution of 1:750-1:1500 in modified blocking medium overnight at 4^0 C. Sections were washed in buffer 1, equilibrated in buffer 3 (100 mM Tris-base, 100 mM NaCl, 50mM $MgCl_2$, adjusted to pH 9.55), and developed in the dark with buffer 3 containing 0.34 mg/ml 4-nitroblue tetrazolium chloride, 0.175 mg/ml 5-bromo-4-chloro-3-indolylphosphate, and 0.25 mg/ml levamisol. Development was stopped by washing with buffer 4 (10mM Tris-HCl, 1mM EDTA, pH 8.0), following which the sections were dried and mounted with DPX (BDH) beneath glass coverslips. The specificity of the hybridisation signal was verified by comparison with the sections processed with sense probe under identical conditions.

The colour development reaction was observed generally to proceed for a maximum of 2-3 days. In the case of some sections processed for CAP-23 ISH, development was extended by removal of the alkaline-phosphatase labelled antibody with 0.01M HCl, which is effective at disrupting antibody-antigen binding with minimal damage to antigenicity (Blanchard et al., 1990), followed by incubation with fresh antibody. Sections were washed once in buffer 1, twice in dH_2O, followed by 3x5 minutes in 0.01M HCl and a final wash in buffer 1. Blocking, antibody incubation and colour development then proceeded as before.

iv. CTB immunohistochemistry

Sections were rinsed in 0.1M PBS and immunoreacted for CTB as described by Campbell et al. (1999). Briefly, after two washes in PBS, sections were incubated for 15 min with 0.3% H_2O_2 in PBS to remove endogenous peroxidase activity. Following another two PBS washes sections were exposed for 30 minutes to blocking buffer (5% normal rabbit serum and 0.3% triton X-100 in PBS) before incubation with polyclonal goat anti-CTB antibody (List Biologicals, Campbell, California) diluted 1 : 80,000 in blocking buffer for 48 hours at 4°C, under a coverslip. Two washes in PBS then preceded incubation for 2 hours with polyclonal rabbit anti-goat IgG diluted 1 : 200 in blocking buffer. Another two washes in PBS were followed by treatment for 2 hours with the avidin-biotin-peroxidase complex (ABC elite kit, Vector, USA) diluted 1 : 200 in PBS. After two further washes in PBS,

immunoreactivity was visualised using a solution consisting of 0.04% 3-3'-diaminobenzidine tetrahydrochloride (DAB), 0.006% nickel chloride and 0.009% H_2O_2 in 0.1M Tris-buffered saline pH 7.6. Sections were then washed twice in PB, counterstained in 0.001% thionine for 10-30 seconds, washed in dH_2O, left overnight to dry and mounted under a coverslip with DPX.

2.9 Studies on transgenic mice overexpressing GAP-43 (Chapter 7)

A. Animals and genotyping

The wt3 strain of transgenic mice described by Aigner et al. (1995) were studied. These overexpress chick GAP-43 under the control of a modified Thy-1 promoter, which drives neuron specific expression commencing postnatally.

Stocks of wt3 mice were maintained as heterozygotes, and animals were genotyped by PCR amplification of transgene from genomic DNA. The following primers, which amplify the ORF of chick GAP-43 (Moss et al., 1990) were used:

Sense: GCC TCG AGG ATC ATG CTG TGC TGT
Antisense: GCC TCG AGG ATC CCC GGG AGT CAT

To guard against false negative results due to failure of the PCR or insufficient genomic DNA in the reaction, positive control primers were included in the genotyping PCR reactions. These were designed to amplify a part of the intronless mouse gene ferritin light chain (corresponding to nucleotides 174-709 of Genbank sequence M73706; Renaudie et al., 1992):

Sense: CAT GAC CTC TCA GAT TCG TCA G
Antisense: TGA GGC GCT CAA AGA GAT ACT C

Genotyping proceeded as follows:

Animals were anaesthetised and approximately 1cm of tail removed, using a new razor for each animal, and samples were stored at -20°C if necessary. Tail samples were digested by incubation in 300µl TNES buffer (50mM Tris pH 8.0, 100mM EDTA, 100mM NaCl, 1% lauryl sulphate) containing 0.5mg/ml proteinase K (Roche) overnight at 55°C. The digests were then centrifuged at 13000rpm for 3 minutes and a sample of the supernatant taken and diluted 1:50. 10µl of this supernatant dilution was used for the subsequent PCR reaction.

Each reaction tube contained 10µl of tail digest, 2µl each deoxynucleotide triphosphate (10mM), 6µl 25mM Mg^{2+}, 10µl Mg^{2+}-free Taq buffer, 0.5µg each primer, 2U Taq polymerase (Promega), in a total of 100µl H_2O overlaid with light mineral oil (Sigma). Reaction temperature cycles were as follows:

94°C 5' x1; 64°C 1', 72°C 1', 94°C 1' x3; 64°C 1', 72°C 1', 94°C 20" x36;
64°C 1', 72°C 10' x1

PCR product was assessed by agarose gel electrophoresis. A successful PCR reaction yielded either one band (indicating a non-transgenic animal) or two bands (indicating a transgenic animal).

B. Surgical procedures

i. Animals

Animals were between 1 and 3 months old when first operated and both males and females were used. Non-transgenic mice used as controls were litter-mates of experimental transgenic animals.

ii. Dorsal column injuries on wt3 mice

A total of 25 wt3 mice received lesions of the ascending dorsal columns. Animals were anaesthetised and an incision made along the dorsal midline and the spinal vertebrae exposed at either midthoracic or lumbar level. A bilateral laminectomy was performed to expose either mid-thoracic or lumbar (L2-L3) spinal cord. An incision was made in the dura and a small area over the midline folded back. Using a 27 gauge needle two puncture holes were made either side of the mid-line to a depth of 1mm. Microscissor points were inserted vertically into the needle holes, and the scissors closed to sever the dorsal columns. Overlying muscle layers were sutured together and the wound closed. Survival times were 2.5-3.5 weeks. Details of animal genotypes, numbers, lesion levels and survival times are given in Table 2.5. Surgery on five of these animals was performed by Prof. Patrick Anderson.

iii. Dorsal root injuries on wt3 mice

Four transgenic wt3 mice and two of their non-transgenic litter-mates received unilateral lumbar dorsal root transections and re-anastomosis. Animals were anaesthetised, and a parasagittal incision was made over the lumbar spinal cord, to the left of the midline. The left L2-L4 vertebral laminae were exposed and the lateral margins of these laminae were removed to expose the L4-L6 dorsal roots. These were severed, taking care to cut all minor branches entering the spinal cord from these roots, and the proximal and distal portions of the L4 and L5 roots were re-anastomosed using 10/O sutures. The wound was closed. The survival time for these animals was 3 months.

iv. Transganglionic labelling

Prior to sacrifice, transganglionic labelling of the central axons of the lumbar primary sensory neurons was performed on all operated wt3 mice, using cholera-toxin B-subunit conjugated to horseradish peroxidase (CT-HRP). Animals were re-anaesthetised and an incision was made over the left tibia and parallel to it. Underlying muscles were separated to reveal the left sciatic nerve. Using a 27 Gauge needle a small hole was made in the epineurium and in the perineurium of the tibial branch of the nerve. A ligature of 5/O suture

thread was placed around the nerve above the incision and 0.5-0.7µl of CT-HRP (14mg/ml) was taken up into the injection device, and the needle carefully inserted into the exposed endoneurium. The ligature was tightened around the part of the nerve containing the needle, and the tracer slowly injected into the nerve over several minutes. The needle was then removed, the ligature tightened further to prevent post-operative leakage of the tracer, and the wound closed. Animals were anaesthetised and killed 2 days later by transcardiac perfusion with 20ml PBS followed by fixative (0.1M phosphate buffer pH 7.2-7.4, 1% paraformaldehyde, 1.25% glutaraldehyde).

In the case of dorsal column injury, a section of the spinal cord containing the lesion site and the dorsal column nuclei were removed. In the case of dorsal root injury, the L4/L5 spinal cord and the dorsal column nuclei were removed. Tissue was stored overnight at 4°C in 30% sucrose in PBS, and frozen in OCT.

Approximately half of all transganglionic labelling procedures were performed by Prof. Patrick Anderson.

Surgical procedure	Genotype	Survival time	Orientation of sections	*n*
Thoracic dorsal column injury	Transgenic	2.5-3 weeks	Horizontal	3
	Transgenic	2.5-3 weeks	Parasagittal	2
	Non-transgenic	2.5-3 weeks	Horizontal	1
	Non-transgenic	2.5-3 weeks	Parasagittal	2
Lumbar dorsal column injury	Transgenic	3-4 weeks	Horizontal	4
	Transgenic	3-4 weeks	Parasagittal	7
	Non-transgenic	3-4 weeks	Horizontal	3
	Non-transgenic	3-4 weeks	Parasagittal	3
L4/L5 dorsal root injury	Transgenic	3 months	Horizontal	4
	Non-transgenic	3 months	Horizontal	2

Table 2.5 Survival times, processing and animal numbers used in studies on transgenic mice overexpressing GAP-43 (Chapter 7).

C. Histochemical procedures

i. Preparation of gelatinised microscope slides

Gelatinised slides were prepared by Julia Winterbottom. Twinfrost glass slides were arranged on racks and dipped for at least 1 minute into a solution of 1.5% gelatin and 0.25% chromium potassium sulphate in dH_2O. The slide racks were wrapped in foil and placed at 50°C overnight.

ii. Visualisation of tracer

CT-HRP was visualised using the method of Mesulam (1982). Parasagittal or horizontal free-floating microtome sections of spinal cord were cut at a nominal thickness of 40µm (see Table 2.5 for details). Dorsal column nuclei were sectioned at 50µm thickness. Sections were stored in PBS during cutting and then washed 6 times for 10-15 seconds in distilled water before being transferred to 0.001M sodium acetate buffer pH 3.3, 1mg/ml sodium nitroferricyanide, 50µg/ml tetramethylbenzidine (dissolved in ethanol before addition to this solution) at 0-4°C. Sections were incubated in this solution on ice for 20 minutes and were then removed. H_2O_2 was added to a final concentration of 0.06µl/ml and the sections reimmersed for 5-20 minutes on ice in the dark. The reaction was halted by transferring the sections to 0.02M acetate buffer, pH 3.3. Sections received five further washes in this solution over half an hour, and were then mounted onto gelatinised slides and allowed to dry overnight before being mounted with DPX under glass coverslips.

iii. Immunohistochemistry

Transgenic wt3 mice express avian GAP-43 mRNA in primary sensory neurons (Aigner et al., 1995; Mason et al., 2000). To confirm that avian GAP-43 protein is produced and transported into the central axon branches, immunohistochemistry was performed using monoclonal antibody 5F10 (gift of P. Caroni), which specifically recognises avian but not mouse GAP-43. The procedure used was that described in Mason et al. (2000). Two transgenic wt3 mice were anaesthetised and transcardially perfused with 4%

paraformaldehyde in phosphate buffer, pH7.4. Cryostat sections were cut at 14-18μm thickness onto gelatinised slides.

A solution consisting of PBS, the monoclonal antibody 5F10 diluted 1:10 and a monovalent goat anti-mouse IgG antibody (Jackson Immunochemicals) diluted 1:500 was incubated for 2 hours at room temperature. (As a control, some sections were processed using a mixture from which the primary antibody had been omitted.) Normal mouse serum was added to a concentration of 2% and the mixture incubated for a further hour, following which normal rabbit serum was added to a concentration of 10%. Sections were incubated with tertiary antibody block (5% normal rabbit serum, 0.5% Triton X-100) for 1 hour to block non-specific staining and then incubated with the antibody mixture at 4°C overnight. Sections were washed and biotinylated rabbit anti-goat IgG was applied, diluted 1:200 in tertiary antibody block, followed by 3 PBS washes, and processing with the ABC kit (Vectastain), using 1:100 dilutions of ABC reagents. Labelling was then visualised with 0.04% DAB and 0.009% H_2O_2 in 0.1M Tris-buffered saline pH 7.6. Sections were then washed twice in PB, washed in dH_2O, dehydrated in ascending ethanols and mounted under a coverslip with DPX.

Chapter 3

Expression of *SCG10*, *CAP-23* and *GAP-43* in neurons regenerating their axons after a sciatic nerve injury

3.1 Introduction

Following crush or cut of the sciatic nerve, injured motor and sensory axons regenerate to their targets, resulting in a degree of functional recovery (Fawcett and Keynes, 1990). Regeneration is accompanied by the upregulation of growth-associated molecules, such as c-Jun, GAP-43 and Tα1 tubulin (Skene and Willard, 1981; van der Zee et al., 1989; Miller et al., 1989; Woolf et al., 1990; Jenkins and Hunt, 1991; Chong et al., 1992). These are normally down-regulated by 5-6 weeks after a crush injury when reinnervation of target tissues is completed, but if reinnervation is prevented by ligation of the nerve, expression persists for at least 6 weeks, although it may decline slightly (Miller et al., 1989; Chong et al., 1994b). Presumably, reconnection with target tissues sends a retrograde signal to the cell bodies of successfully regenerated neurons for the downregulation of growth associated proteins. In contrast, injury to the central branches of the axons of primary sensory neurons results in minimal regeneration and little or no increase in expression of growth-associated molecules (Schreyer and Skene, 1993; Jenkins et al., 1993b; Zhang et al., 2000). The growth-cone molecules SCG10 and CAP-23 are good candidates for molecules which may be important for axon regeneration. To determine whether they are upregulated by DRG and motor neurons regenerating their axons after peripheral nerve injury, expression of *SCG10* and *CAP-23* mRNAs was examined by in situ hybridisation in lumbar motor neurons and L4/L5 DRG neurons after a unilateral (left) sciatic nerve crush. Expression of these mRNAs was also examined following cut and ligation to the sciatic nerve or dorsal root injury, to determine if the regulation of these molecules parallels that of other growth-associated molecules after these procedures. Expression of *GAP-43* mRNA was also examined in all cases, as a positive control for the expression of growth-associated molecules by the injured neurons and to enable comparison of the temporal and spatial patterns of expression of all three molecules. Animal survival times and numbers are shown in Table 3.1.

Surgical procedure	Survival time	*n*
Sciatic nerve crush	1 day	3
	3 days	3
	7 days	3
	14 days	3
	5 weeks	1
	6-7 weeks	3
Sciatic nerve cut and ligation	14 days	1
	5 weeks	2
	6-7 weeks	3
Dorsal root injury	3 days	1
	7 days	1
	24 days	1

Table 3.1 Survival times and number of animals used for studies of *SCG10*, *CAP-23* and *GAP-43* expression following injury to the sciatic nerve or lumbar dorsal roots.

3.2 Results

i. Expression of SCG10, CAP-23 and GAP-43 in spinal motor neurons of unoperated animals

To examine the constitutive expression of *SCG10*, *CAP-23* and *GAP-43* in the spinal motor neurons, ISH was performed on the lumbar spinal cord of two unoperated animals. Expression of *SCG10* and *CAP-23* in the intact lumbar spinal cord was found to be identical to that on the unoperated side of the cord following sciatic nerve injury, which may be seen on the contralateral (right) side of the spinal cord sections shown in Figs. 3.1 and 3.2. *SCG10* was strongly expressed by the majority of motor neurons, with weak or moderate expression in the remaining motor neurons. Many other neurons throughout the grey matter showed weak or moderate expression, with the exception of neurons in laminae I and II of the dorsal horn, where no expression was found. *CAP-23* was weakly

expressed by all motor neurons, and by the great majority of other neurons in the grey matter. Motor neurons expressed very low levels of *GAP-43* message.

ii. Expression of SCG10, CAP-23 and GAP-43 in spinal motor neurons following sciatic nerve injury

Animals were examined at time points between 1 day and 6-7 weeks after sciatic nerve crush (see Tables 2.1, 3.1) and ISH signals for *SCG10*, *CAP-23* and *GAP-43* in motor neurons ipsilateral to the injury were compared to those on the contralateral side. Sciatic nerve crush or cut and ligation provoked upregulation of *SCG10*, *CAP-23* and *GAP-43* in the injured motor neurons (Figs. 3.1, 3.2). No upregulation of either *SCG10* or *CAP-23* was observed at 1 day, although adjacent sections showed upregulation of *GAP-43*, but *SCG10* and *CAP-23* were clearly upregulated in ipsilateral motor neurons from 3 days onwards (Fig. 3.1). Expression of both *SCG10* and *CAP-23* was increased in magnitude at 6 days and further increased at 2 weeks (Fig. 3.2). The increases in expression in *SCG10* and *CAP-23* appeared to be similar to each other but both showed a less marked increase than did *GAP-43*. When serial sections from animals surviving 6 days or longer after injury were hybridised with probes for *SCG10*, *GAP-43* and *CAP-23* mRNAs, the same cells were shown to have upregulated all three molecules (Fig. 3.3, left). At two weeks after surgery, no difference was observed in the magnitude of upregulation of *SCG10* and *CAP-23* between a sciatic nerve crush and a cut and ligation injury (not shown). At 6-7 weeks after sciatic nerve crush, both *SCG10* and *CAP-23* had returned to normal levels (Fig. 3.4, right column), although the signal for *GAP-43* in the injured motor neurons remained slightly elevated, albeit significantly reduced from that seen at two weeks. At five weeks and 6-7 weeks after cut and ligation of the sciatic nerve, all three molecules were still significantly upregulated (Fig. 3.4, left column).

iii. Expression of SCG10, CAP-23 and GAP-43 in lumbar dorsal root ganglion neurons of the unoperated animal

Constitutive expression of *SCG10*, *CAP-23* and *GAP-43* in lumbar primary sensory neurons was examined by ISH on L4 and L5 dorsal root ganglia (DRG) from unoperated animals. Expression was found to be indistinguishable from that in the DRG on the

unoperated side of experimental animals, shown in Figs. 3.5-3.7 (right-hand columns). Moderate to strong *SCG10* expression was found in all DRG neurons, and a few small to medium sized cells showed very high levels (Fig. 3.5, right column). Low levels of *CAP-23* were found in nearly all small to medium diameter DRG neurons, with some cells showing stronger expression (Fig. 3.6, right column). A minority of large diameter DRG neurons also showed weak expression, but the remainder were devoid of *CAP-23*. Most large diameter neurons did not have detectable levels of *GAP-43*, although a minority had moderate levels. Many of these neurons were negative for both *GAP-43* and *CAP-23* (Fig. 3.8, lower panel). Some *GAP-43* was found in nearly all small to medium sized DRG neurons (Fig. 3.7, right column; Fig. 3.8, lower panel) with most having only a low level, and a subset having relatively high levels. Where it could be determined by studying adjacent sections, this subset of cells appeared to be the same neurons as those expressing high levels of *SCG10* and *CAP-23* (Fig. 3.8, lower panel).

iv. Expression of SCG10, CAP-23 and GAP-43 in lumbar dorsal root ganglion neurons following sciatic nerve injury

At time points of between 1 day and 6-7 weeks after a crush injury or a transection and ligation of the sciatic nerve, ISH for *SCG10*, *CAP-23* and *GAP-43* was performed on lumbar primary sensory neurons and the hybridisation signals in neurons ipsilateral to the injury were compared to those on the contralateral side. One day after a sciatic nerve crush, no increase in *SCG10* or *CAP-23* mRNAs could be detected (Figs. 3.5, 3.6), although *GAP-43* was clearly increased (Fig. 3.7). From 3 days until at least 2 weeks after injury, levels of *SCG10*, *CAP-23* were increased in neurons of all sizes in ipsilateral DRGs (Figs. 3.5, 3.6) and the higher levels of *GAP-43* were maintained (Fig. 3.7). The increases in expression in *SCG10* and *CAP-23* appeared to be similar to each other, and similar to that seen in motor neurons. Again, both these mRNAs showed a less marked increase than did *GAP-43*.

Where individual neurons could be identified in more than one consecutive serial section, those neurons which had upregulated one of these molecules were found also to have upregulated the molecule probed for on the adjacent section. Some neurons could be identified on three consecutive sections and had clearly upregulated all three molecules

(Fig. 3.8). Two weeks after injury no difference was observed in the magnitude of upregulation of *SCG10* and *CAP-23* in injured DRG neurons between animals which had received a cut and ligation of the sciatic nerve and those which had received a crush (not shown). By 6-7 weeks after sciatic nerve crush, the expression of both *SCG10* and *CAP-23* had declined to normal levels (Fig. 3.9, lower panels in each set), but after sciatic nerve cut and ligation injuries, the expression of *SCG10*, *CAP-23* and *GAP-43* was seen to remain elevated in DRG neurons 5 weeks and 6-7 weeks following injury (Fig. 3.9, upper panels in each set), although the signals were somewhat reduced compared with animals killed after two weeks.

v. Expression of SCG10, CAP-23 and GAP-43 in lumbar dorsal root ganglion neurons following dorsal root injury

Three animals received a transection and re-anastomosis of the dorsal roots of the L4 and L5 spinal nerves. At 3, 7 and 24 days after injury, no obvious increase in the number of *CAP-23*, *SCG10* or *GAP-43* mRNA-positive cells, nor in the intensity of hybridisation signals, was observed in the axotomised neurons (not shown).

Figs. 3.1, 3.2. In situ hybridisation (ISH) with digoxigenin-labelled probes for *SCG10*, *CAP-23* and *GAP-43* in the lumbar spinal cord between 1 and 14 days following unilateral sciatic nerve crush. In the motor neurons on the contralateral (right) side of each section, a moderate constitutive level of expression of *SCG10* and *CAP-23* can be seen. Upregulation of both these mRNAs is visible in the motor neurons on the crush (left) side from 3 days onwards, while *GAP-43* is upregulated from 1 day onwards.

Bar 500μm.

FIGURE 3.1

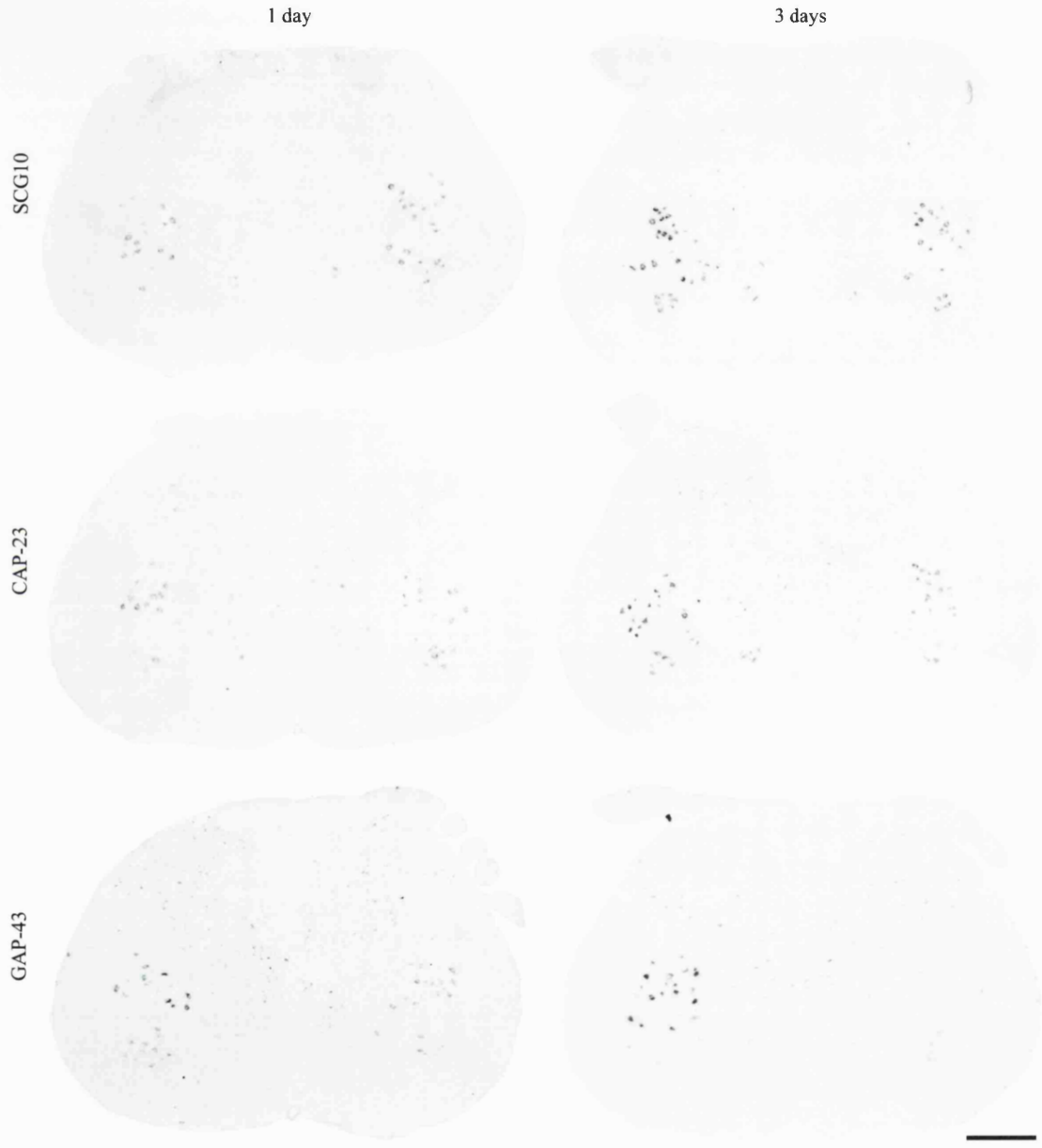


FIGURE 3.2

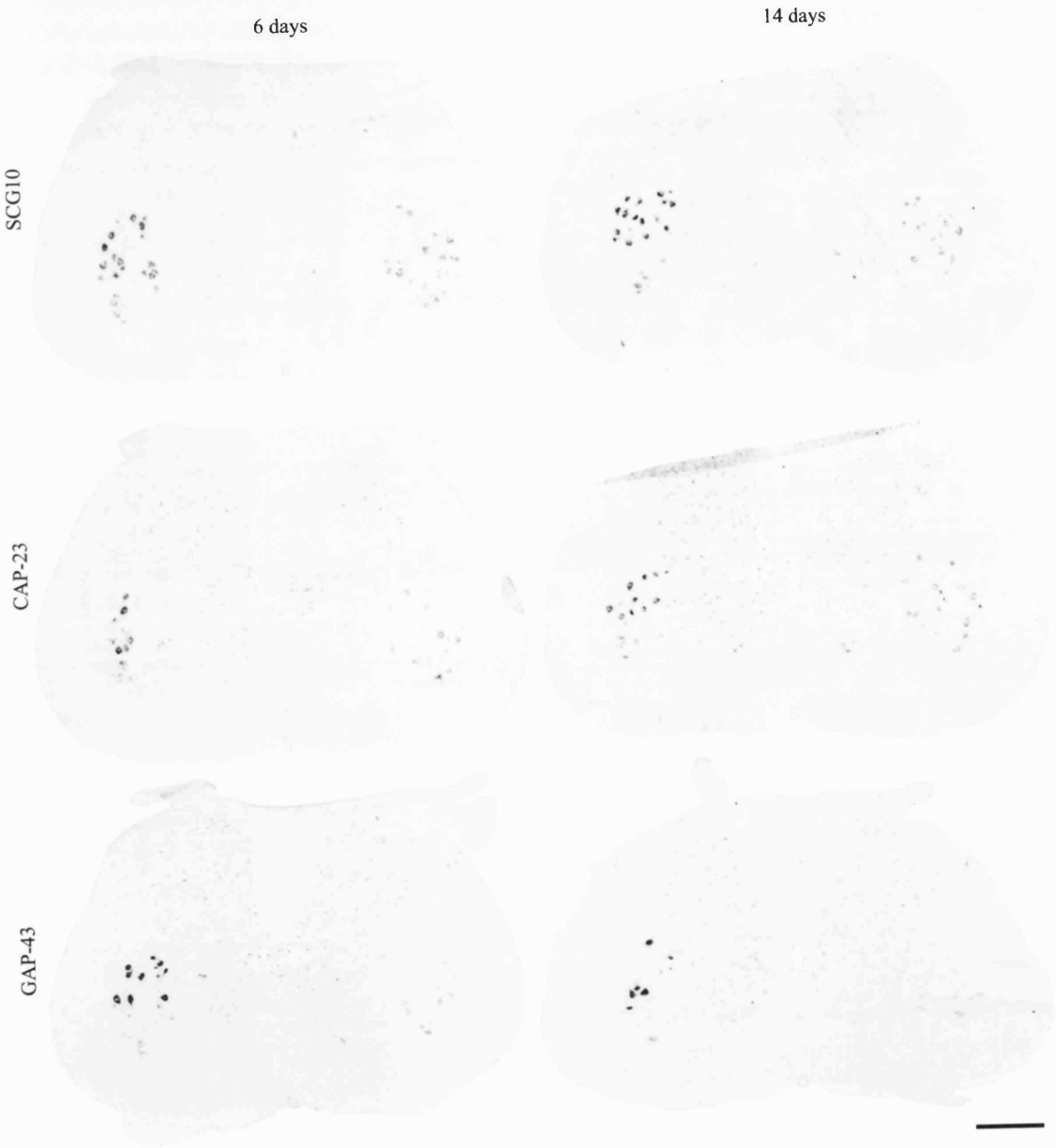


Fig. 3.3. ISH for *SCG10*, *GAP-43* and *CAP-23* on serial sections through lumbar spinal cord 6 days after sciatic nerve crush. On the side of the injury the same motor neurons have upregulated all three molecules (the numbers1-7 identify corresponding profiles of individual neurons in the three serial sections). In the motor neurons on the contralateral side, *SCG10* and *CAP-23* are expressed at low levels and *GAP-43* expression is barely detectable (the two lower panels on the right show the contralateral motorneurons of the sections shown on the left, but the upper panel shows another section from the same series).

Bar 50 μm.

FIGURE 3.3

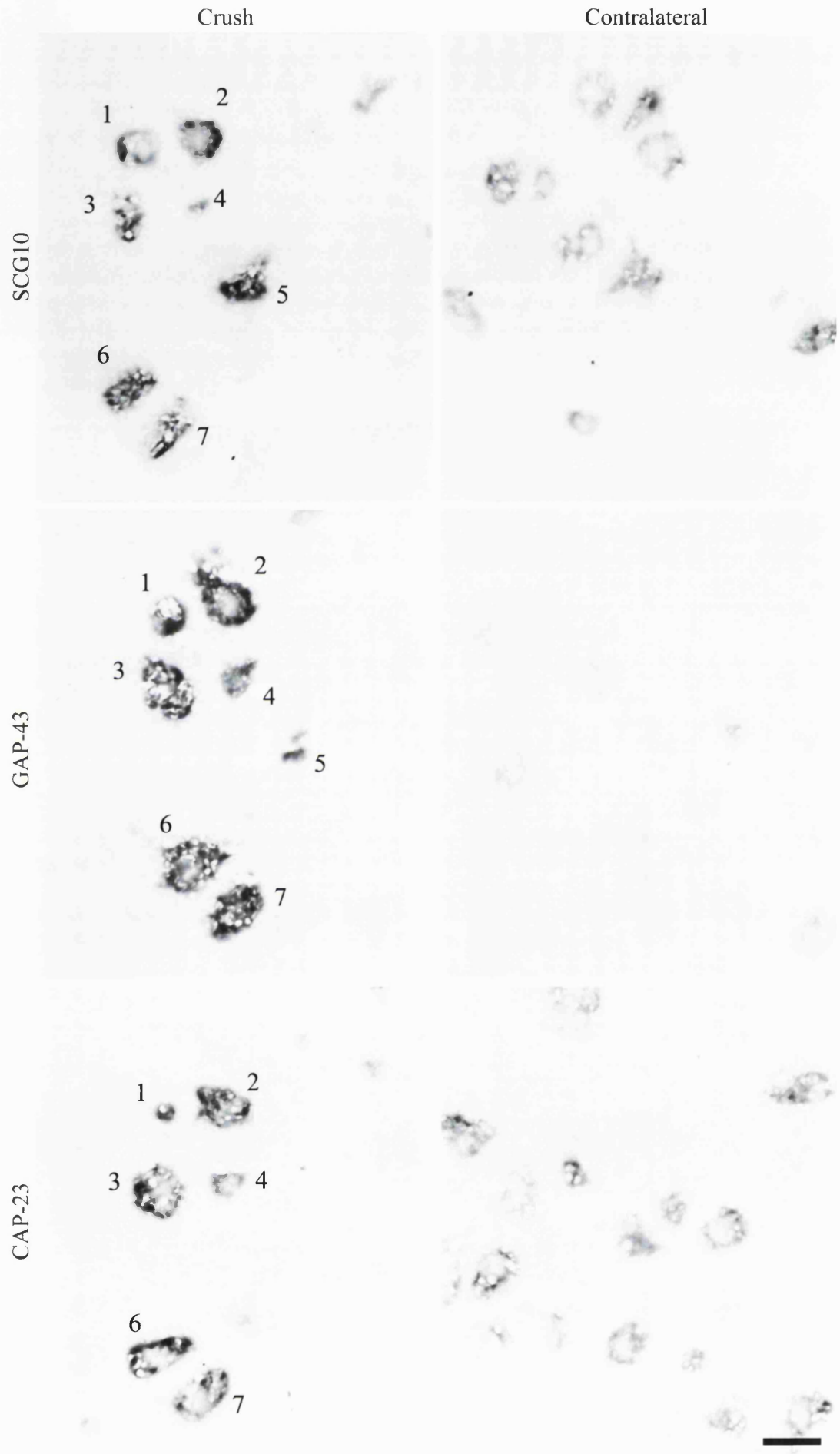


Fig. 3.4. Comparison of expression of *SCG10* and *CAP-23* in lumbar motor neurons 6-7 weeks after crush or cut and ligation of the sciatic nerve. By this time afer a crush injury, *SCG10* and *CAP-23* expression has returned to basal levels **(right column).** In contrast, cut and ligation of the nerve, which prevents axons from regenerating to and reinnervating their targets, also prevents the downregulation of these two molecules **(left column).**

Bar 500μm.

FIGURE 3.4

Fig. 3.5. Expression of *SCG10* in L5 dorsal root ganglion neurons 1 day, 3 days, 6 days and 14 days following unilateral sciatic nerve crush. On the contralateral side (right column), constitutive expression is found in all neurons and stronger expression is found in a subset of small and medium-sized cells. On the crush side (left column), upregulation of *SCG10* is visible in neurons of all sizes from 3 days onwards but is most marked in the smaller neurons.

Bar 100 μm.

FIGURE 3.5

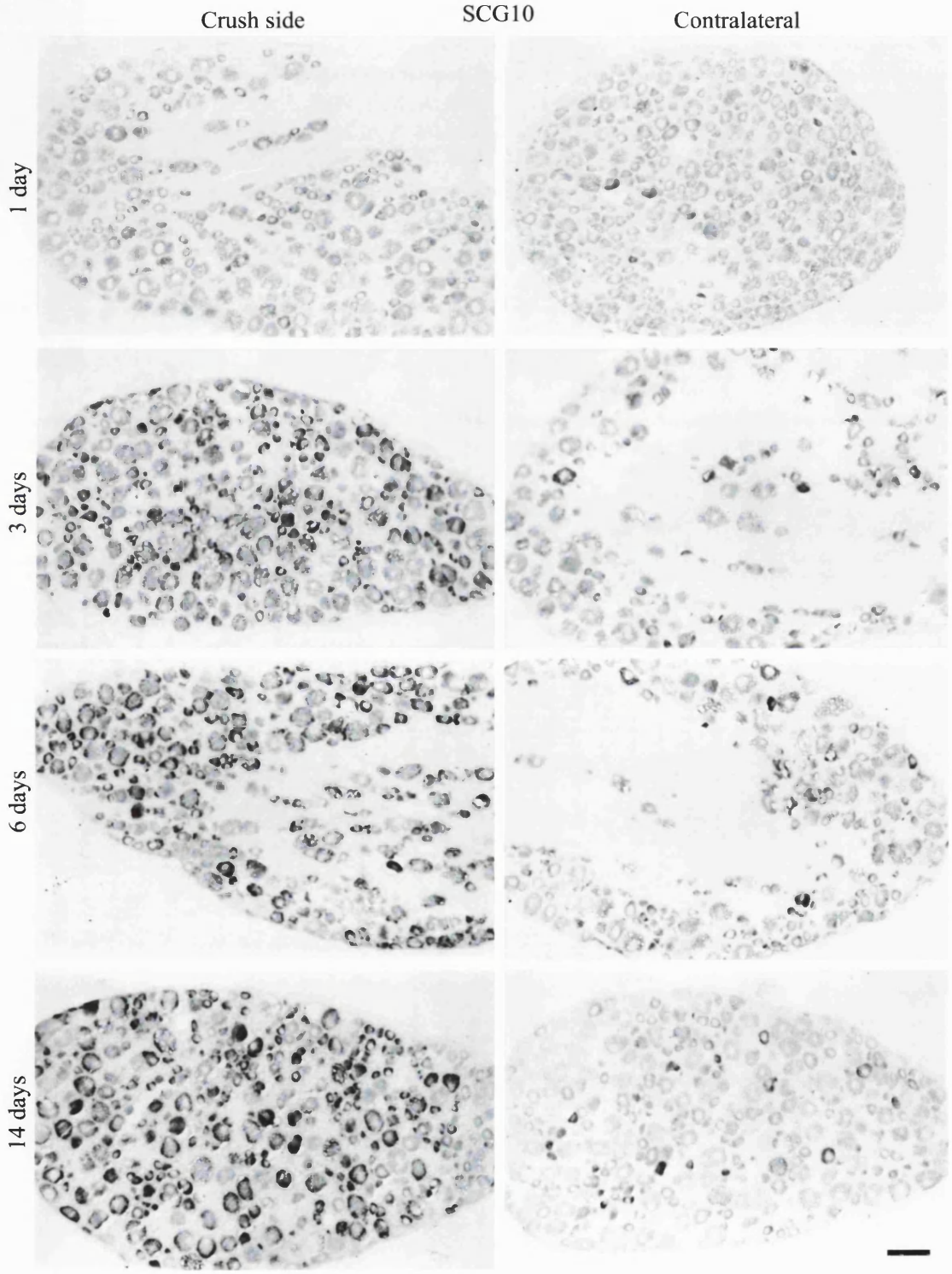


Fig. 3.6. Expression of *CAP-23* in L5 dorsal root ganglion neurons 1 day, 3 days, 6 days and 14 days following unilateral sciatic nerve crush. On the contralateral side (right column), a low level of constitutive expression is apparent in nearly all small to medium sized neurons and some large diameter neurons, but some small to medium neurons show stronger expression. On the crush side (left column), upregulation of *CAP-23* is visible in neurons of all sizes from 3 days onwards.

Bar 100 μm.

FIGURE 3.6

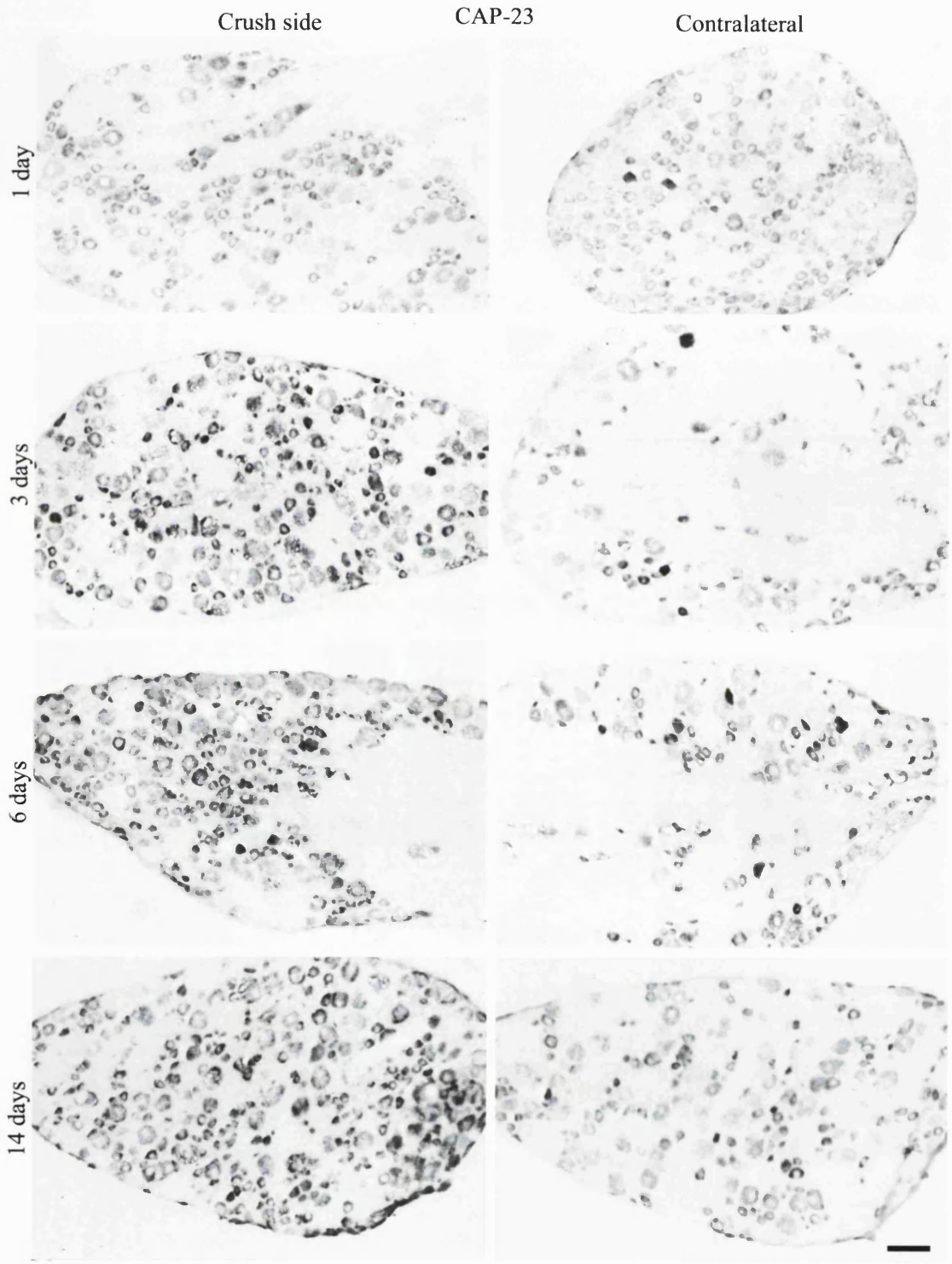


Fig. 3.7. Expression of *GAP-43* in L5 dorsal root ganglion neurons 1 day, 3 days, 6 days and 14 days following unilateral sciatic nerve crush. On the contralateral side (right column) some *GAP-43* expression is found in most small to medium neurons and some large diameter cells, with a subset of the former having higher levels. On the crush side, upregulation of both *GAP-43* is visible in neurons of all sizes from 1 day onwards.

Bar 100 μm.

FIGURE 3.7

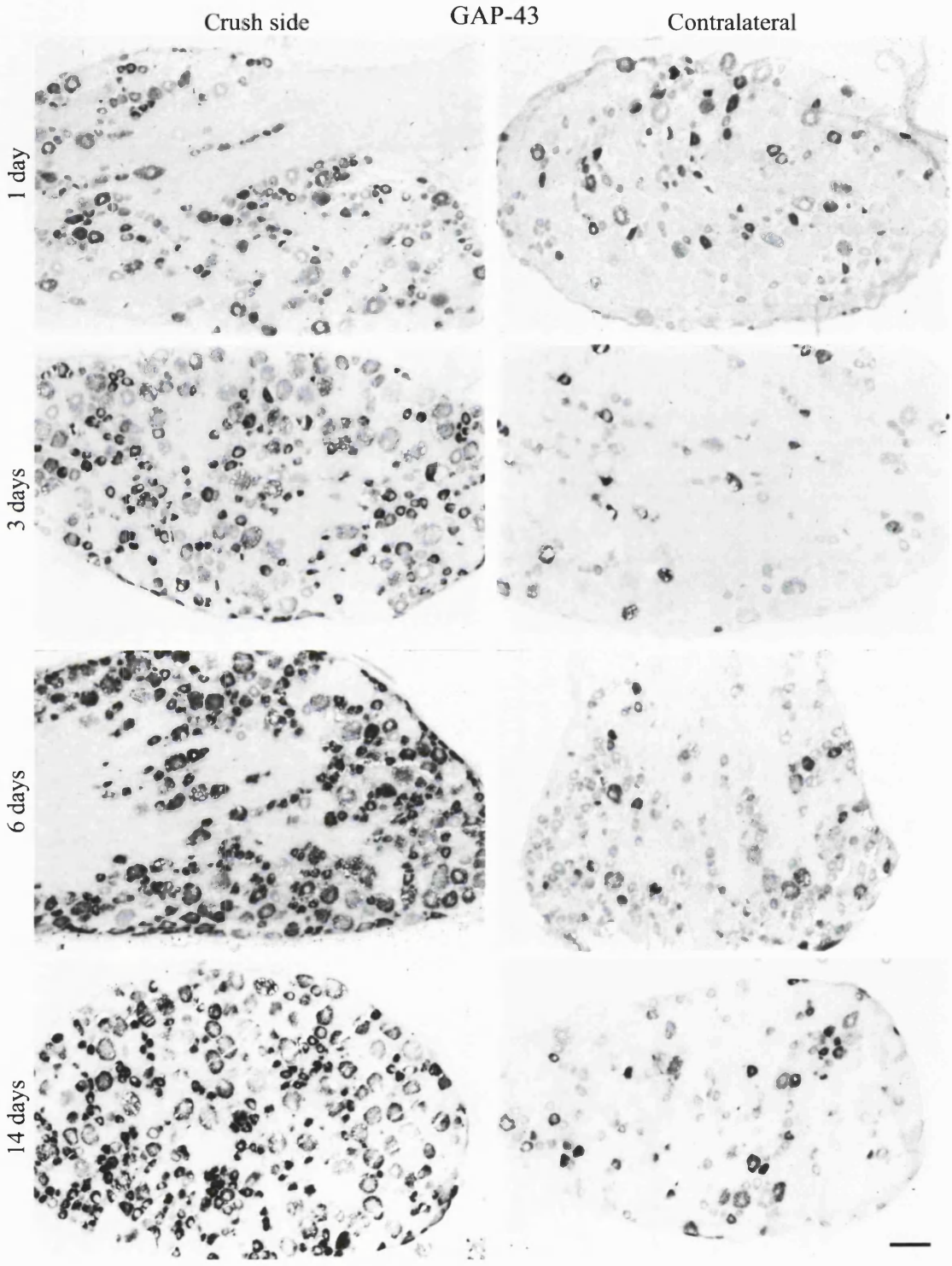


Fig. 3.8. Serial sections of L5 dorsal root ganglia, ipsilateral (top row) and contralateral (bottom row) to a 2 week sciatic nerve crush, probed by ISH for *SCG10*, *GAP-43* and *CAP-23*. On the crush (ipsilateral) side, the same cells have upregulated *SCG10*, *GAP-43* and *CAP-23*, as shown for some of the 13 cells which are identified by corresponding numbers. On the control (contralateral) side, one small neuron which strongly expresses all three molecules is present in the field of view and several other cells show light expression of all three mRNAs.

Bar 20μm.

FIGURE 3.8

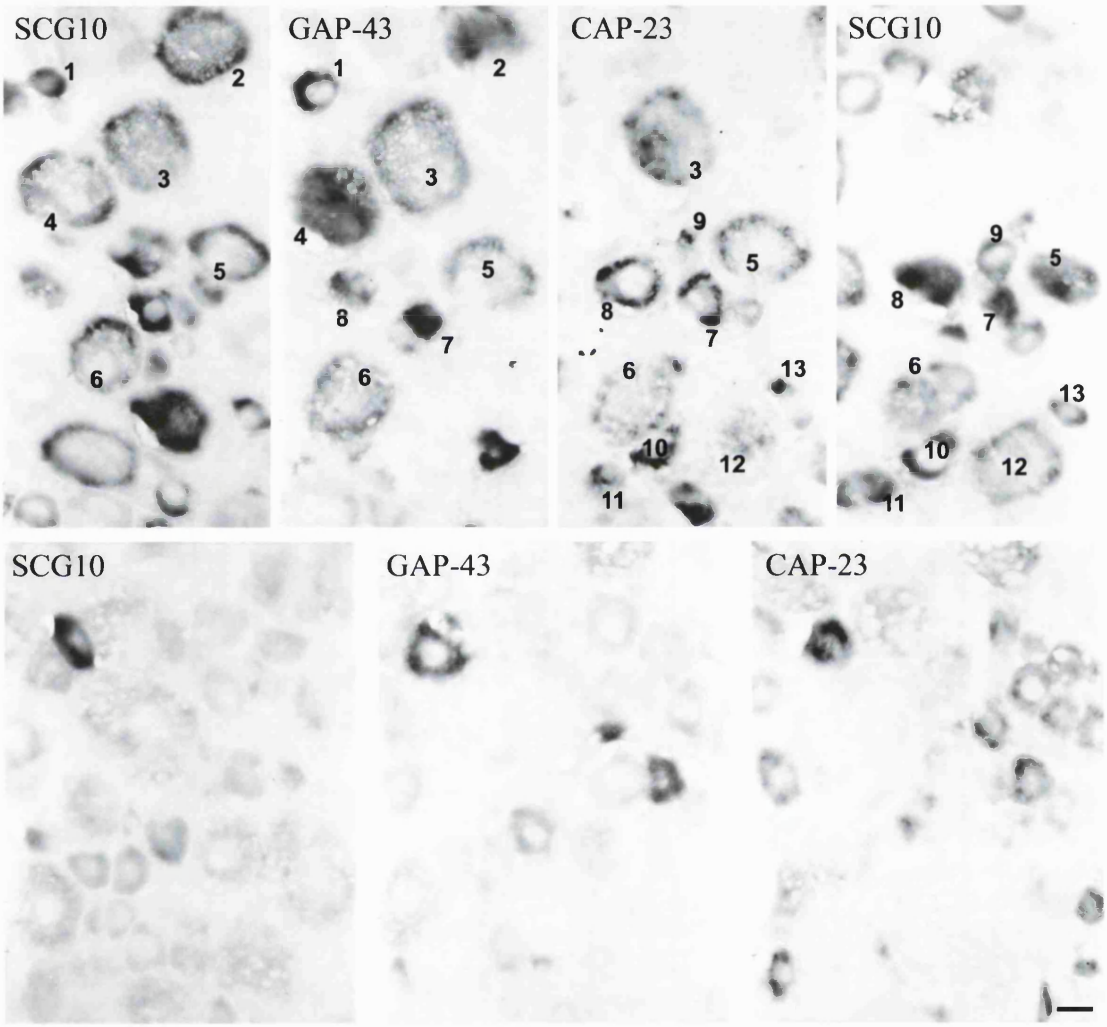
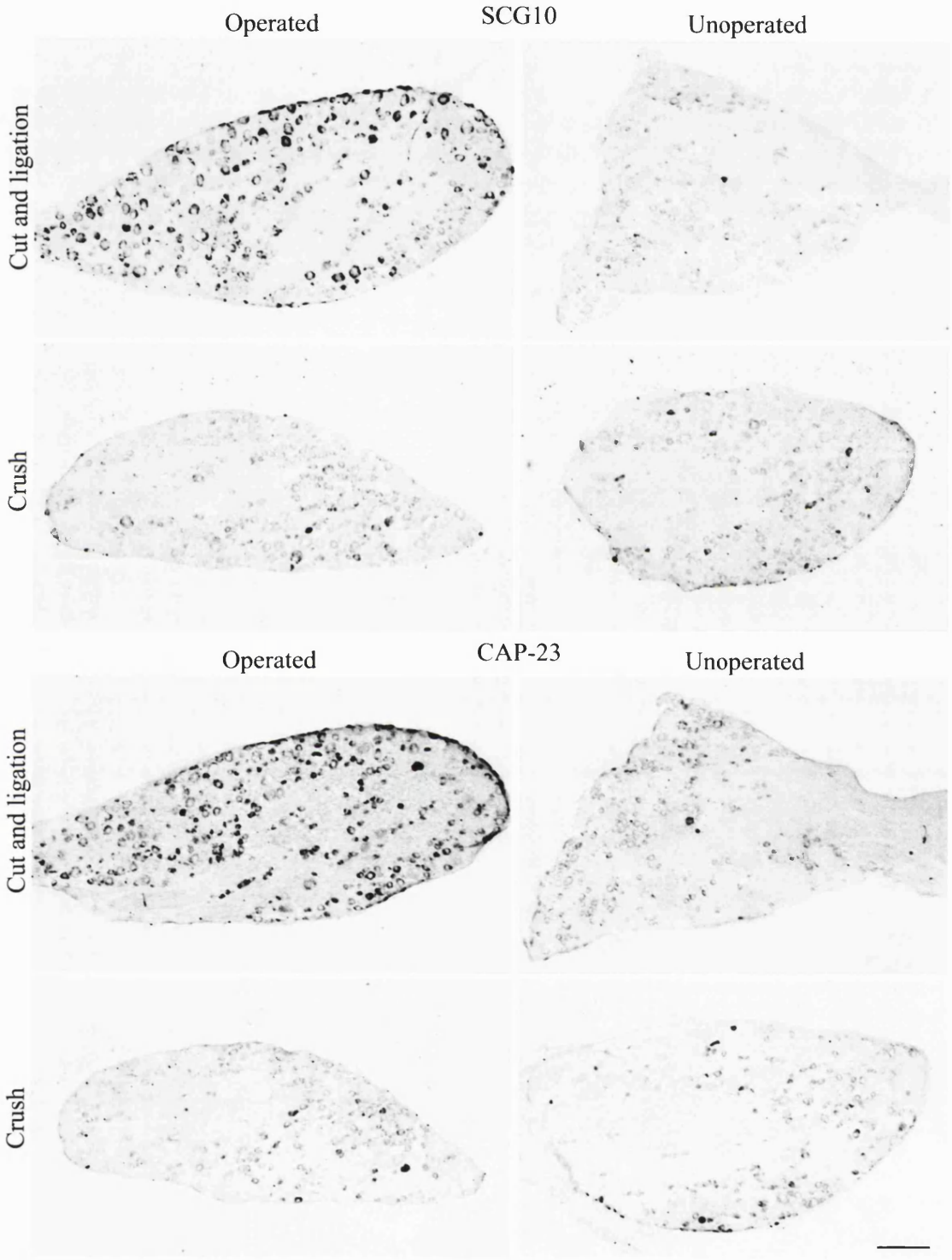


Fig. 3.9. Comparison of the expression of *SCG10* (upper four panels) and *CAP-23* (lower four panels) in L5 dorsal root ganglion neurons 6-7 weeks after crush or cut and ligation of the sciatic nerve. After a crush injury *SCG10* and *CAP-23* expression has returned to basal levels (2nd and 4th rows), but after cut and ligation of the nerve, which prevents the regeneration of axons to their targets, expression of both molecules remains elevated (1st and 3rd rows).

Bar 100μm.

FIGURE 3.9


Operated
SCG10
Unoperated
Cut and ligation
Crush
Operated
CAP-23
Unoperated
Cut and ligation
Crush

Chapter 4

Expression of *SCG10*, *CAP-23* and *GAP-43* in the thalamus after implantation of a tibial nerve graft

4.1 Introduction

Many CNS neurons will, after an axotomy, regenerate axons into grafts of peripheral nerve tissue. Following implantation of a segment of peripheral nerve into thalamus, neurons of the TRN regenerate axons into the graft in large numbers, whereas axotomised neurons of the dorsal thalamus largely fail to do so. This model, therefore, allows gene expression in regenerating neurons to be compared to that in nearby neurons which are not regenerating. The regenerating neurons in the TRN upregulate several growth-associated molecules after axotomy and during regeneration, in which respect their response mirrors that of neurons axotomised by peripheral nerve injury. They have previously been shown to upregulate GAP-43, c-Jun, L1 and CHL1, and this upregulation lasts for many weeks (Vaudano et al., 1995; Zhang et al., 1995; Vaudano et al., 1998; Chaisuksunt et al., 2000b). Conversely, upregulation of these molecules is not seen in the non-regenerating dorsal thalamic neurons. Implantation of nerve grafts in which the cells have been killed by freeze-thawing do not support regeneration and do not elicit lasting upregulation of these molecules. *SCG10* and *CAP-23* mRNAs were shown in Chapter 3 to be upregulated by neurons regenerating their axons after peripheral nerve injury. In order to investigate whether this is consistently associated with axon regeneration, the regulation of these molecules was studied in CNS neurons regenerating axons into nerve grafts. Tibal nerve grafts were implanted into the thalamus of adult rats and expression of *SCG10* and *CAP-23* was then examined in thalamic neurons by ISH. *GAP-43* expression was also studied both as a positive control (as it should be detectable in neurons mounting a regenerative response to injury), and to allow comparison with the expression of *SCG10* and *CAP-23*. In some animals, retrograde labelling from the distal part of the graft (which remains outside the skull) was also used to identify neurons which had grown axons into the grafts. Further animals received freeze-killed grafts to examine expression of these molecules following an identical injury but under conditions when regeneration into the grafts does not occur. Animal survival times and numbers are shown in Table 4.1.

Surgical procedure	Survival time	*n*
Tibial nerve autograft in thalamus	3 days	3
	2 weeks	5
	4 weeks	3
	6 weeks	3
Tibial nerve autograft in thalamus, followed by CTB injection into the distal graft	4 weeks	3
	6 weeks	1
Freeze-killed tibial nerve autograft in thalamus	2 weeks	3

Table 4.1 Animal usage and survival times for studies of *SCG10*, *CAP-23* and *GAP-43* expression following tibial nerve autografting in the thalamus.

4.2 Results

i. Expression of SCG10, CAP-23 and GAP-43 in the intact thalamus

The patterns of expression of *CAP-23*, *SCG10* and *GAP-43* were examined in the intact thalamus of two animals, by ISH on serial sections. While the expression of each mRNA varied markedly between thalamic nuclei, a broadly similar pattern was observed for all three molecules (Fig. 4.1). In particular, high levels of mRNAs for all three molecules were expressed in the intralaminar nuclear complex, especially the paraventricular thalamic nuclei and the central medial nuclei and expression was notably weak in the submedial nucleus (nucleus gelatinosus) and mediodorsal nuclei. A major difference between the three RNAs was that the TRN was devoid of expression of *GAP-43* and displayed expression of *CAP-23* only in its dorsal pole, but all neurons in the TRN were positive for *SCG10*, with the dorsal pole displaying a stronger signal than other parts of the nucleus (Fig. 4.1, upper panel). Differences were also seen in the expression of these mRNAs in the hipppocampal formation: whereas *CAP-23* and *SCG10* were expressed throughout the pyramidal layer of CA1- CA4 and in the granule cell layer of the dentate gyrus, *GAP-43* was expressed relatively less strongly in CA1-CA2 and is not detectable in the granule cell layer of the dentate gyrus (Fig. 4.1, middle panel).

ii. Expression of SCG10, CAP-23 and GAP-43 in the thalamus following implantation of a living tibial nerve graft.

Animals were killed at survival times of 3 days, 2 weeks, 4 weeks and 6 weeks (see Tables 2.2; 4.1) following graft implantation. There was little evidence of increased expression of *SCG10*, *CAP-23* or *GAP-43* in neurons of the dorsal thalamus at any survival time (Figs. 4.2-4.5), with the exception of two animals with survival times of 2 weeks, in which a few neurons of the dorsal thalamus close to the graft tip had upregulated *SCG10* and *GAP-43* (not illustrated). The expression of *SCG10*, *CAP-23* and *GAP-43* was, however, increased in neurons of the TRN, ipsilateral to the grafts. Moderate upregulation of these mRNAs was seen at 3 days post-implantation (Fig. 4.2) and a more marked increase was seen at 2 weeks (Figs. 4.3, 4.4), 4 weeks (not shown) and 6 weeks (Figs. 4.5, 4.6). For all three molecules, fewer neurons showed upregulation at 2-6 weeks than at 3 days, although the magnitude of the increase was greater at the later time points (compare Fig. 4.2 with Figs. 4.3-4.6). In most animals killed at 2 weeks or more after surgery, neurons with a detectable increase in mRNA expression were confined to the dorsal-most part of the TRN; in these animals the grafts generally terminated in the dorsolateral part of the dorsal thalamus. Those animals where upregulation of the mRNAs examined was found in more ventral parts of the TRN appeared to be those where the grafts had been inserted more deeply or in a more medial positon (data not shown).

At all time points the upregulation of *SCG10* appeared to be more marked than that of either *CAP-23* or *GAP-43* (Figs. 4.2-4.6) and from 2 weeks onwards the *SCG10* was more strongly expressed in the presumably regenerating neurons of the TRN than in any other cells in the forebrain.

In some animals, hybridisation signals for *CAP-23* and *GAP-43*, but never *SCG10*, were observed in Schwann cell columns in the graft. *CAP-23* could be seen at three days and 2 weeks post grafting while *GAP-43* was not seen at 3 days but was clearly visible at 2 weeks; this can be seen in Figs. 4.3 and 4.4. Both mRNAs were only faintly detectable or not detectable at 6 weeks; signal is absent in the graft shown in Figs. 4.5 and 4.6.

In two animals (survival times 4 and 6 weeks) analysis was performed to identify neurons on consecutive serial sections hybridised with different probes. Many neurons could be identified on more than one section and where this was the case *SCG10*, *CAP-23* and *GAP-43* were found to be upregulated by the same neurons (Fig. 4.7, 4.8).

iii. Retrograde labelling of neurons with axons in the grafts, and expression of SCG10 and CAP-23 visualised on adjacent sections

In four further animals, CTB retrograde tracer was injected into the distal part of the graft in order to identify neurons which had grown axons into the grafts. These were examined after survival times of 4 weeks (n=3) and 6 weeks (n=1). Almost all of the retrogradely labelled neurons were found in the TRN (Fig. 4.9), although in one animal (4 weeks survival) a few labelled neurons were found in the dorsal thalamus very close to the graft tip (not shown). Alternate sections were reacted for ISH and CTB immunohistochemistry (Fig. 4.9) and subsequent analysis allowed the identification of individual neurons on consecutive sections. Where this was successful, all retrogradely labelled neurons showed increased expression of *SCG10* and the great majority had upregulated *CAP-23* (Figs. 4.10-4.13). Thus in the left-hand columns of Figs. 4.10-4.13, 43 retrogradely labelled neurons are identified, 42 of which are also identifiable on adjacent sections and have upregulated *SCG10*. Similarly, in the right-hand columns 63 retrogradely labelled neurons are identified, 60 of which are identifiable on adjacent sections and 58 of which have clearly upregulated *CAP-23*. Those neurons which have not clearly upregulated *CAP-23* were all found in the dorsal pole of the TRN where a moderate basal level of expression is found.

iv. Control retrograde labelling of dorsal thalamic neurons

As a control to ensure that the absence of CTB labelling of thalamocortical projection neurons in the grafted animals was not because dorsal thalamic neurons are unable to take up and retrogradely transport CTB, the thalamus was examined in two animals in which CTB had been injected into various areas of neocortex. CTB immunohistochemistry on sections of thalamus of these animals revealed scattered retrograde labelling in neurons throughout the dorsal thalamus (Fig. 4.14).

v. *Expression of SCG10, CAP-23 and GAP-43 in the thalamus following implantation of a freeze-killed tibial nerve graft.*

Three animals received grafts of freeze-killed tibial nerve (which do not support axonal regeneration) into the thalamus (survival time 14 days). In these animals, neurons of the TRN did not show upregulation of *SCG10*, *CAP-23* or *GAP-43* expression (Fig. 4.15), indicating that axotomy alone was insufficient to produce prolonged upregulation of any of these molecules.

Fig. 4.1. Expression of *SCG10*, *GAP-43* and *CAP-23* in the thalamus of an unoperated adult control rat. These three molecules show a strikingly similar pattern of expression in the dorsal thalamus. Note strong expression of all three mRNAs in the paraventricular nucleus (PV), central medial nucleus (CM), and other components of the intralaminar complex, the low expression of all three mRNAs in the submedial nucleus (SM) and complete absence of *GAP-43* expression in the thalamic reticular nucleus (TRN: outlined on the left side of all three panels). Note, however, that there is some *CAP-23* expression in the most dorsal part of the TRN, and that *SCG10* expression is seen throughout the TRN and is especially strong dorsally.

Bar 500 μm.

FIGURE 4.1

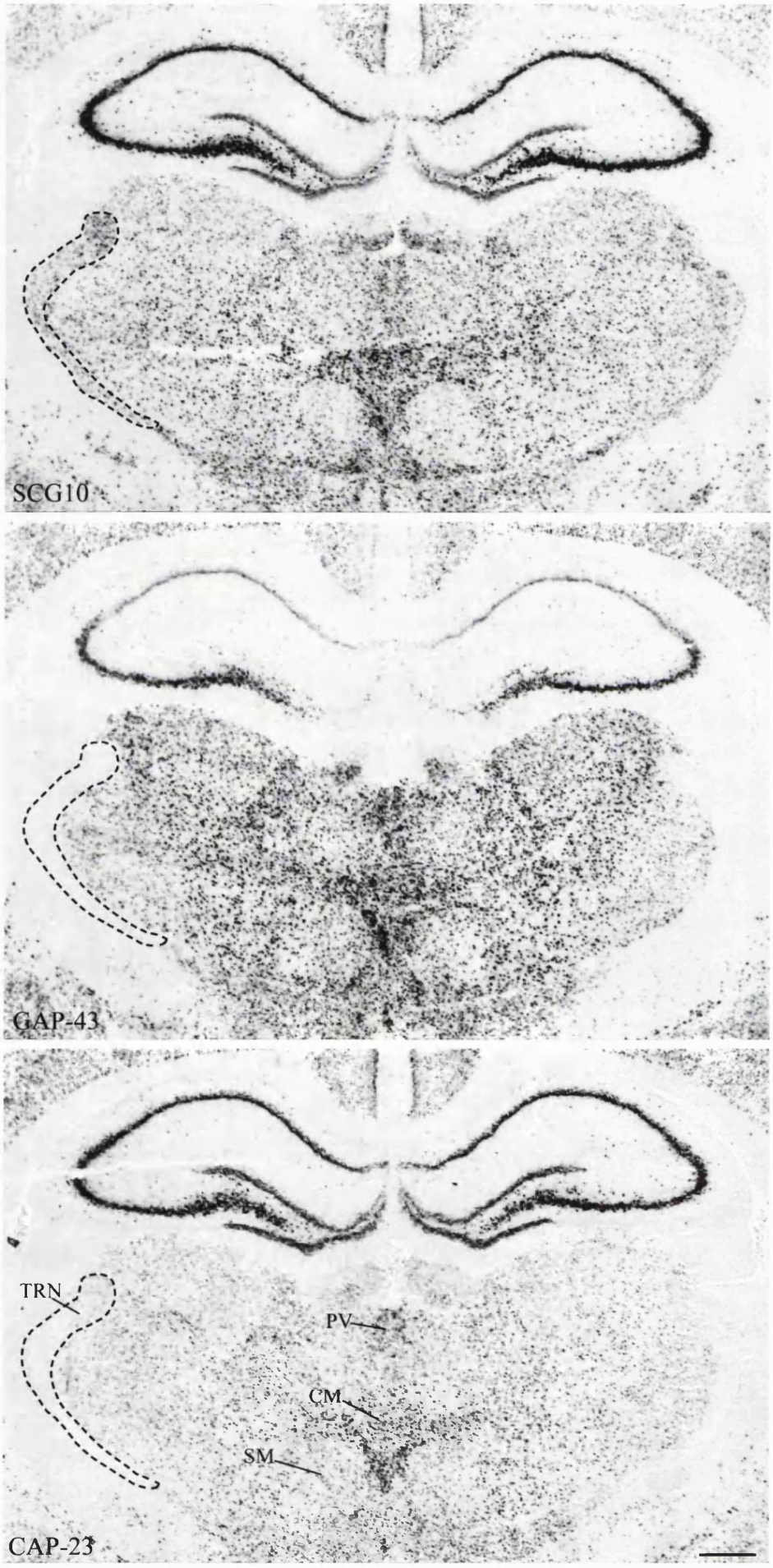


Fig. 4.2. Expression of *SCG10*, *CAP-23* and *GAP-43* in the thalamus 3 days after implantation of a tibial nerve graft. Upregulation of all three mRNAs can be seen in neurons throughout the portion of the TRN seen (indicated by arrows). No upregulation is found in neurons of the dorsal thalamus. Note the graft itself is not visible on this section, but the insertion of the graft has resulted in an area of damage in the region of the left hippocampus.

Bar 500μm.

FIGURE 4.2

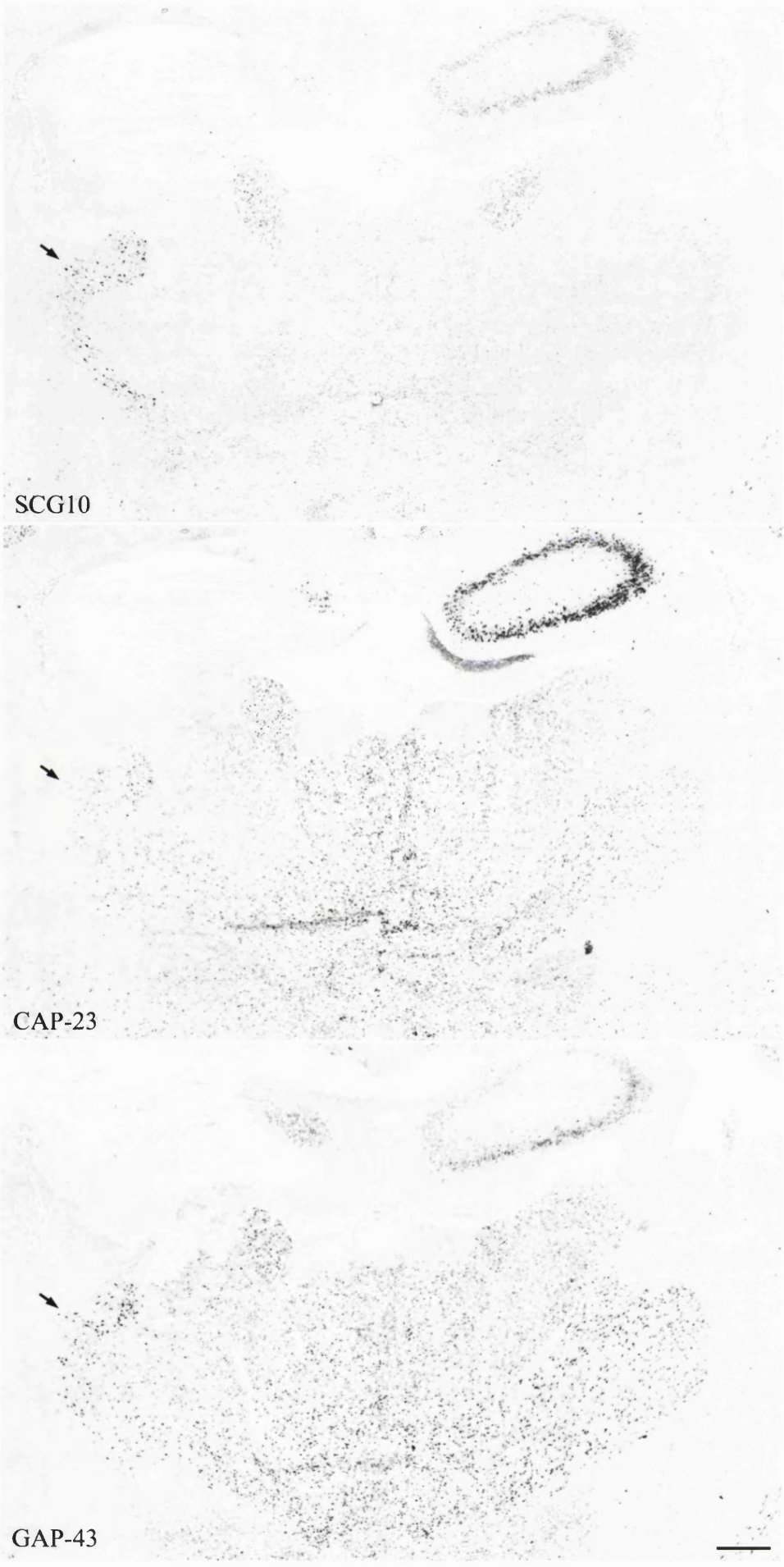


Fig. 4.3. Expression of *SCG10* and *CAP-23* in the thalamus 2 weeks after implantation of a tibial nerve graft (outlined by dashed lines). Upregulation of both molecules can be seen in neurons of the rostrodorsal TRN (arrows), shown at higher magnification in the lower panels. Fewer neurons are positive than at three days post-implantation, but the degree of upregulation is greater (compare with Fig. 4.2). Neurons of the dorsal thalamus show no detectable change in expression. Note also that some *CAP-23* hybridisation signal is visible in the Schwann cell columns of the graft.

Bars 500μm (upper panels) and 50 μm (lower panels).

FIGURE 4.3

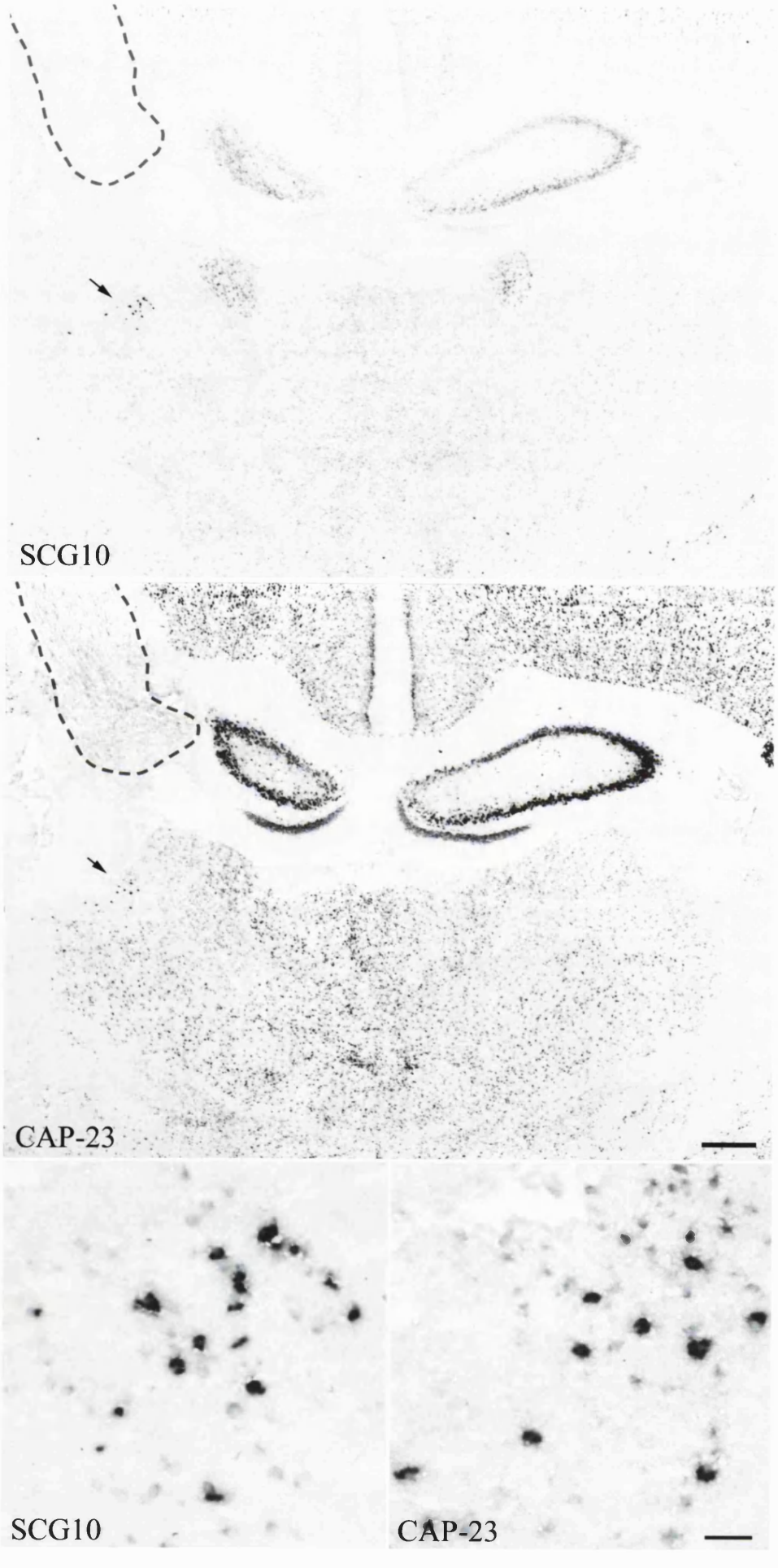
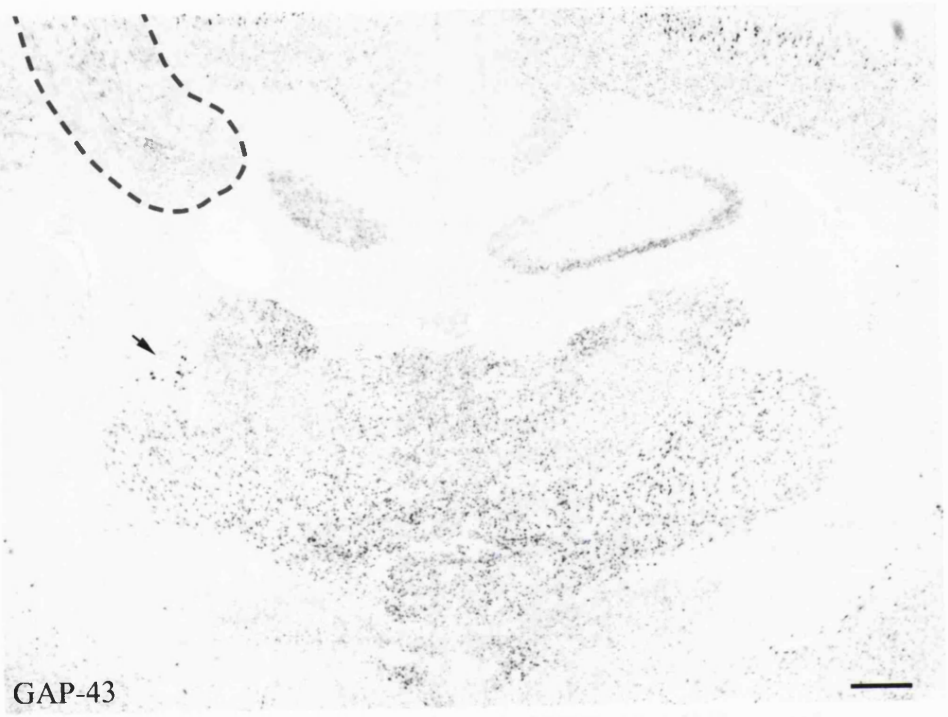


Fig. 4.4. Expression of *GAP-43* in the thalamus 2 weeks after implantation of a tibial nerve graft (outlined by dashed line). *GAP-43* upregulation can be seen in neurons of the rostrodorsal TRN (arrows), shown at higher magnification in the lower panel. Neurons of the dorsal thalamus show no detectable change in expression. *GAP-43* hybridisation signal is also visible in Schwann cell columns in the graft.

Bars 500μm (upper panel) and 50 μm (lower panel).

FIGURE 4.4



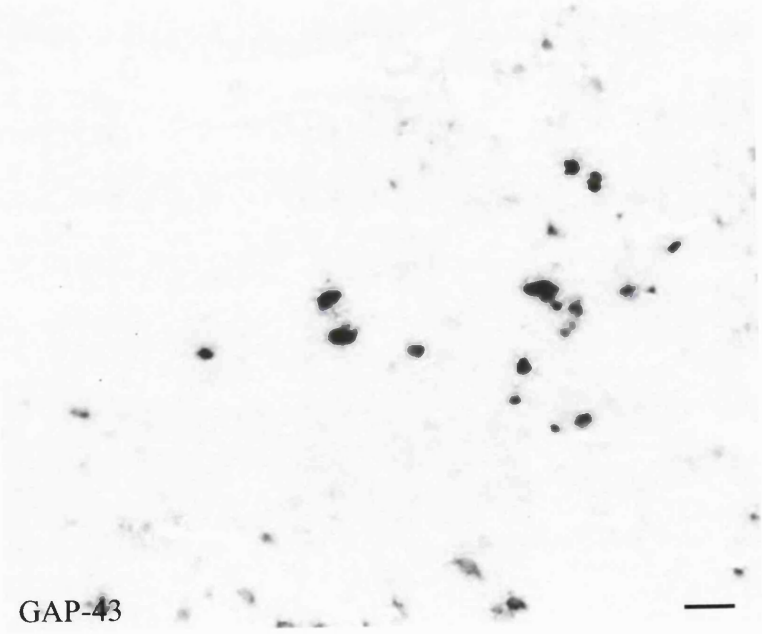


Fig. 4.5. Expression of *SCG10* and *CAP-23* in the thalamus 6 weeks after implantation of a tibial nerve graft (outlined by dashed lines). Neurons of the rostrodorsal TRN (shown at higher magnification in the lower panels) continue to express both molecules.

Bars 500 μm (upper panels) and 50 μm (lower panels).

FIGURE 4.5

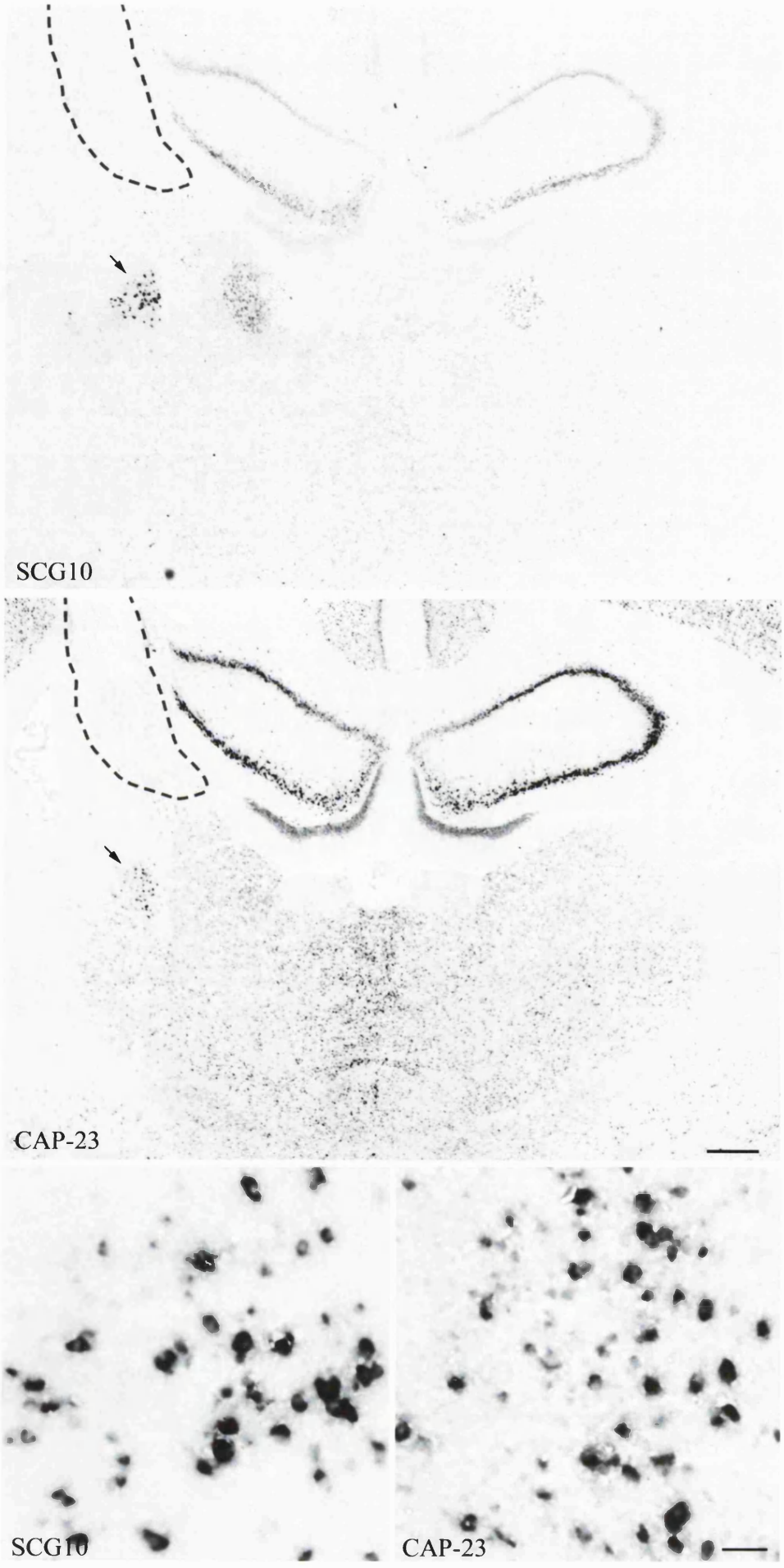


Fig. 4.6. Expression of *GAP-43* in the thalamus 6 weeks after implantation of a tibial nerve graft (outlined). Neurons of the rostrodorsal TRN (shown at higher magnification in the lower panel) continue to express both molecules.

Bars 500 μm (upper panel) and 50 μm (lower panel).

FIGURE 4.6

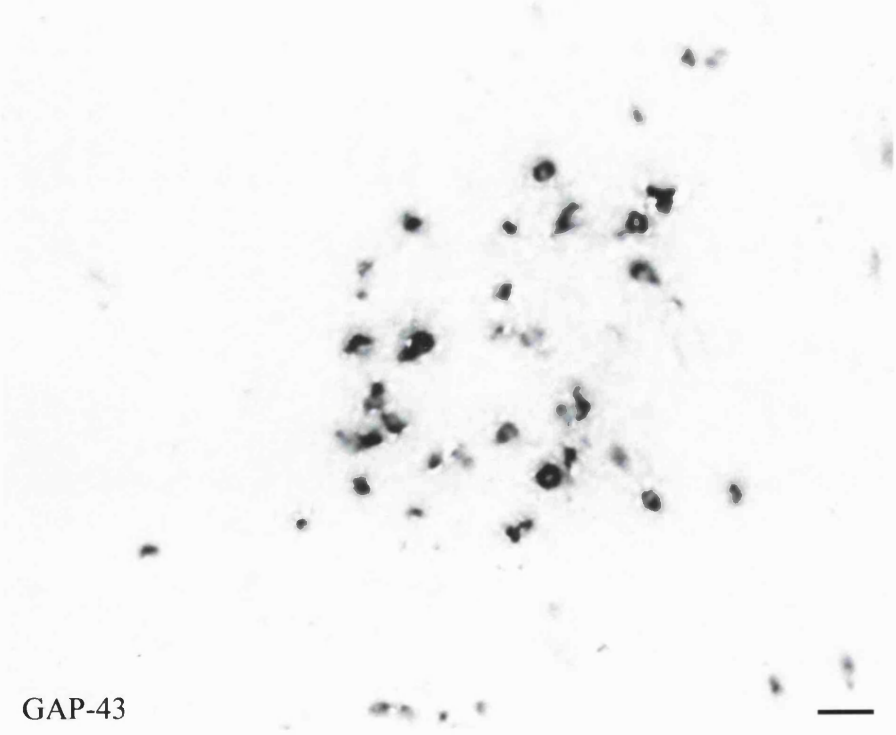


Fig. 4.7. Co-expression of *SCG10*, *CAP-23* and *GAP-43* in presumptive regenerating TRN neurons. Consecutive serial sections from the ipsilateral TRN of two animals 4 weeks (left) and 6 weeks (right) after implantation of a tibial nerve graft, reacted for ISH. Neurons identified on the middle of three serial sections (centre panels) are numbered and many can be identified on the adjacent sections seen above and below. Most neurons so identified can be seen to have upregulated two of the three molecules (compare with Fig. 4.8); some can be seen to express all three.

Bar 50 μm.

FIGURE 4.7

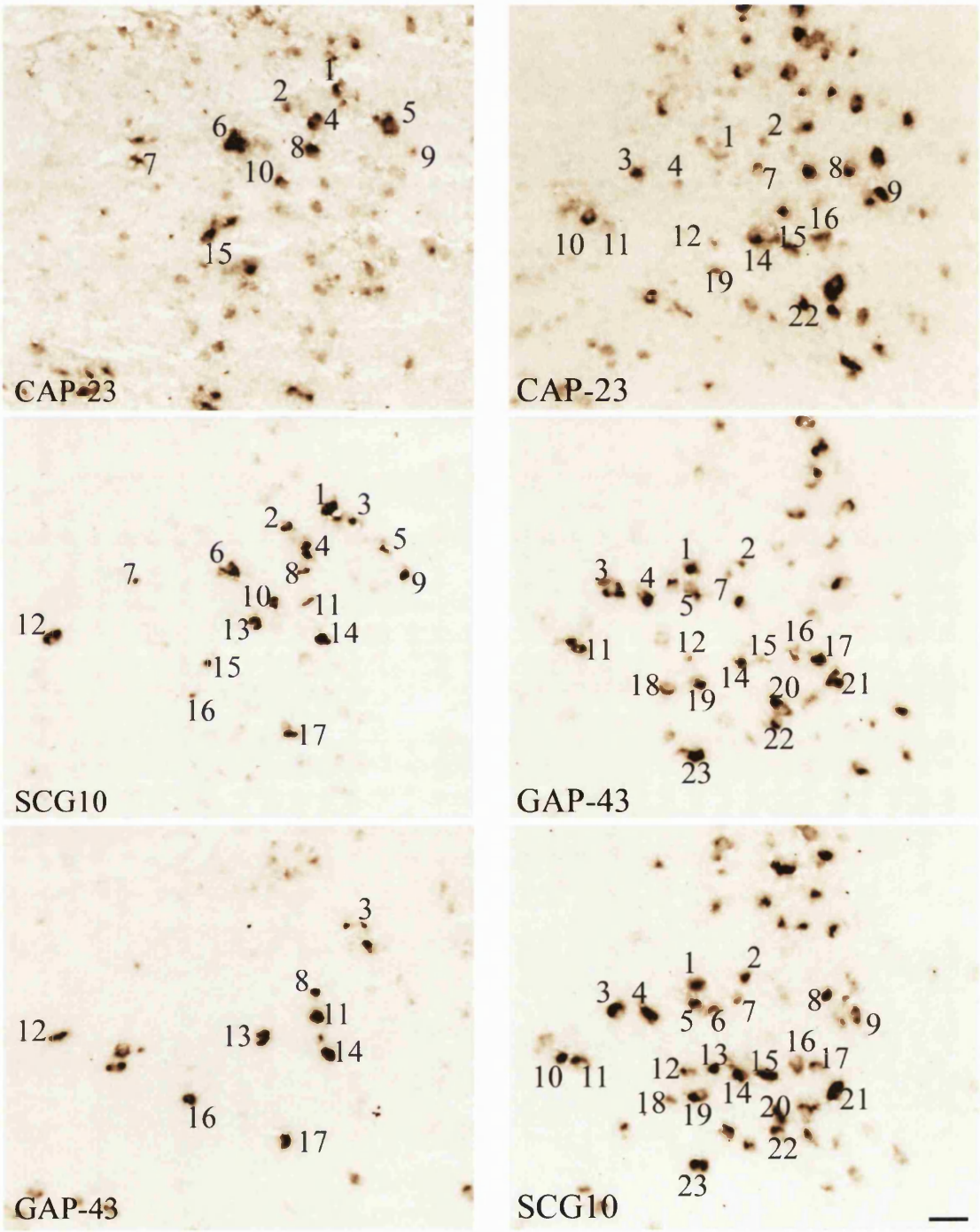


Fig. 4.8. Expression of *SCG10*, *CAP-23* and *GAP-43* in the contralateral TRN of the sections shown in Fig. 4.7 (left column), for comparison. Little or no expression of *SCG10*, *CAP-23* or *GAP-43* is visible.

Bar 50μm.

FIGURE 4.8

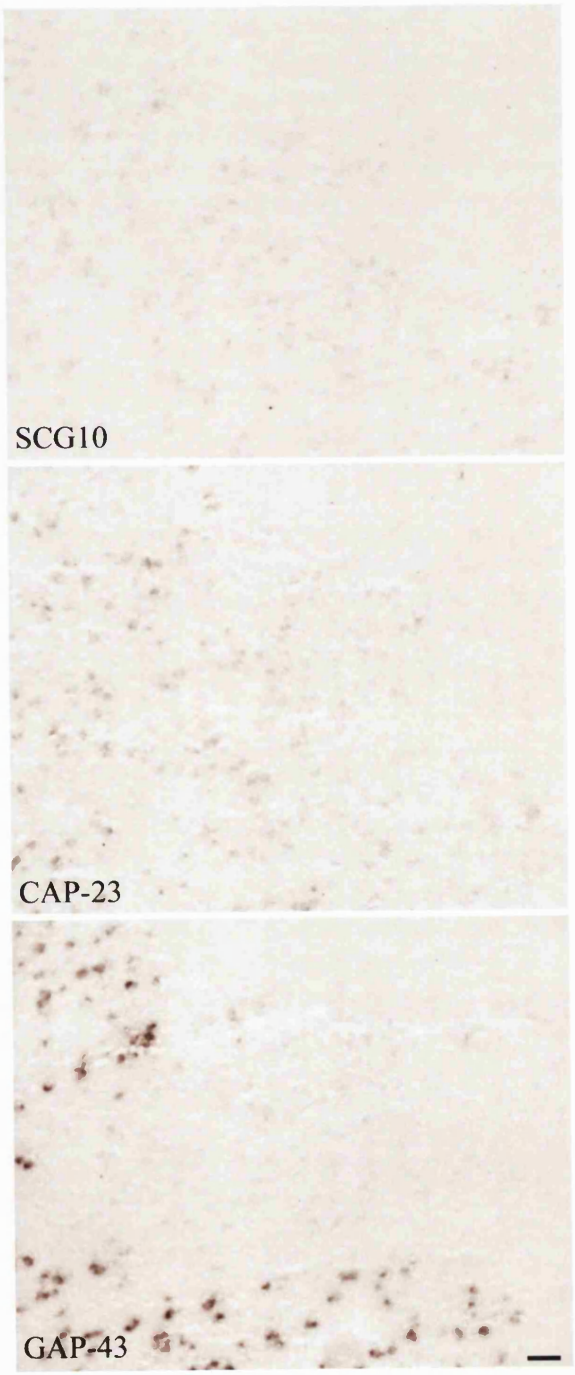
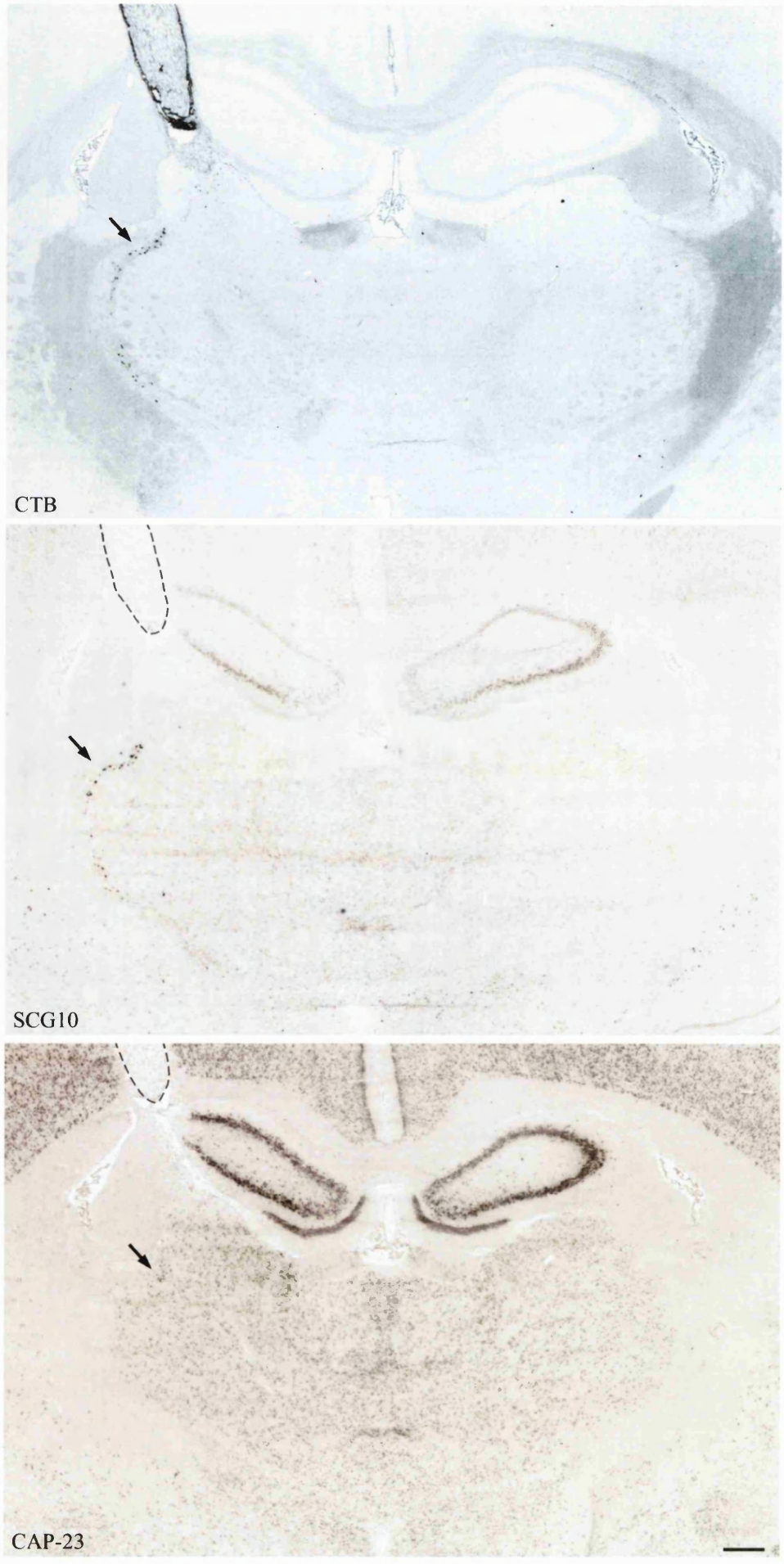


Fig. 4.9. Low power images of sections of thalamus reacted for CTB immunohistochemistry or ISH for *SCG10* or *CAP-23*. This animal was killed 6 weeks after implantation of a graft (outlined in the lower two panels) and 24 hours after injection of tracer into the distal graft. The TRN, indicated by an arrow in each panel, contains CTB-positive neurons and neurons with hybridisation signal for *SCG10* and *CAP-23*.

Bar 500μm.

FIGURE 4.9



Figs. 4.10 - 4.13. Each figure shows retrograde labelling with CTB and ISH for either *SCG10* or *CAP-23* on consecutive serial coronal sections of the thalamus of one animal. Survival times were 4 weeks (Figs. 4.10, 4.11, 4.13) and 6 weeks (Fig. 4.12) after the implantation of a graft in the thalamus. Each column shows a set of three consecutive sections, the central section having been reacted for immunohistochemistry for CTB (centre panels) and so identifying neurons which have regenerated axons into the graft. Most of these neurons can also be identified on the adjacent hybridised sections. All neurons so identified on sections hybridised with an *SCG10* probe can be seen to have upregulated *SCG10* (left-hand columns) and the great majority of such neurons identified on sections hybridised with a *CAP-23* probe have upregulated *CAP-23* mRNA (right-hand columns).

Bars: 50 μm (Figs. 4.10, 4.11); 20 μm (Figs. 4.12, 4.13)

FIGURE 4.10

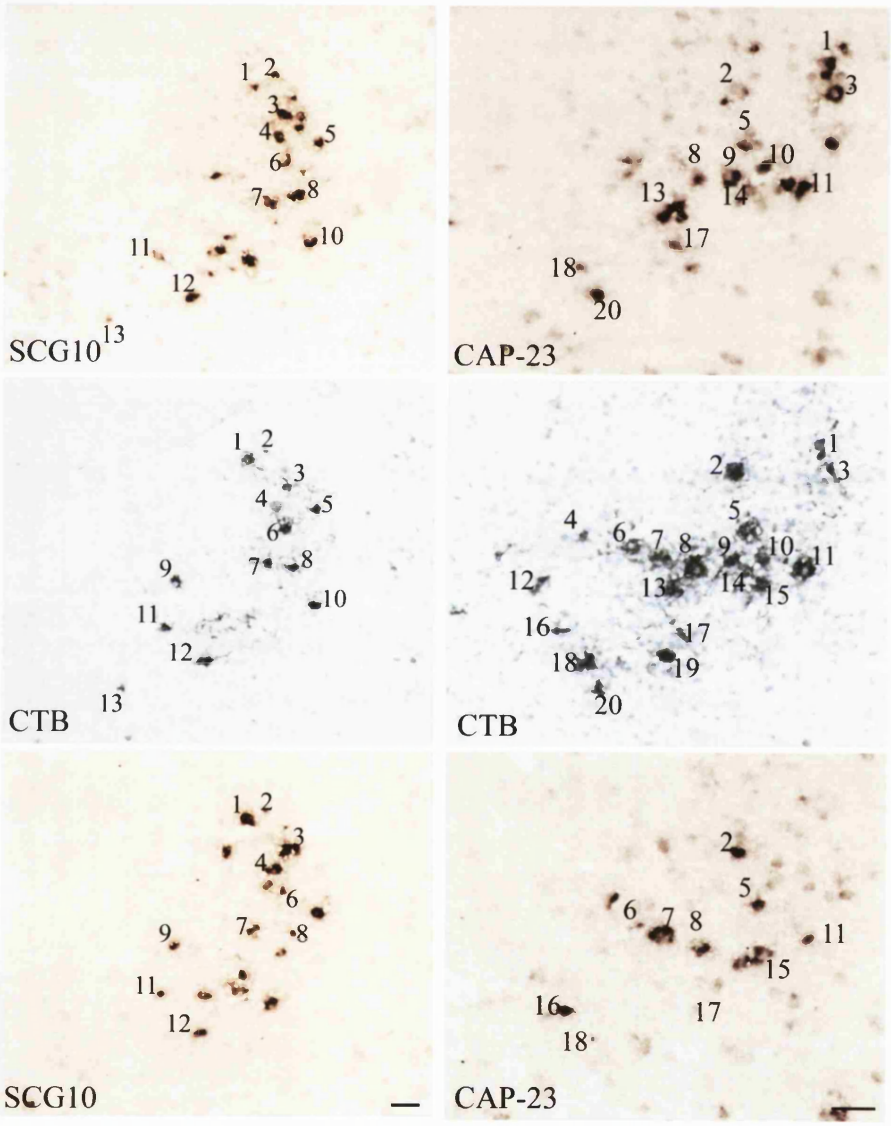


FIGURE 4.11

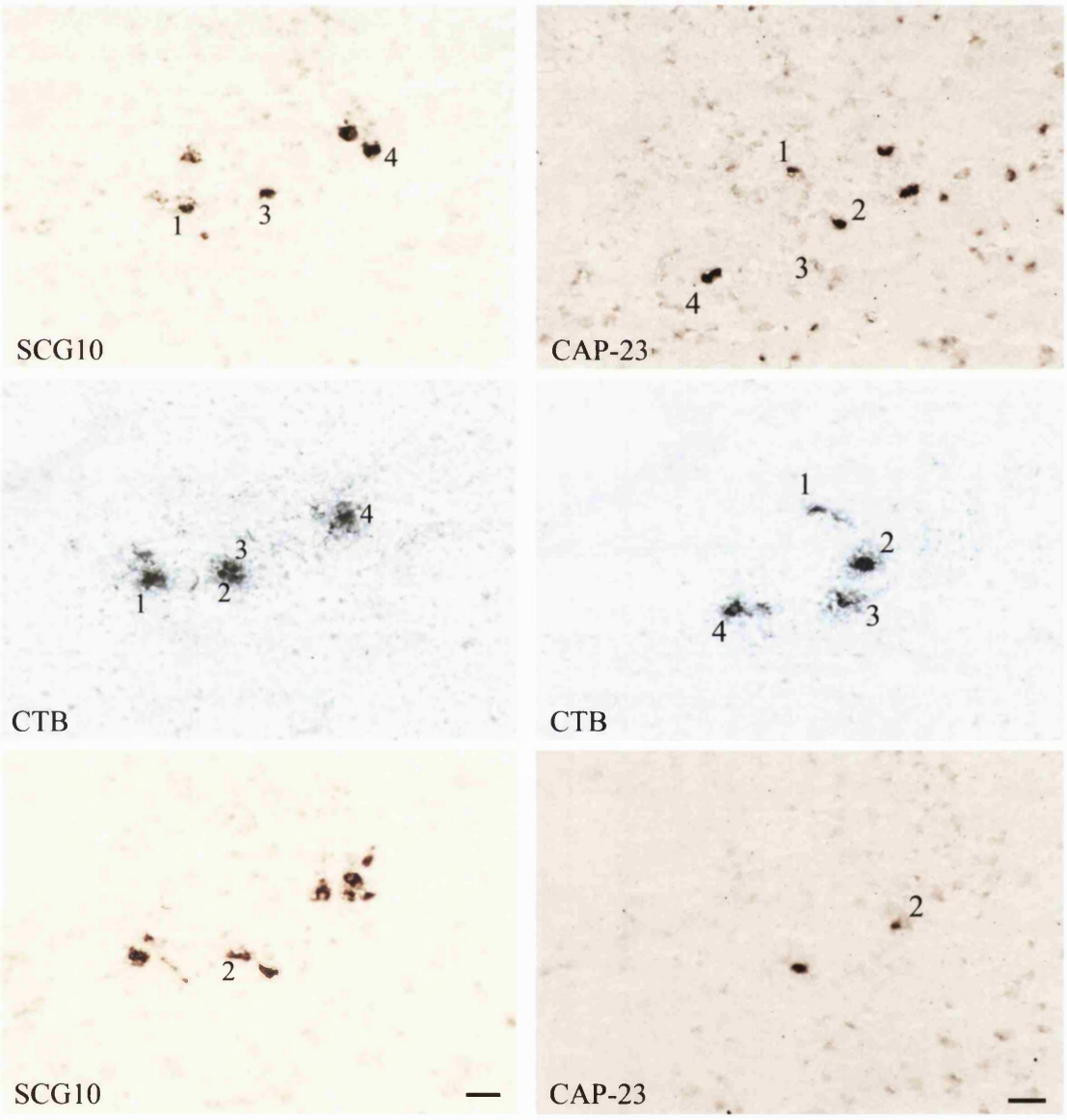


FIGURE 4.12

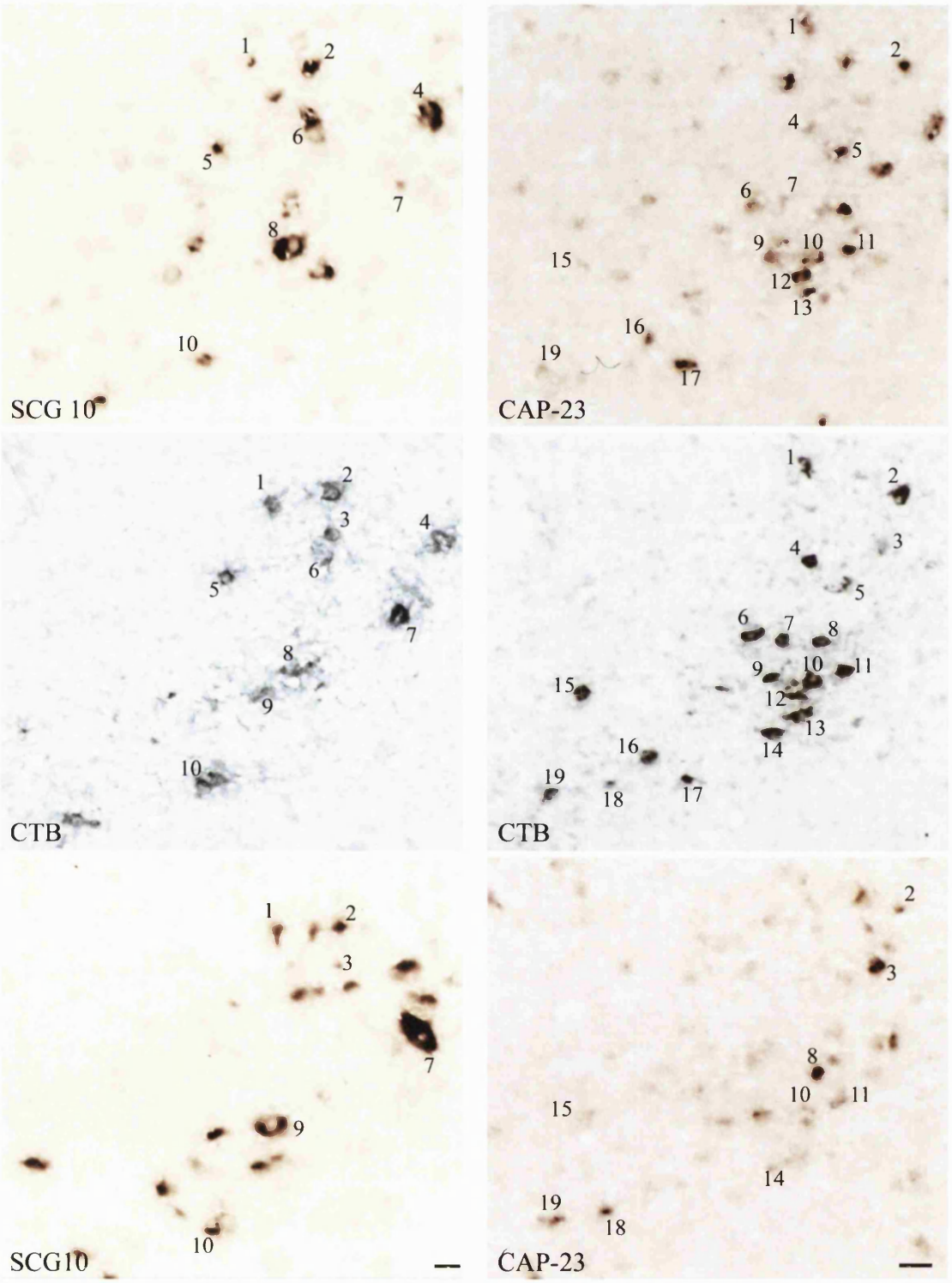


FIGURE 4.13

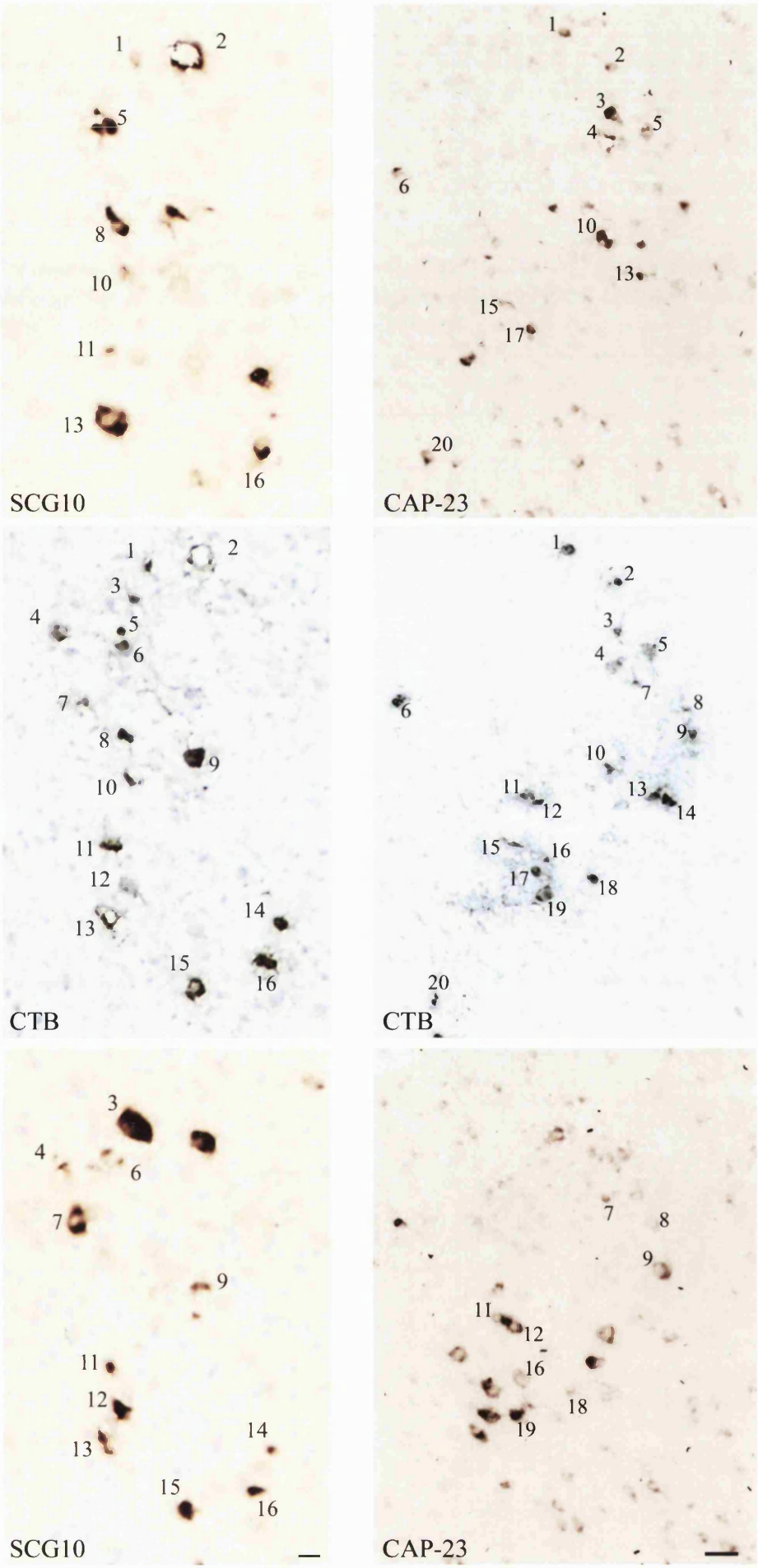


Fig. 4.14. Retrograde labelling with CTB of dorsal thalamic neurons from cortex. Seven days after multiple injections of CTB into neocortex, retrogradely labelled thalamocortical projection neurons are visible throughout the dorsal thalamus, indicating that these neurons readily take up and retrogradely transport this tracer. No labelled neurons are seen in the TRN (outlined).

Bar 250μm.

FIGURE 4.14

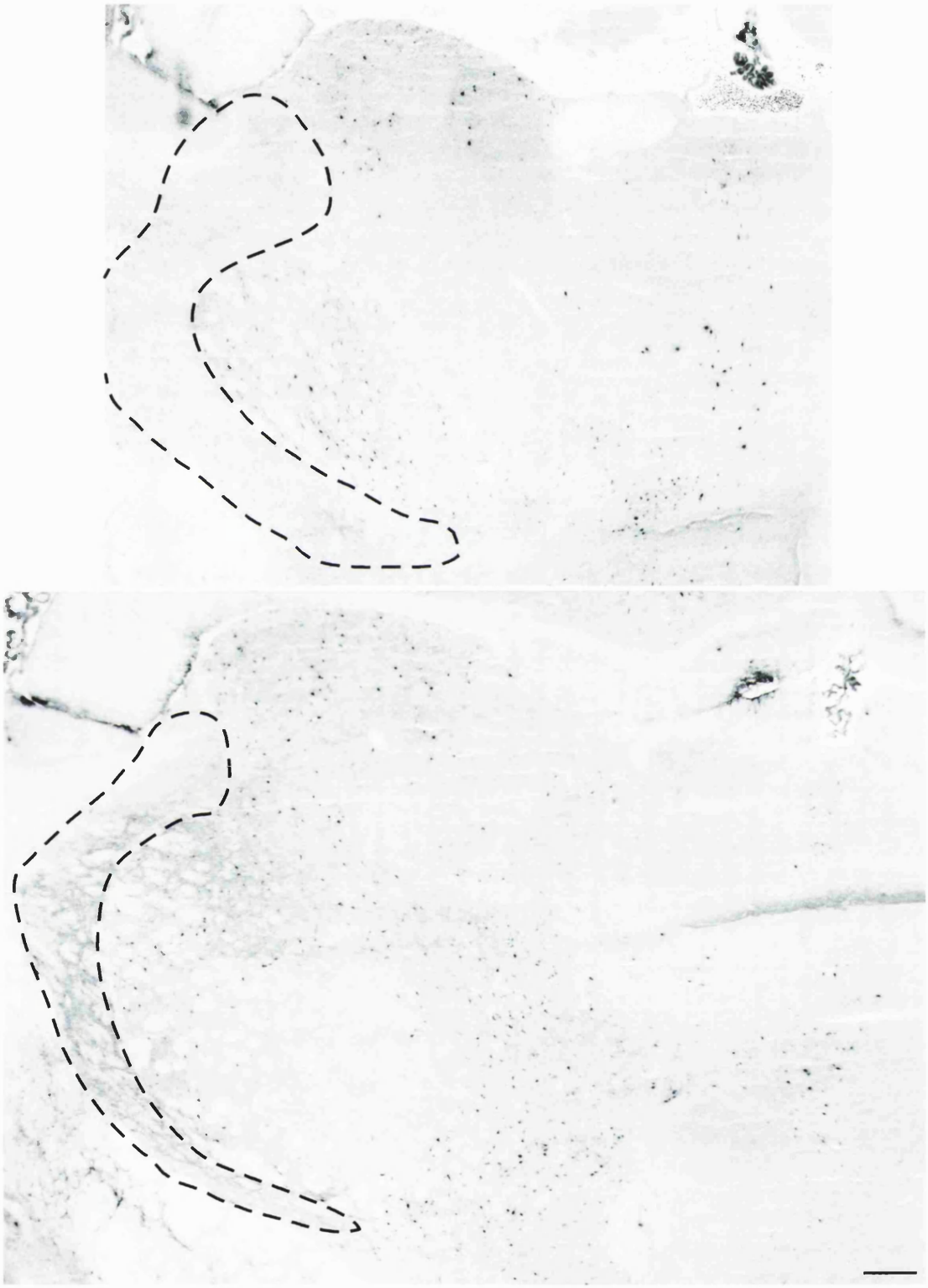
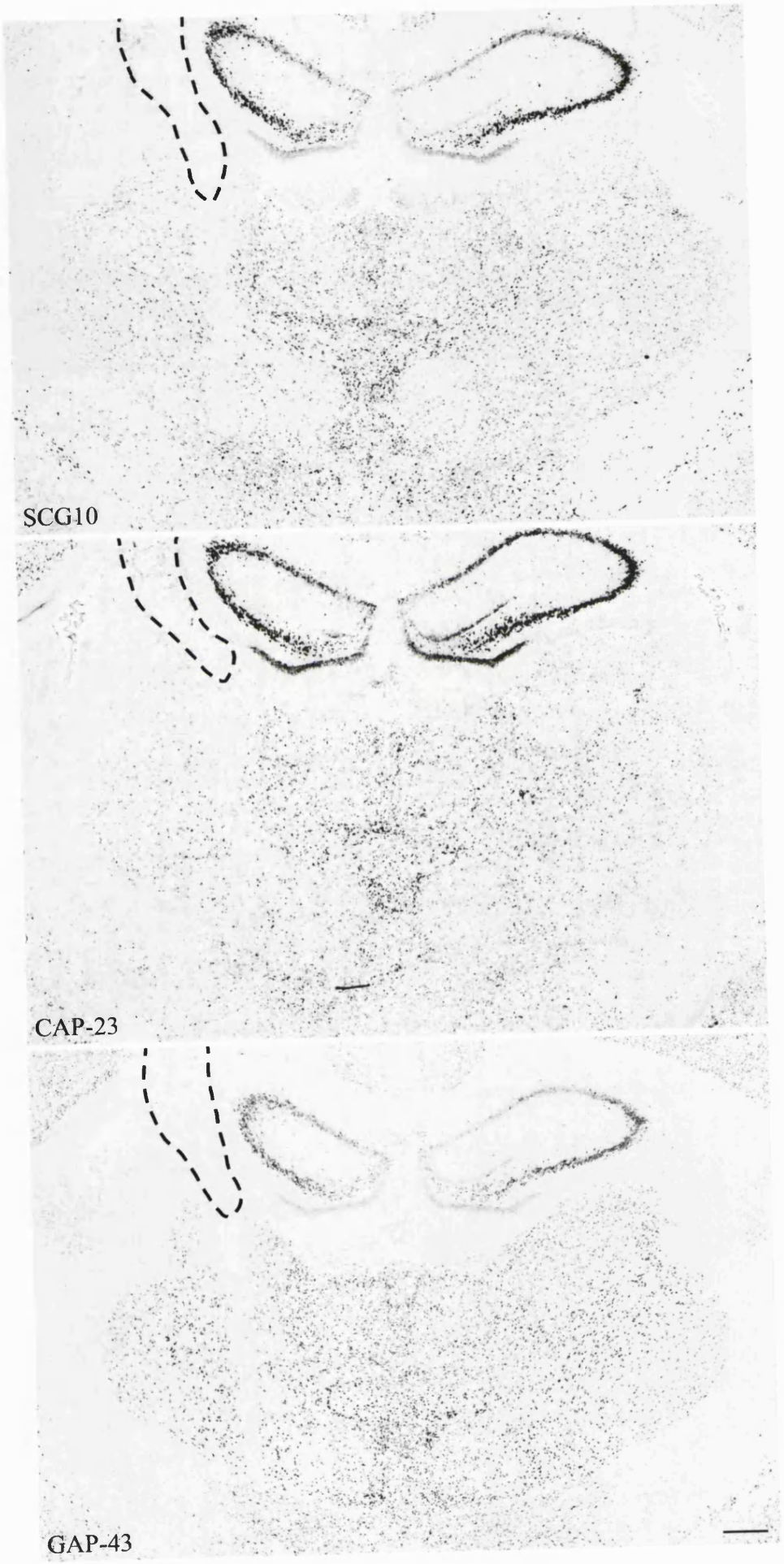


Fig. 4.15. Expression of *SCG10* and *CAP-23* in the thalamus 2 weeks after implantation of a tibial nerve graft (outlined) in which the cells have been killed by freeze-thaw cycles, and which therefore fails to support axon regeneration. No upregulation of *SCG10* or *CAP-23* is visible in the ipsilateral TRN. The graft position is similar to those of the living grafts shown in Figs. 4.3-4.6.

Bar 500μm.

FIGURE 4.15



Chapter 5

Expression of *SCG10*, *CAP-23* and *GAP-43* in the cerebellum after implantation of a tibial nerve graft

5.1 Introduction

Implantation of nerve grafts in the cerebellum provides another model to examine gene expression in CNS neurons regenerating their axons. Neurons of the cerebellar deep nuclei regenerate axons into the graft, but neurons of the cerebellar cortex, including Purkinje cells, fail to do so (Dooley and Aguayo, 1982; Chaisuksunt et al., 2000a). Again, this experimental model allows gene expression to be compared in neurons which can be induced to regenerate their axons and those which cannot. Regenerating neurons in the deep nuclei upregulate GAP-43, c-Jun, L1 and CHL1 and other molecules but Purkinje cells generally upregulate none of these unless very proximally injured (Vaudano et al., 1998; Chaisuksunt et al., 2000a). To determine if *SCG10* and *CAP-23* are also upregulated in regenerating neurons of the deep cerebellar nuclei, to compare their expression to that of *GAP-43*, and to further characterise the responses of Purkinje cells to axotomy, ISH for *SCG10*, *CAP-23* and *GAP-43* was performed on sections of cerebellum following peripheral nerve implantation in the cerebellum. Retrograde labelling from the distal part of the graft was used to identify neurons which had grown axons into the grafts, and animals which had received freeze-killed grafts were examined as controls. Animal usage and survival times are summarised in Table 5.1.

Surgical procedure	Survival time	*n*
Tibial nerve autograft in cerebellum	3 days	3
	2 weeks	4
	4 weeks	2
	6 weeks	5
Tibial nerve autograft in cerebellum, followed by CTB injection into the distal graft	4 weeks	4
Freeze-killed tibial nerve autograft in cerebellum	2 weeks	3

Table 5.1. Animal usage and survival times for studies of *SCG10*, *CAP-23* and *GAP-43* expression following tibial nerve autografting in the cerebellum.

5.2 Results

i. SCG10 and CAP-23 expression in the intact cerebellum

Expression of *CAP-23*, *SCG10* and *GAP-43* was examined in the intact cerebellum of two animals. Moderate levels of *SCG10* were expressed in neurons of the deep cerebellar nuclei (Fig. 5.1), in the granule cell layer of the cerebellar cortex and by most Purkinje cells (Fig. 5.2). There was also moderate constitutive expression of *SCG10* by adjacent brainstem nuclei (not shown). *CAP-23* was also expressed in all the deep cerebellar nuclei, with stronger expression in the parvocellular part of the lateral cerebellar nucleus and the medial cerebellar nucleus (Fig. 5.1). Some Purkinje cells in the paraflocculus and in some parts of the vermis, for example lobule X, expressed *CAP-23* (Fig. 5.2), but it was not expressed by the great majority of Purkinje cells in the cerebellar hemispheres. Some Purkinje cells in the paraflocculus also showed slightly stronger expression of *SCG10* than Purkinje cells in the rest of the cerebellar cortex, but they did not appear to be the same cells that expressed *CAP-23* (Fig. 5.2).

ii. Expression of SCG10, CAP-23 and GAP-43 in the cerebellum after implantation of a living tibial nerve graft

Animals were examined 3 days, 2 weeks, 4weeks, and 6 weeks (see Tables 2.2; 5.1) following implantation of a living tibial nerve graft into the cerebellum. In all animals with living grafts *SCG10*, *CAP-23* and *GAP-43* were upregulated in neurons in the cerebellar deep nuclei, ipsilateral to the graft, from 3 days onwards (Figs. 5.3-5.6). The numbers of neurons showing increased expression of the molecules appeared to be maximal at 3 days (compare Fig. 5.3 with Figs. 5.4-5.6). In animals in which the graft tip was medially located (especially at 2 weeks), some neurons in the contralateral medial deep nucleus had also upregulated all three molecules. At 2 weeks or longer post-grafting, *GAP-43* appeared to be upregulated in more neurons than were *SCG10* or *CAP-23*. Fewer neurons showing upregulated expression of *SCG10* and *CAP-23* in the deep nuclei were visible at 6 weeks compared to 2 weeks. At 3 days after grafting a few Purkinje cells close to the graft of one animal were found to have increased levels of *CAP-23* (Fig. 5.7). At later time points, no

upregulation of *CAP-23* was seen in Purkinje cells (Figs. 5.5, 5.6) and these neurons did not upregulate *SCG10* at any time point (Figs. 5.3, 5.4, 5.6).

iii. Retrograde labelling of neurons with axons in the grafts, and expression of SCG10 and CAP-23 visualised on adjacent sections

In three additional animals, CTB was injected into the distal graft to retrogradely label neurons which had regenerated axons into the grafts (survival time 4 weeks). All retrogradely labelled neurons were found in the cerebellar deep nuclei; an example is shown in Fig. 5.8. Alternate sections were reacted for ISH and CTB immunohistochemistry and subsequent analysis allowed the identification of individual neurons on consecutive sections. Where this was successful, all retrogradely labelled neurons had upregulated *SCG10*, and nearly all had upregulated *CAP-23*. Thus in Figs. 5.9 and 5.10, 19 retrogradely labelled neurons are identified, 16 of which are also identifiable on the adjacent sections, and all of these have upregulated *SCG10* mRNA. In Figs. 5.11-5.13, 22 retrogradely labelled neurons are identified, 18 of which are also identifiable on adjacent sections, and all of these have upregulated *CAP-23*.

iv. Expression of SCG10, CAP-23 and GAP-43 in the cerebellum following implantation of a freeze-killed tibial nerve graft

Three animals received autografts in the cerebellum of tibial nerve in which the cells had been killed by freeze-thaw cycles. 14 days post-operation, little or no upregulation of *SCG10* or *CAP-23* was observed in the deep cerebellar nuclei (Fig. 5.14), although in one animal 2-3 neurons showed a minimal upregulation of *SCG10* and *CAP-23*.

v. Neuronal cell bodies found in the implanted nerve grafts

A curious feature of the peripheral nerve grafts in the cerebellum was that they exhibited a high incidence of neuronal cell bodies ectopically located within the implanted nerve (Fig. 5.15). Five out of 19 living grafts in the cerebellum contained ectopic neurons, usually in clusters, compared with none of the 18 living nerve grafts placed in the thalamus (see Chapter 4). These cells expressed *SCG10* (Fig 5.15*a,b*), *CAP-23* (Fig. 5.15*d*) and *GAP-43*

(Fig, 5.15*c*). In animals used for retrograde labelling the ectopic neurons also contained retrograde tracer (Fig. 5.15 *e*,*f*) showing that they had axons extending to the distal graft.

Fig. 5.1. Expression of *SCG10* and *CAP-23* in the deep cerebellar nuclei of a control (unoperated) animal. Both molecules are expressed at a moderate level in neurons throughout the deep nuclei. *SCG10* expression is almost uniform in these cells, but stronger expression of *CAP-23* can be seen in the medial cerebellar nucleus (M) and the parvocellular part of the lateral cerebellar nucleus (L).

M:	Medial cerebellar nucleus	L:	Lateral cerebellar nucleus
CC:	Cerebral cortex	CP:	Choroid plexus of 4^{th} ventricle
BS:	Brainstem		

Bar 100μm.

FIGURE 5.1

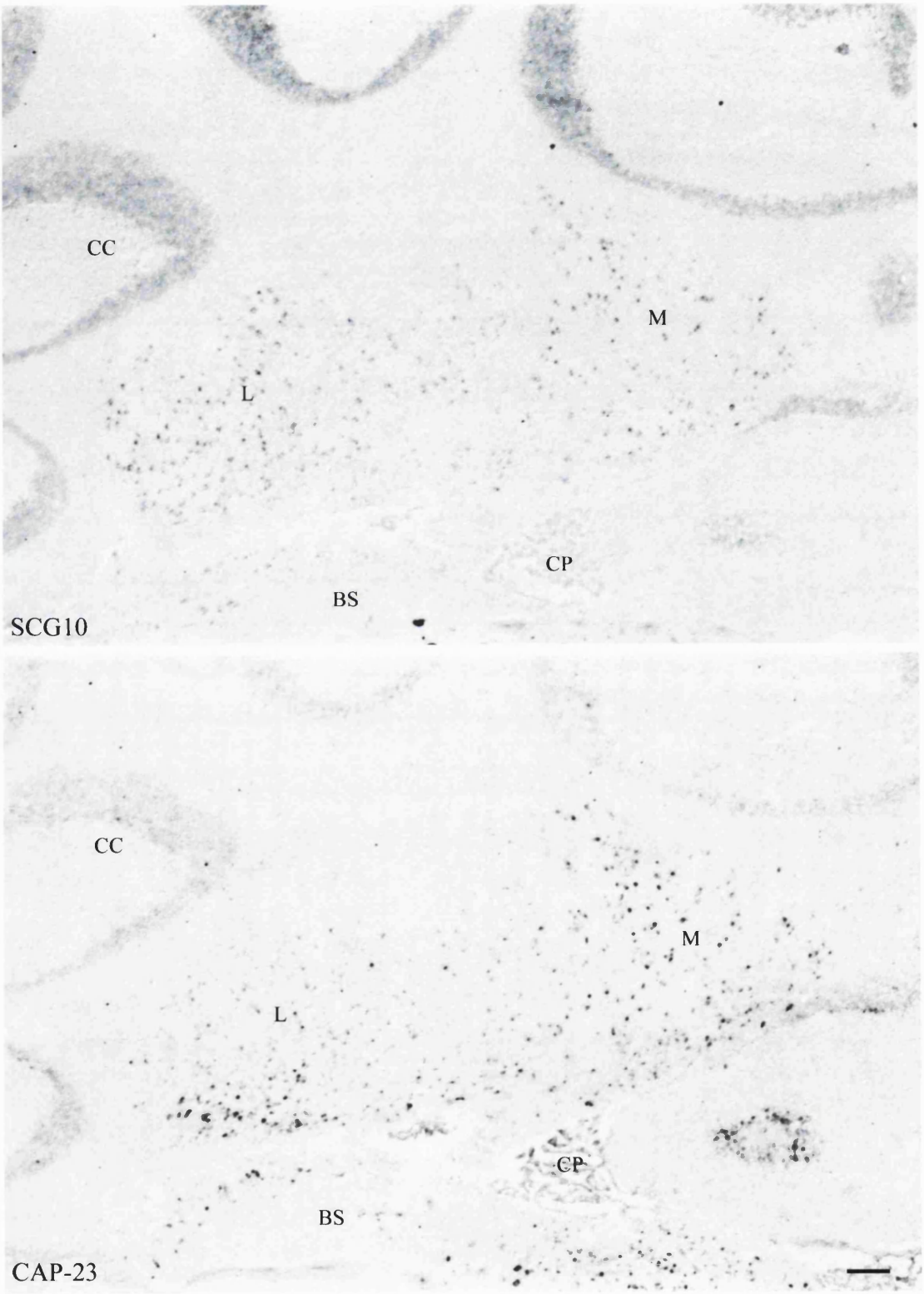


Fig. 5.2. Expression of *SCG10* and *CAP-23* in the cerebellar cortex of an unoperated animal. *a*, *b*: *SCG10* is expressed by Purkinje cells (arrows in *a*) throughout the cerebellar cortex with some cells in the paraflocculus (PF) showing stronger expression (arrows in *b*). *c*, *d*: *CAP-23* expression is found in a subset of Purkinje cells, indicated by arrows, in areas corresponding to vestibulocerebellar cortex, particularly lobule X of the vermis (*c*) and part of the paraflocculus (*d*).

DN:	Deep nuclei	CP:	Choroid plexus of 4th ventricle
BS:	Brainstem	PF:	Paraflocculus

Bars: 50μm (*a*); 250μm (*b-d*)

FIGURE 5.2

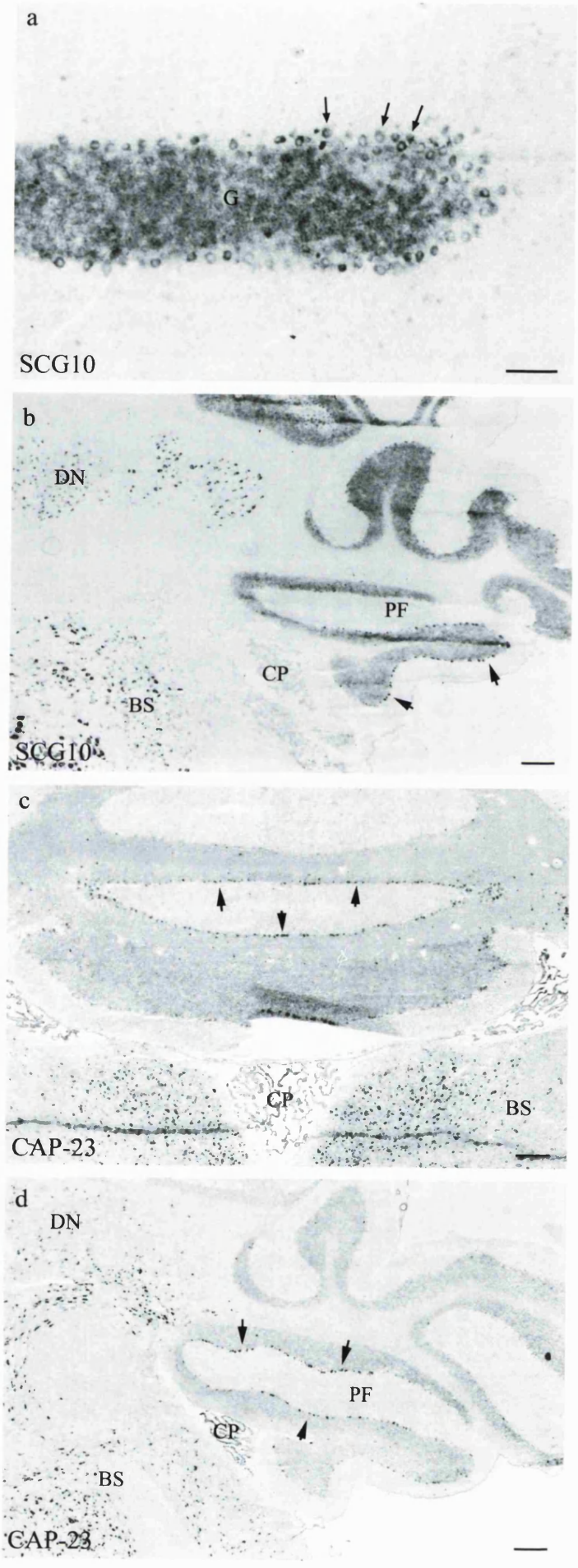


Fig. 5.3. Expression of *SCG10*, *CAP-23* and *GAP-43* in the cerebellum 3 days after implantation of a tibial nerve graft (outlined). Many neurons in the ipsilateral medial cerebellar nucleus, next to the graft tip, have upregulated both molecules (compare with the contralateral side and with Fig. 5.1).

Bar 500μm.

FIGURE 5.3

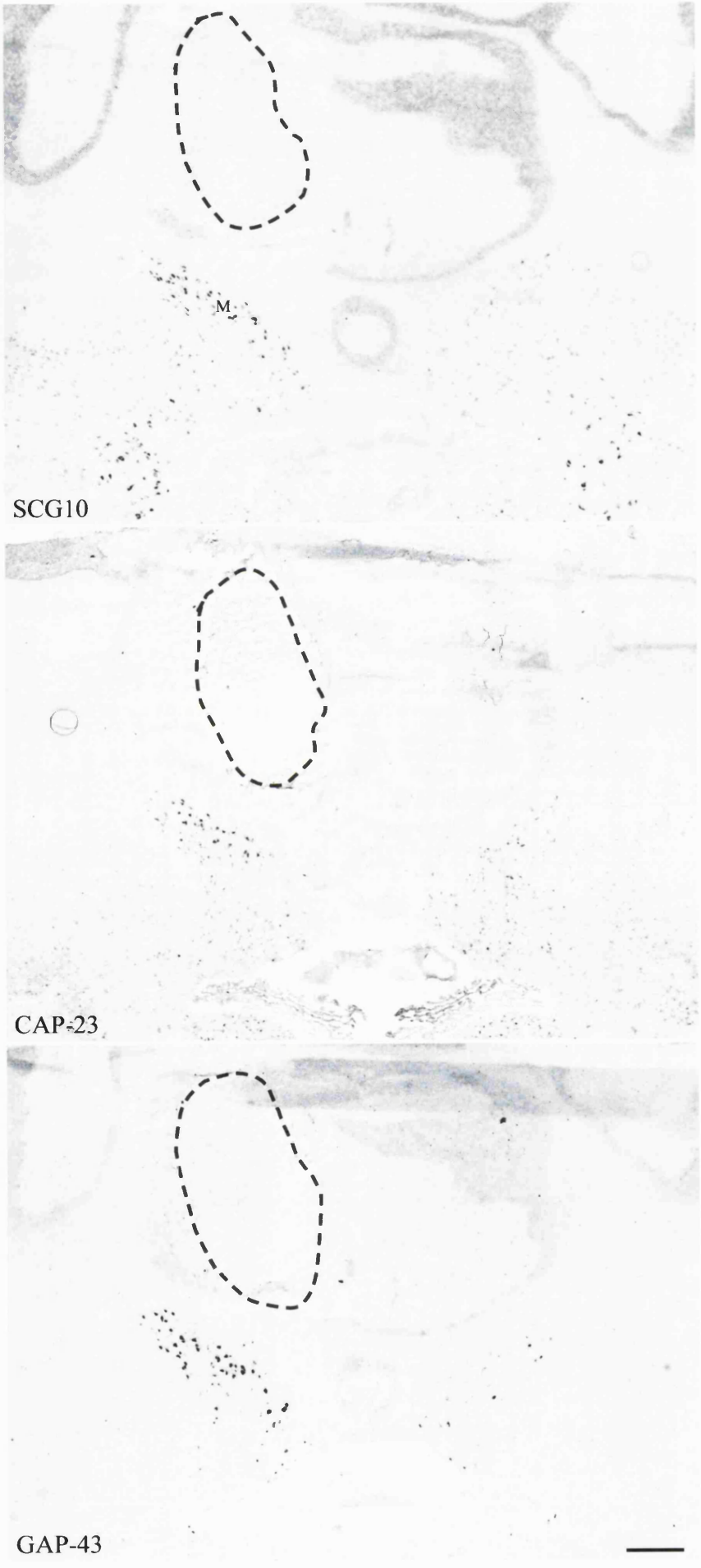


Fig. 5.4. Expression of *SCG10* in the cerebellum two weeks after the implantation of a tibial nerve graft (outlined). *SCG10* is clearly upregulated in several neurons of the ipsilateral medial cerebellar nucleus close to the graft (shown at higher magnification in the lower panel) and in one neuron in the contralateral medial cerebellar nucleus. No upregulation is seen in any neurons of the cerebellar cortex. Compare with Fig. 5.1.

Bars: 250μm (top); 50μm (bottom).

FIGURE 5.4

Fig. 5.5. Expression of *CAP-23* in the cerebellum two weeks after the implantation of a tibial nerve graft (outlined). *CAP-23* is clearly upregulated in several neurons of the ipsilateral medial cerebellar nucleus close to the graft (shown at higher magnification in the lower panel), but no upregulation is seen in any neurons of cerebellar cortex. Compare with Fig. 5.1.

Bars: 250μm (top); 50μm (bottom).

FIGURE 5.5

CAP-23

Fig. 5.6. Expression of *SCG10*, *CAP-23* and *GAP-43* in the cerebellum six weeks after the implantation of a tibial nerve graft (outlined). All three molecules are still upregulated in several neurons (indicated by arrows) in the medial cerebellar nuclei. (Compare with Fig. 5.1).

Bar 500µm.

FIGURE 5.6

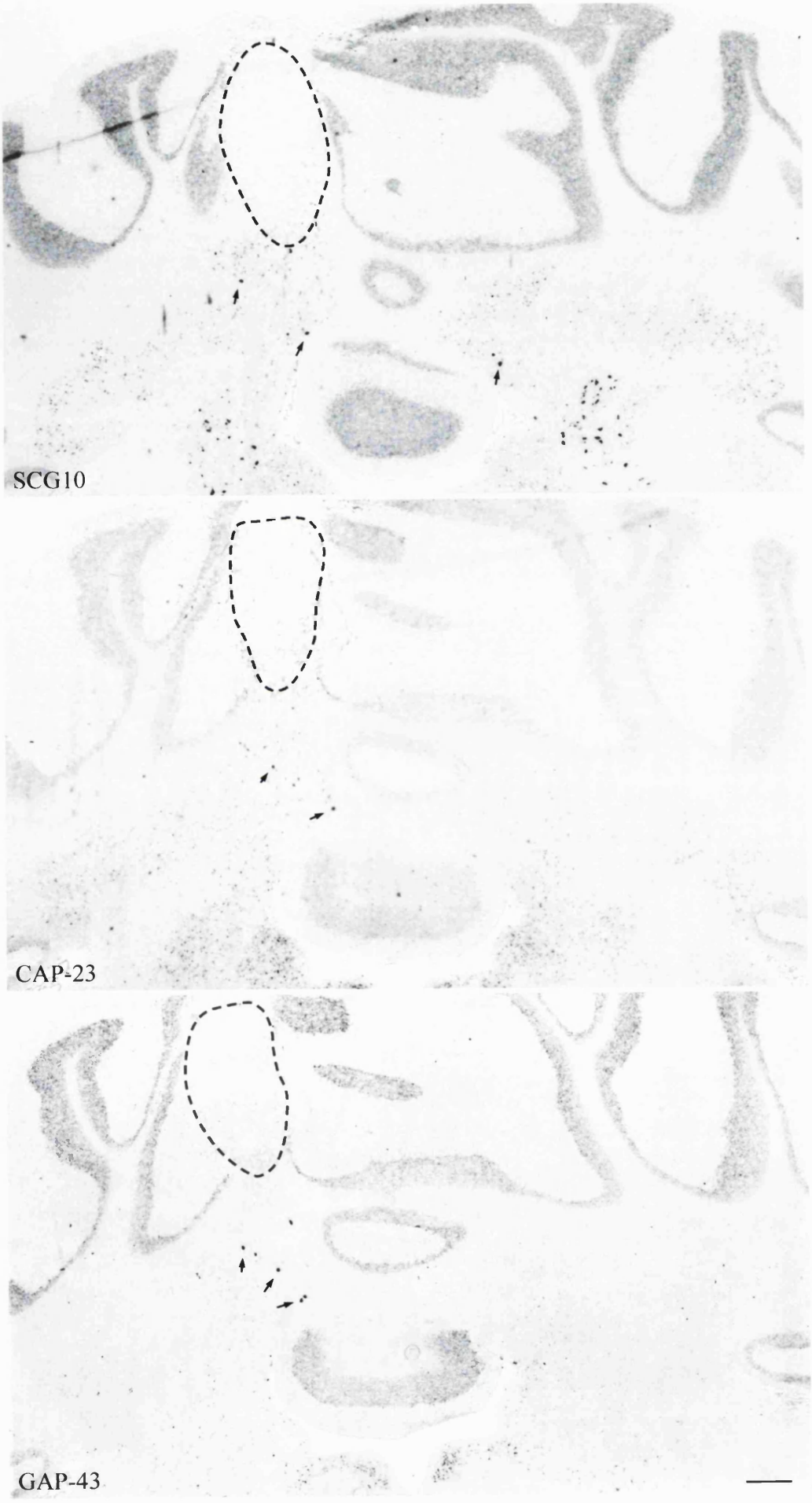


Fig. 5.7. Expression of *CAP-23* in Purkinje cells three days after graft implantation. A small number of Purkinje cells, indicated by arrows, show upregulation of *CAP-23* mRNA. These are located close to the graft (G) in a region where no constitutive expression of *CAP-23* was found in unoperated animals, and the majority of Purkinje cells on the part of cerebellar cortex shown here do not express *CAP-23*.

Bar 50μm.

FIGURE 5.7

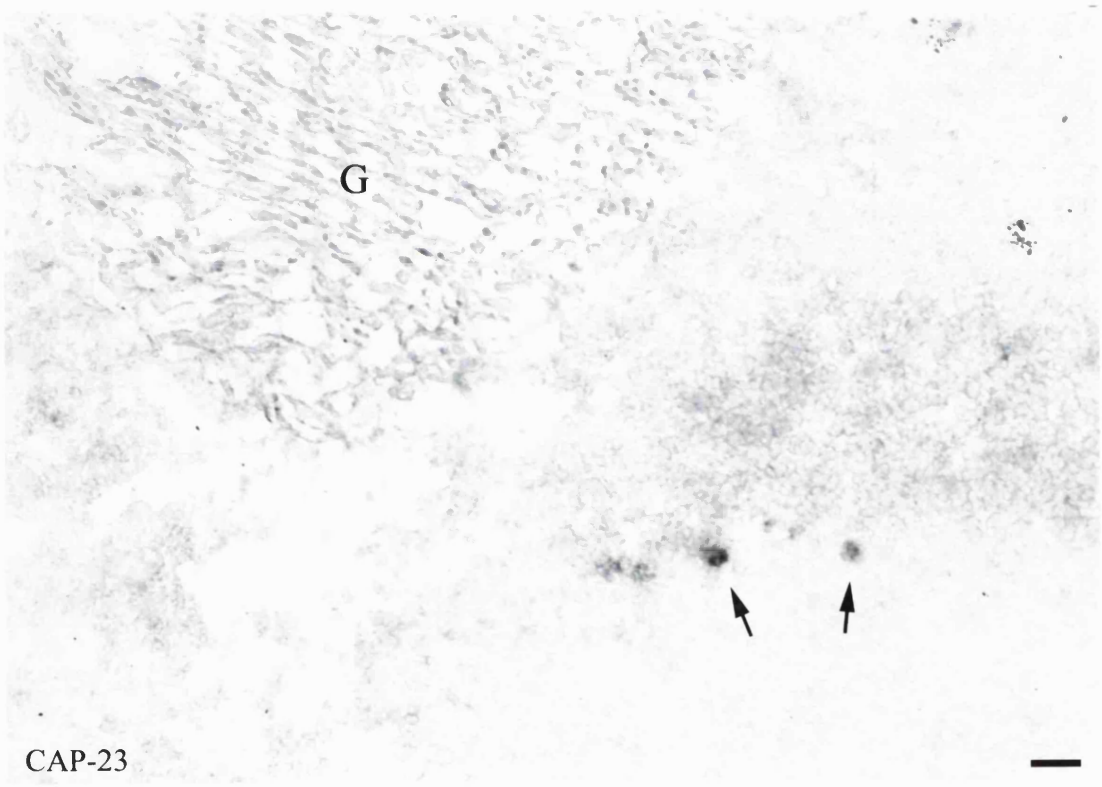


Fig. 5.8. Retrograde labelling of neurons with axons in the grafts with CTB. CTB was applied to the distal graft 24 hours before sacrifice (4 weeks after graft implantation). In this low power image, 4 neurons (indicated by the arrow) can be seen in the medial cerebellar nucleus which are CTB-positive, indicating they have axons in the distal part of the graft.

Bar 1mm.

FIGURE 5.8

Figs. 5.9, 5.10. Retrograde labelling with CTB applied to the graft tip and ISH for *SCG10* on consecutive serial sections of cerebellum , 4 weeks after implantation of a tibial nerve graft. Each column shows a set of three consecutive sections, the central section having been reacted for immunohistochemistry for CTB (centre panels) and so identifying neurons which have regenerated axons into the graft. All but two retrogradely labelled neurons can be identified on the adjacent hybridised sections and all of these can be seen to have upregulated *SCG10* mRNA. A total of four examples are shown, taken from 3 animals.

Bar 100μm.

FIGURE 5.9

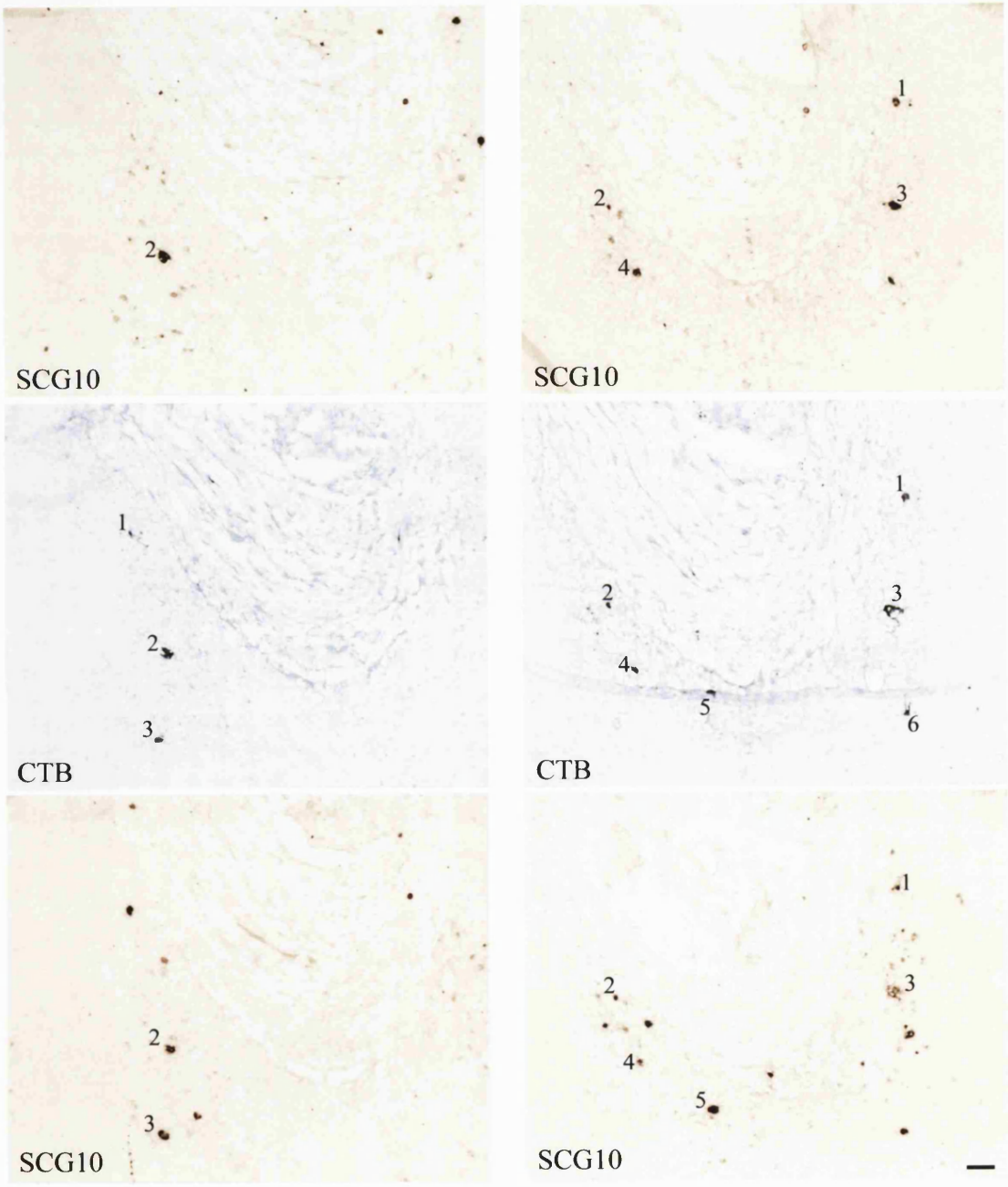
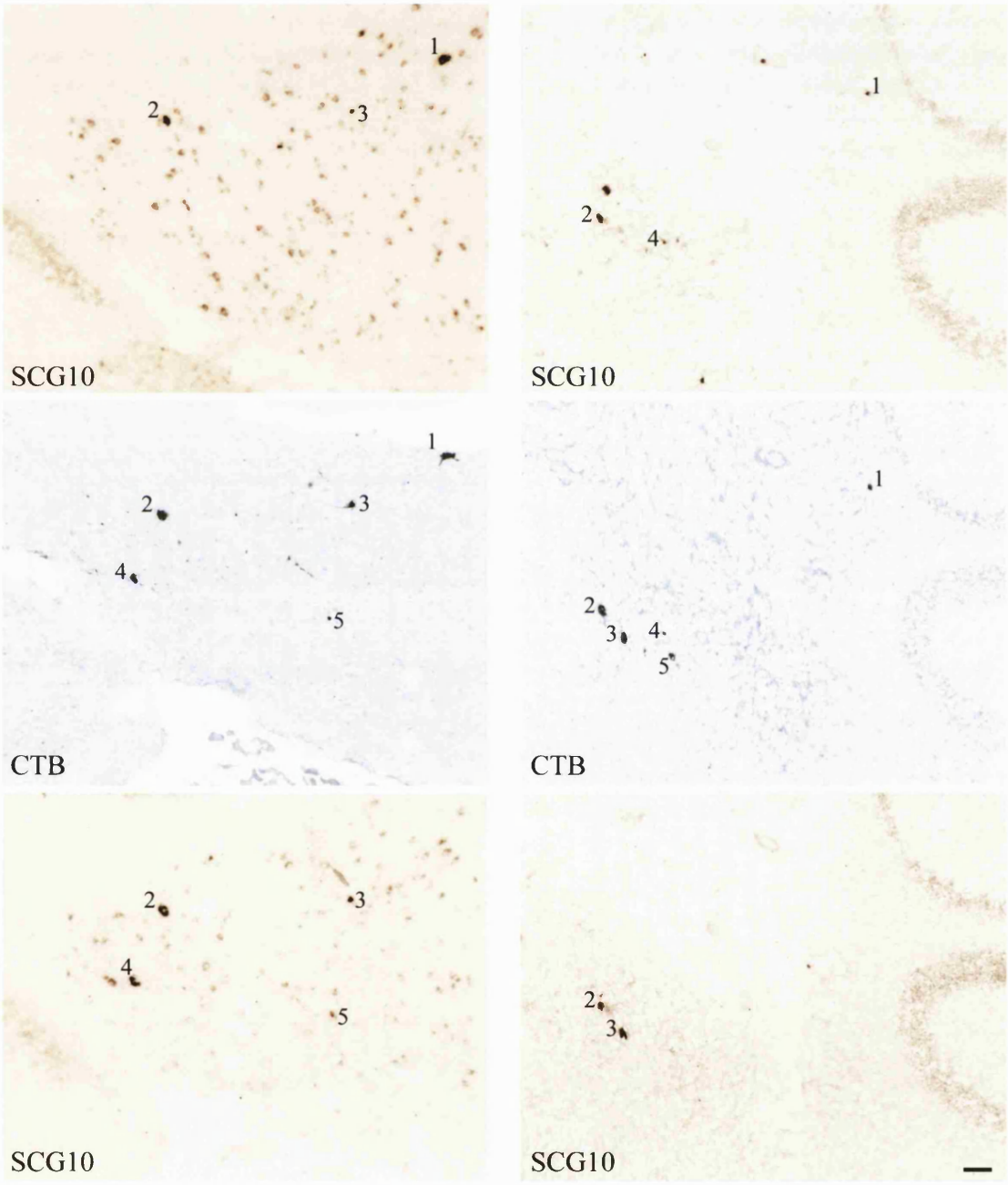


FIGURE 5.10



Figs. 5.11, 5.12, 5.13. Retrograde labelling with CTB applied to the graft tip and ISH for *CAP-23* on consecutive serial sections of cerebellum, 4 weeks after implantation of a tibial nerve graft. Each column in Figs. 5.11 and 5.12 shows a set of three consecutive sections, the central section having been reacted for immunohistochemistry for CTB (centre panels) and so identifying neurons which have regenerated axons into the graft. In Fig. 5.13 pairs of adjacent sections are shown, one of each pair (in the lower panels) having been reacted for CTB immunohistochemistry. Most retrogradely labelled neurons can be identified on the adjacent hybridised sections and all of these can be seen to have upregulated *CAP-23* mRNA. A total of five sets of sections are shown, taken from 3 animals.

Bars 100μm.

FIGURE 5.11

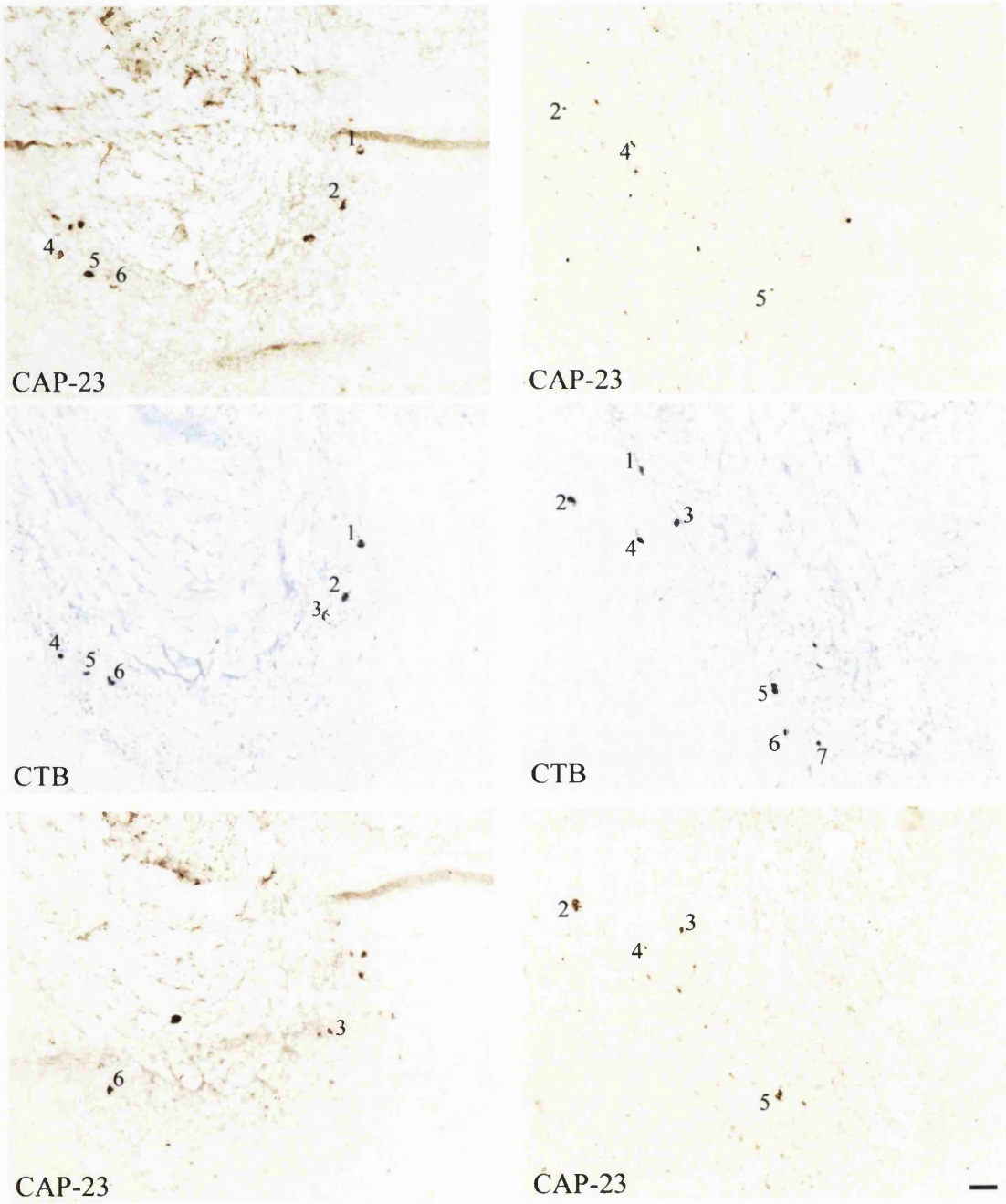

1
2
4
5
6
CAP-23
2
4
5
CAP-23
1
2
3
4
5
6
CTB
1
2
3
4
5
6
7
CTB
3
6
CAP-23
2
3
4
5
CAP-23

FIGURE 5.12

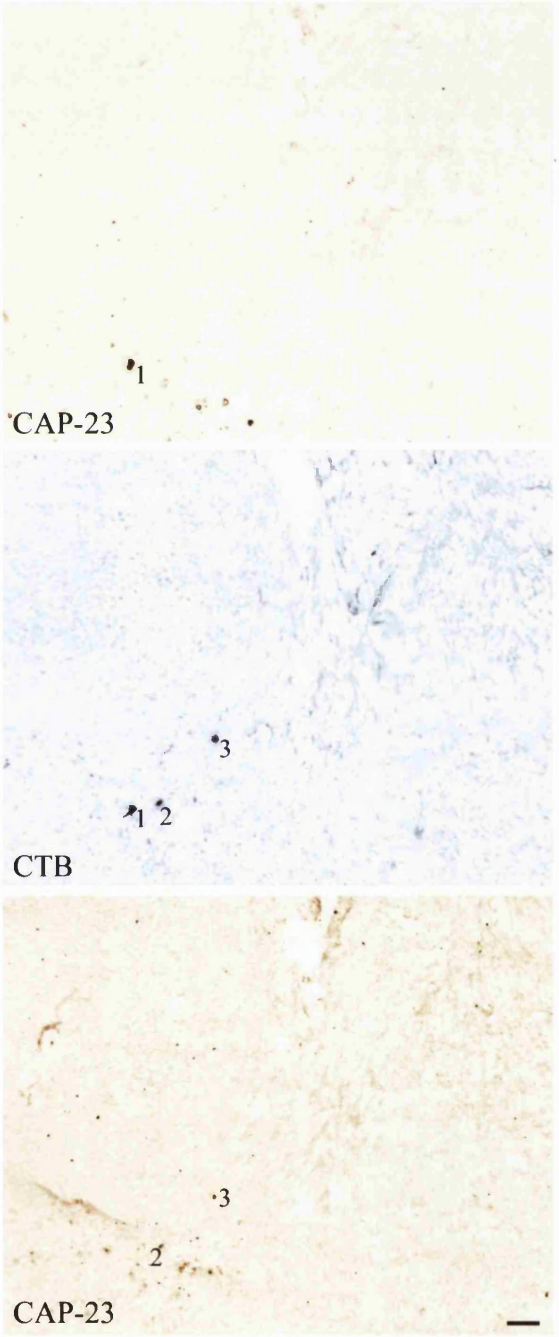


FIGURE 5.13

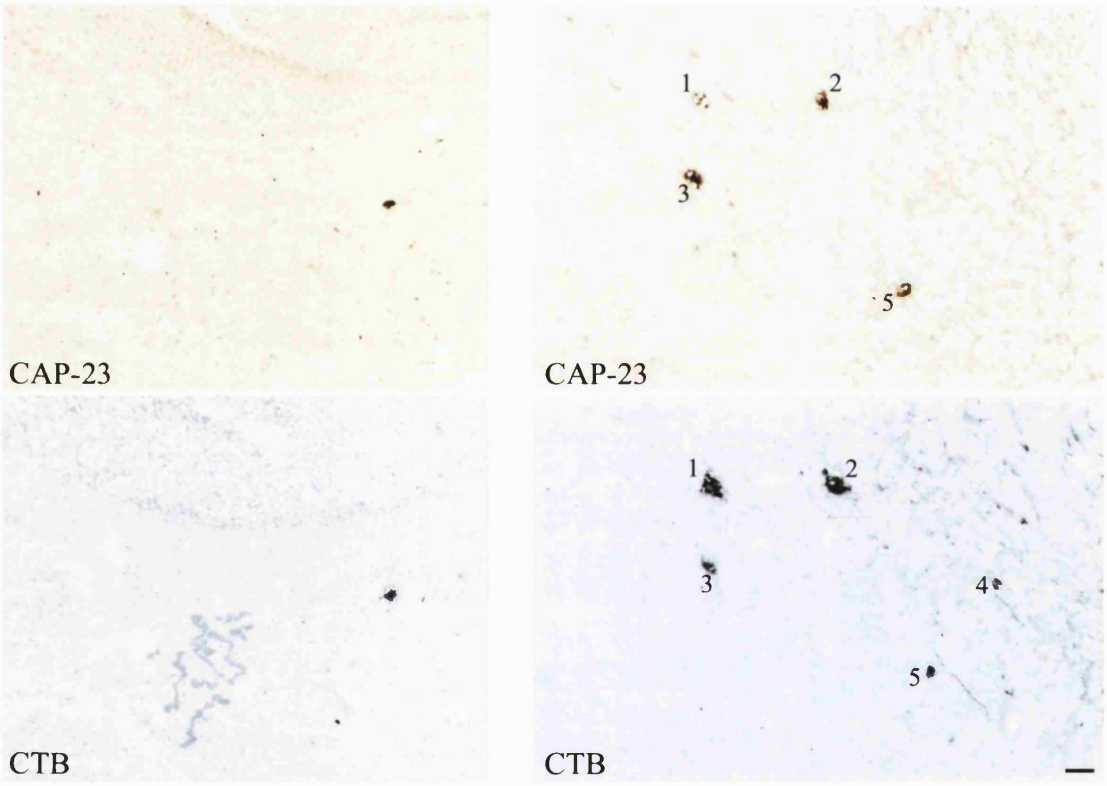


Fig. 5.14. Expression of *SCG10* and *CAP-23* in the cerebellum 2 weeks after implantation of a tibial nerve graft (outlined) in which the cells have been killed by freeze-thaw cycles, and which therefore fails to support axon regeneration. No upregulation of *SCG10* or *CAP-23* is visible in the cerebellar deep nuclei. The graft position is similar to that of the living graft shown in Figs. 5.3- 5.6.

Bar 500μm

FIGURE 5.14

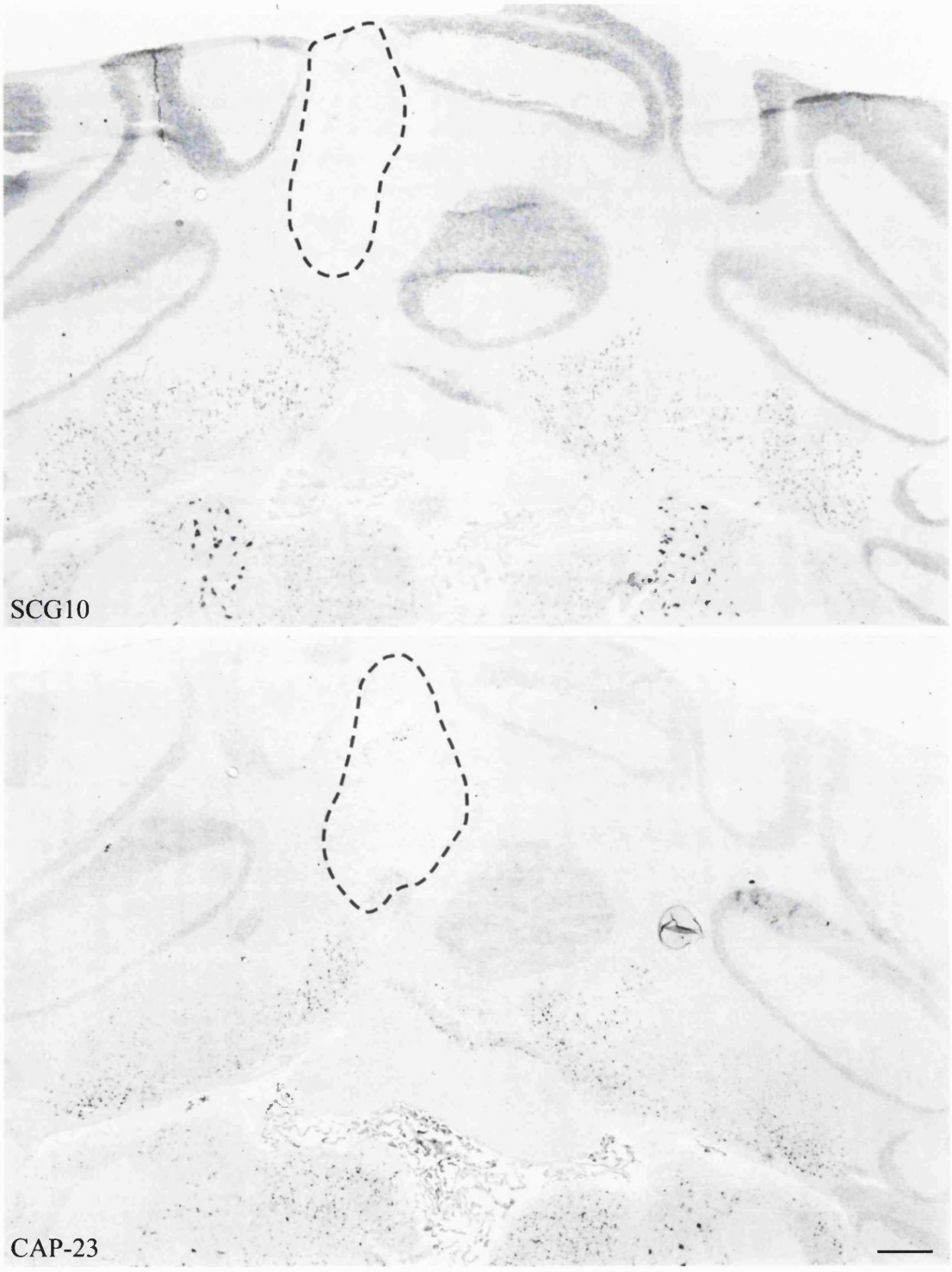
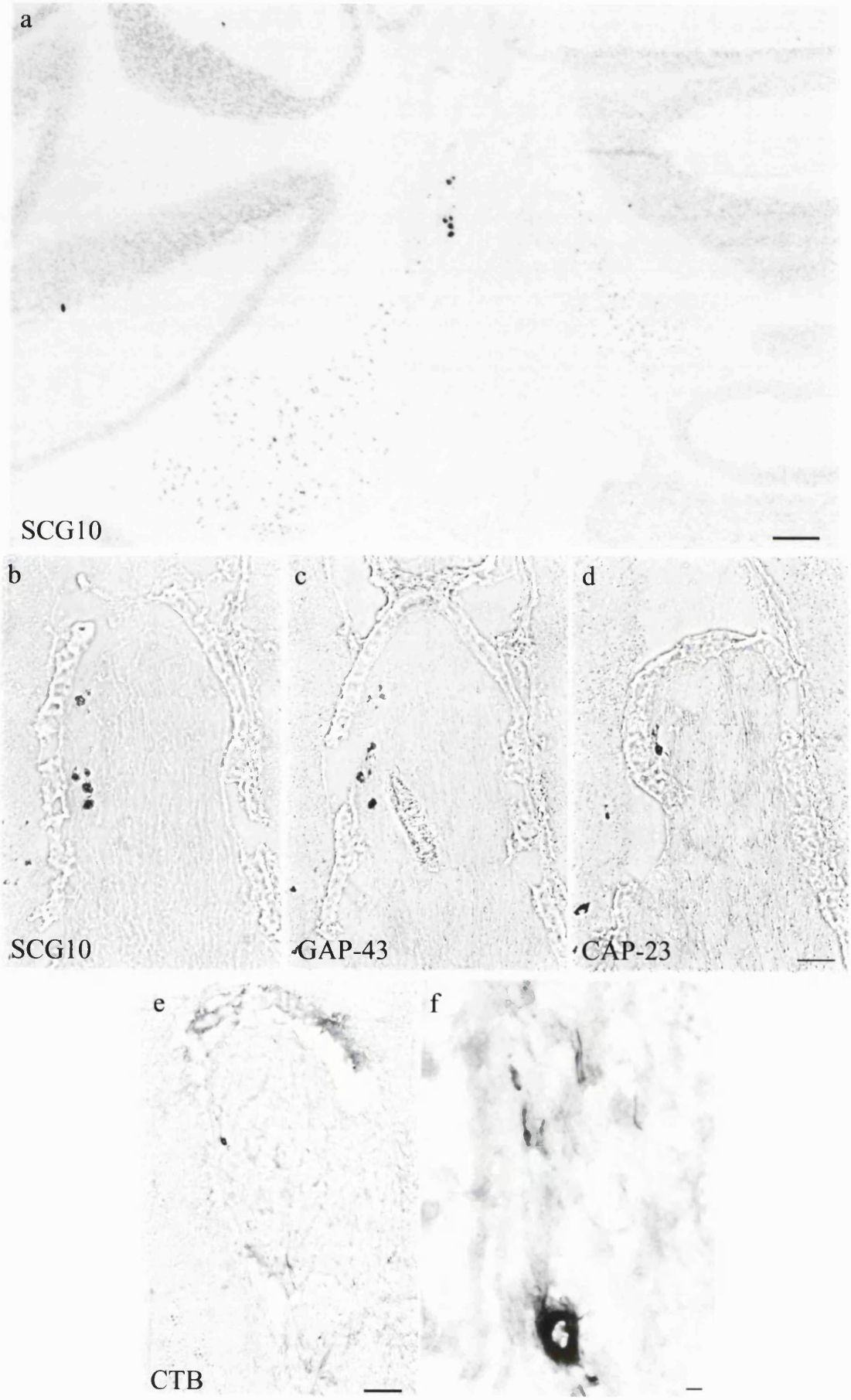


Fig. 5.15. Ectopic neurons within a tibial nerve graft in the cerebellum. In *a-d*, 2 weeks after graft implantation a cluster of neurons is visible in the graft. These neurons express for *SCG10* (*a*,*b*), *GAP-43* (*c*) and *CAP-23* (*d*). The boundaries of the graft are more easily seen in the phase contrast images of the serial sections presented in panels *b-d*. In *e* and *f*, 4 weeks after graft implantation and following application of CTB to the distal graft, the graft contains an ectopic neuron which has been retrogradely labelled with CTB. At higher magnification, the neuronal characteristics of the retrogradely labelled cell and the presence of CTB-labelled axons in the graft are apparent.

Bar 250μm (*a*); 100μm (*b-d*); 200μm (*e*); 10μm (*f*).

FIGURE 5.15



Chapter 6

Expression of *FKBP12* in axotomised neurons after implantation of a tibial nerve graft in the thalamus or cerebellum

6.1 Introduction

The immunophilin FKBP12 has previously been shown to be upregulated in axotomised neurons following peripheral nerve injury, and may mediate neurotrophic effects of FK506 (Gold et al., 1994; Lyons et al., 1994; Gold et al., 1995; Lyons et al., 1995). To determine whether its upregulation is consistently associated with axon regeneration, expression of *FKBP12* mRNA was studied by ISH in CNS neurons regenerating into peripheral nerve grafts. Animals with grafts in thalamus or cerebellum were studied. Nearly all these animals were also used for the studies of expression of *SCG10*, *CAP-23* and *GAP-43* mRNAs presented in Chapters 4 and 5, and the experimental outline is therefore identical. The data on expression of *FKBP12* mRNA was, however, analysed separately. *FKPB12* expression was examined between 3 days and 6-7 weeks after living tibial nerve autografts were placed in the thalamus or cerebellum, and retrograde labelling was also used in some animals to identify neurons which had regenerated axons within the graft. Freeze-killed nerve graft controls were also examined. Animal usage and survival times are detailed in Table 6.1.

6.2 Results

i. Expression of FKBP12 in the intact thalamus

The pattern of expression of *FKBP12* was examined in the intact thalamus. Expression appeared to be largely confined to neurons, and was found in the great majority of neurons throughout the dorsal thalamus with only minor variation. *FKBP12* was expressed at a much lower level in the TRN, although it was present (Fig.6.1).

ii. Expression of FKBP12 in the thalamus after implantation of a tibial nerve graft

FKBP12 expression was examined in the thalamus 3 days, 2 weeks, 4 weeks, and 6 weeks after implantation of a nerve graft (see Table 6.1). Upregulation of *FKBP12* was observed in neurons of the TRN ipsilateral to the grafts. A slight increase in *FKBP12* was visible at 3 days (Fig.6.2), and a greater increase was clearly visible at 2 weeks (Fig.6.3), 4 weeks

Surgical procedure	Survival time	*n*
Tibial nerve autograft in thalamus	3 days	3
	2 weeks	5
	4 weeks	3
	6 weeks	3
Tibial nerve autograft in thalamus, followed by CTB injection into the distal graft	4 weeks	3
	6 weeks	1
Freeze-killed tibial nerve autograft in thalamus	2 weeks	3
Tibial nerve autograft in cerebellum	3 days	3
	4 weeks	2
	6 weeks	2
Tibial nerve autograft in cerebellum, followed by CTB injection into the distal graft	4 weeks	3
Freeze-killed tibial nerve autograft in cerebellum	2 weeks	3

Table 6.1. Animal usage and survival times for studies of *FKBP12* expression following tibial nerve autograft implantation into the thalamus or cerebellum.

(not shown) and 6 weeks (Fig.6.3). Many more neurons were seen to upregulate *FKBP12* at 3 days than at later time points (compare Fig. 6.2 with Fig. 6.3).

In four animals regenerating neurons were identified by retrograde labelling with CTB applied to the distal end of the graft. These were examined after survival times of 4 weeks (n=3) and 6 weeks (n=1). As described in Chapter 4, retrogradely labelled neurons were largely confined to the ipsilateral TRN. Alternate sections were processed for *FKBP12* ISH or CTB immunohistochemistry. Many individual neurons identified by retrograde labelling could also be identified on adjacent sections processed for ISH and nearly all identified regenerating neurons expressed high levels of *FKBP12* (Figs. 6.4-6.6): of 50 cells identified as CTB positive in Figs. 6.4-6.6 (centre panels), 47 are also identifiable on the adjacent sections reacted for ISH, and 46 of these have upregulated *FKBP12* mRNA.

Three animals received freeze-killed tibial nerve grafts (which do not support axonal regeneration) in the thalamus. In these animals (survival time 14 days), no neurons in the TRN showed upregulation of *FKBP12* expression (Fig.6.7).

iii. FKBP12 expression in the intact cerebellum

FKBP12 expression was examined in the intact cerebellum, where it was found to be expressed at a moderate level in neurons of the deep cerebellar nuclei, and in the granule cell layer of the cerebellar cortex. High levels were expressed by Purkinje cells throughout the cerebellar cortex and by neurons in adjacent brainstem nuclei (Fig.6.8).

iv. Expression of FKBP12 in the cerebellum after implantation of a tibial nerve graft

Animals were examined 3 days, 4 weeks and 6 weeks after implantation of living grafts into the cerebellum (see Table 6.1). *FKPB12* was upregulated in neurons in the cerebellar deep nuclei, ipsilateral to the graft, at all these time points (Figs.6.9, 6.10). At 3 days the number of neurons showing increased expression was greater than at later time points, but the strength of the signal for FKBP 12 mRNA in individual neurons was higher at the later time points (compare Figs. 6.9 and 6.10). The numbers of neurons showing upregulated expression of *FKBP12* in the deep nuclei were similar at 6 weeks and 4 weeks. In three further animals, neurons which had regenerated axons into the graft were identified by retrograde labelling with CTB (survival time 4 weeks). All retrogradely labelled neurons were found in the deep cerebellar nuclei. Adjacent sections were processed for CTB immunohistochemistry and ISH for *FKBP12* and analysis of these allowed individual neurons to be identified on adjacent sections. Nearly all retrogradely labelled neurons had upregulated *FKBP12* (Figs. 6.11-6.12). Of 15 cells identified as CTB positive in Figs. 6.11-6.12 (centre panels), 13 are identifiable on the adjacent sections processed for ISH, and 12 of these have upregulated *FKBP12*.

Three rats received freeze-killed grafts in the cerebellum. 14 days post-operation, no upregulation of *FKBP12* could be seen in deep cerebellar neurons in these animals (Fig. 6.13).

Fig. 6.1. Expression of *FKBP12* in the thalamus of an unoperated animal. Expression is found throughout the dorsal thalamus with only minor variation, whereas in the TRN (outlined) expression is much lower.

Bar 250μm.

FIGURE 6.1

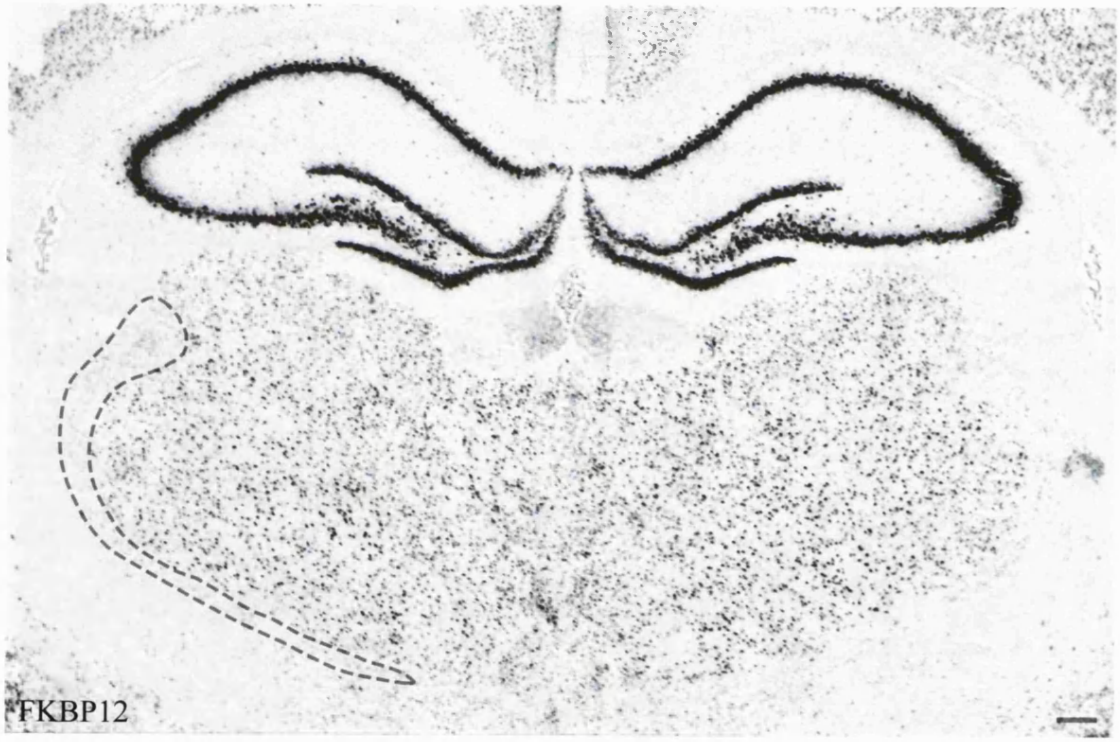


Fig. 6.2. Expression of *FKBP12* 3 days after implantation of a tibial nerve graft into the thalamus. Upregulation of *FKBP12* is visible in most neurons of the TRN ipsilateral to the graft seen on this section. The outlines of the TRN on both sides are marked by a dashed line. No upregulation can be seen in neurons of the dorsal thalamus. The graft itself is not visible on this section, but graft implantation has resulted in tissue damage where the ipsilateral hippocampus would normally be found.

Bar 250μm.

FIGURE 6.2

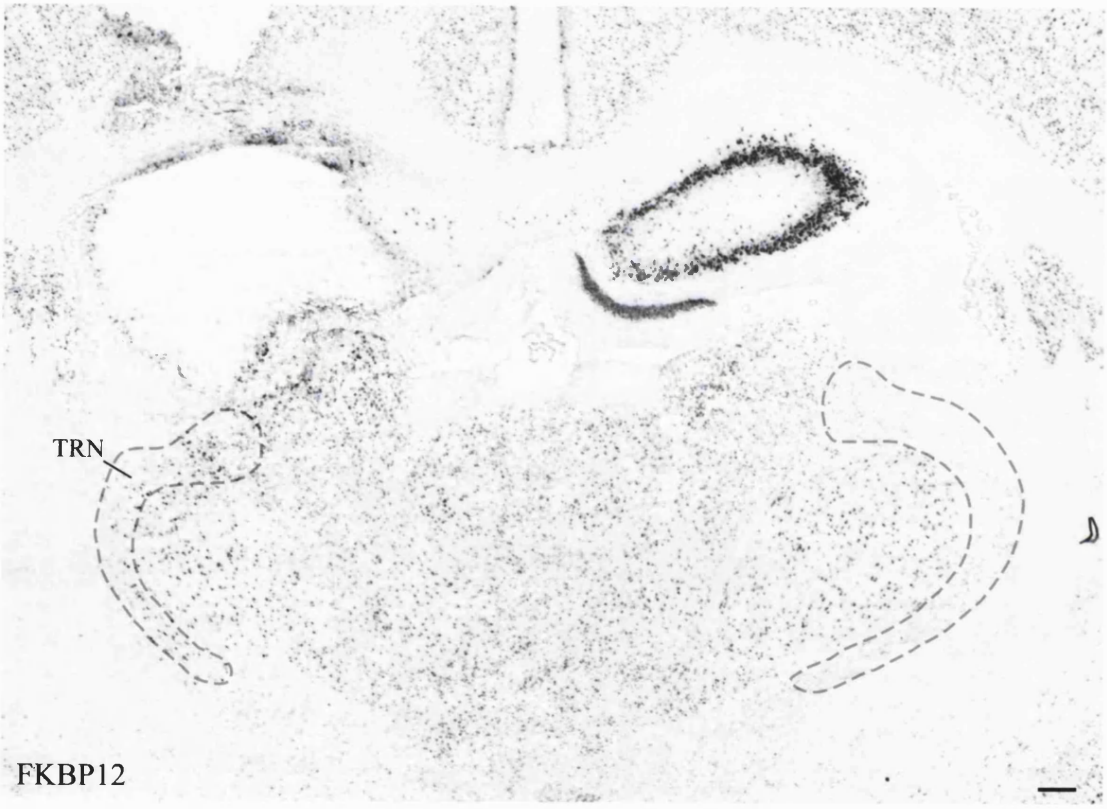


Fig. 6.3. Expression of *FKBP12* 2 weeks (*a*,*d*) and 6 weeks (*b*,*c*) after implantation of a nerve graft (outlined) into the thalamus. Neurons in the rostrodorsal part of the TRN, indicated by arrows in a and b, have upregulated *FKBP12* and are shown at higher magnification in *c* and *d*. No upregulation can be seen in neurons of the dorsal thalamus.

Bars 500μm (*a*,*b*); 50μm (*c*,*d*).

FIGURE 6.3

Figs. 6.4, 6.5, 6.6. Retrograde labelling with CTB applied to the distal graft and *FKBP12* ISH on consecutive serial sections of thalamus 4 weeks (Figs. 6.5 and 6.6) and 6 weeks (Fig. 6.4) after graft implantation. Each column shows a set of three consecutive sections, the central section having been immunohistochemically reacted for CTB (centre panels) and so identifying neurons which have regenerated axons into the graft. Nearly all retrogradely labelled neurons (47 out of 50) are identifiable on the adjacent hybridised sections. All but one of the neurons so identified show upregulation of *FKBP12* mRNA.

Bar 20μm (Fig. 6.4); 50μm (Figs. 6.5, 6.6).

FIGURE 6.4

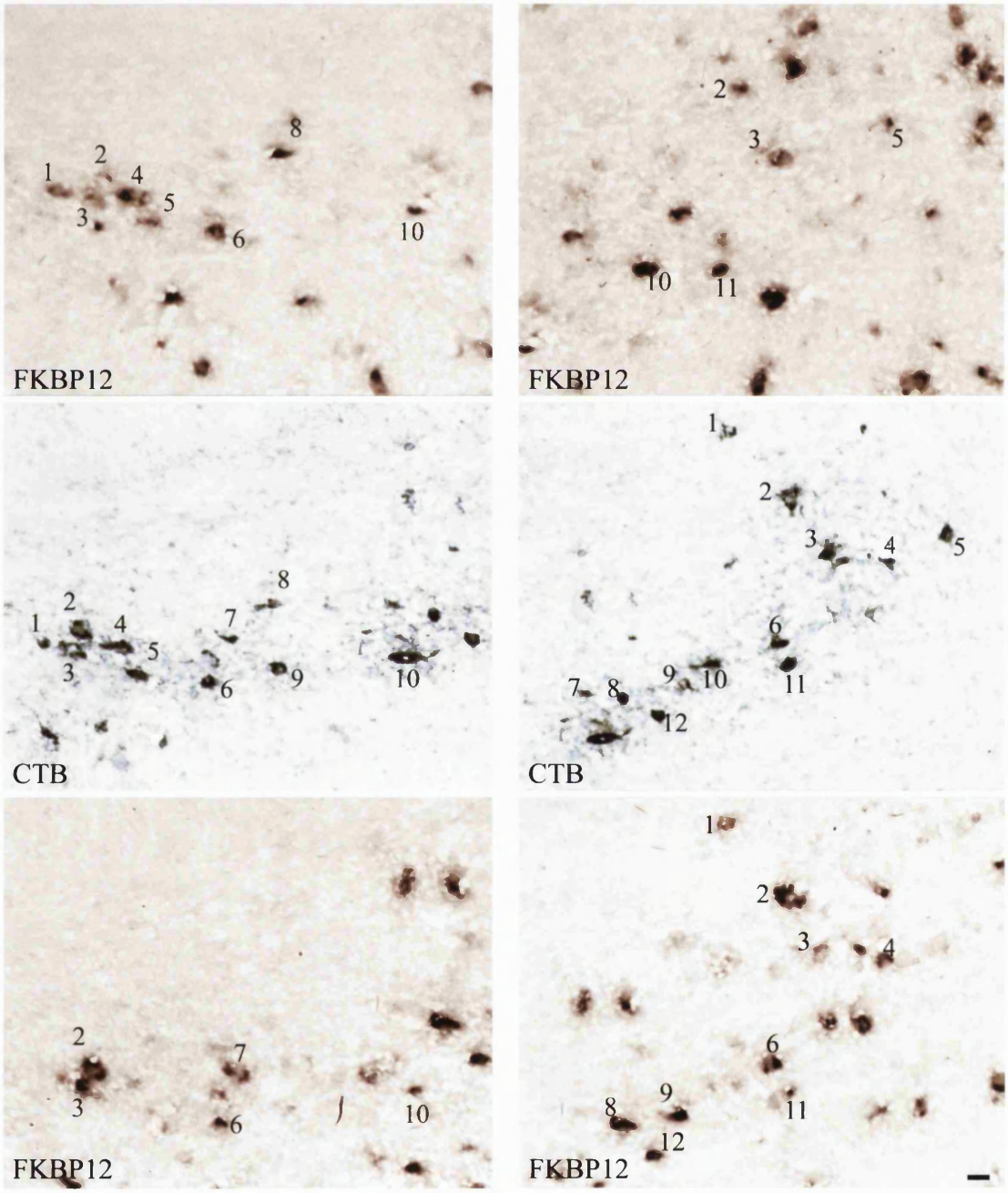


FIGURE 6.5

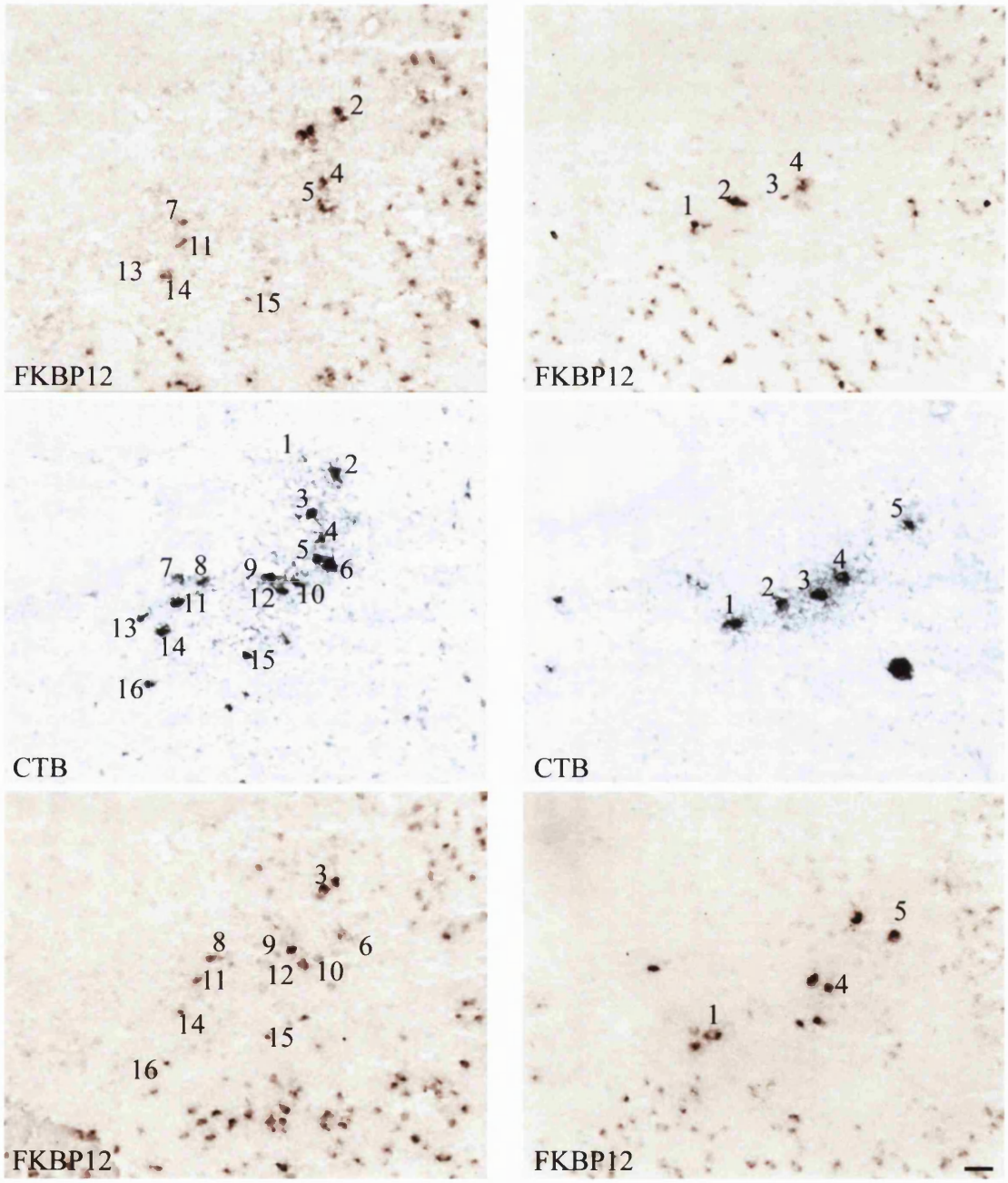


FIGURE 6.6

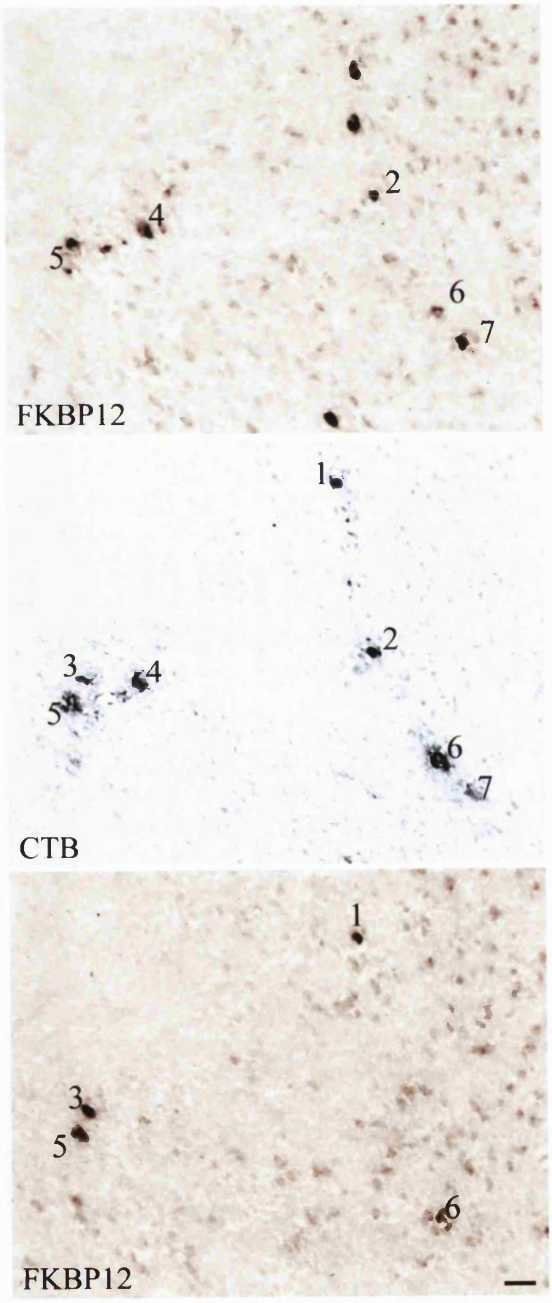


Fig. 6.7. Expression of *FKBP12* in the thalamus following implantation of a tibial nerve graft (outlined) in which the cells have been killed by repeated freeze-thaw cycles. No upregulation of *FKBP12* can be seen in the ipsilateral TRN or in the dorsal thalamus. The graft is in a similar position to that of the living grafts seen in Figs. 6.2 and 6.3.

Bar 500μm.

FIGURE 6.7

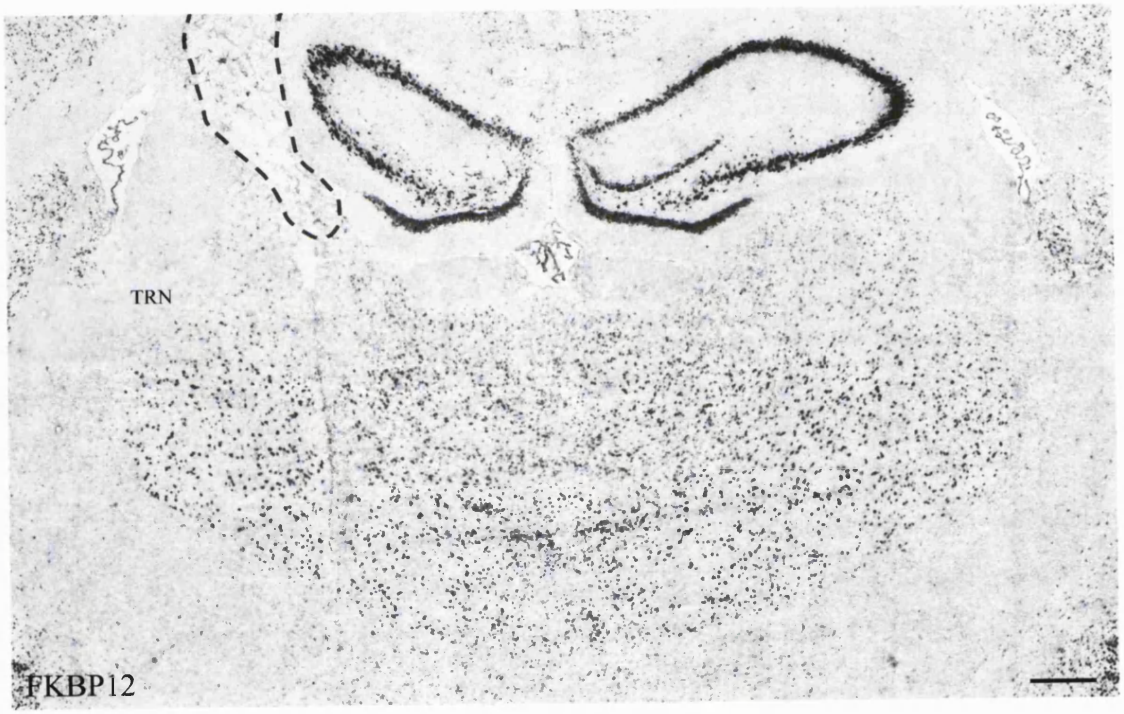


Fig. 6.8. Expression of *FKBP12* in the cerebellum of an unoperated animal. Moderate expression is seen throughout the medial, interposed and lateral cerebellar nuclei (indicated by M, I and L respectively), and in the granule cell layer of cerebellar cortex. Strong expression is seen in all Purkinje cells.

Bar 500μm.

FIGURE 6.8

Fig. 6.9. Expression of *FKBP12* in the cerebellum three days after implantation of a nerve graft. Slight upregulation of *FKBP12* is visible in many neurons of the medial cerebellar nucleus ipsilateral to the graft (compare with the contralateral side and with Fig. 6.8).

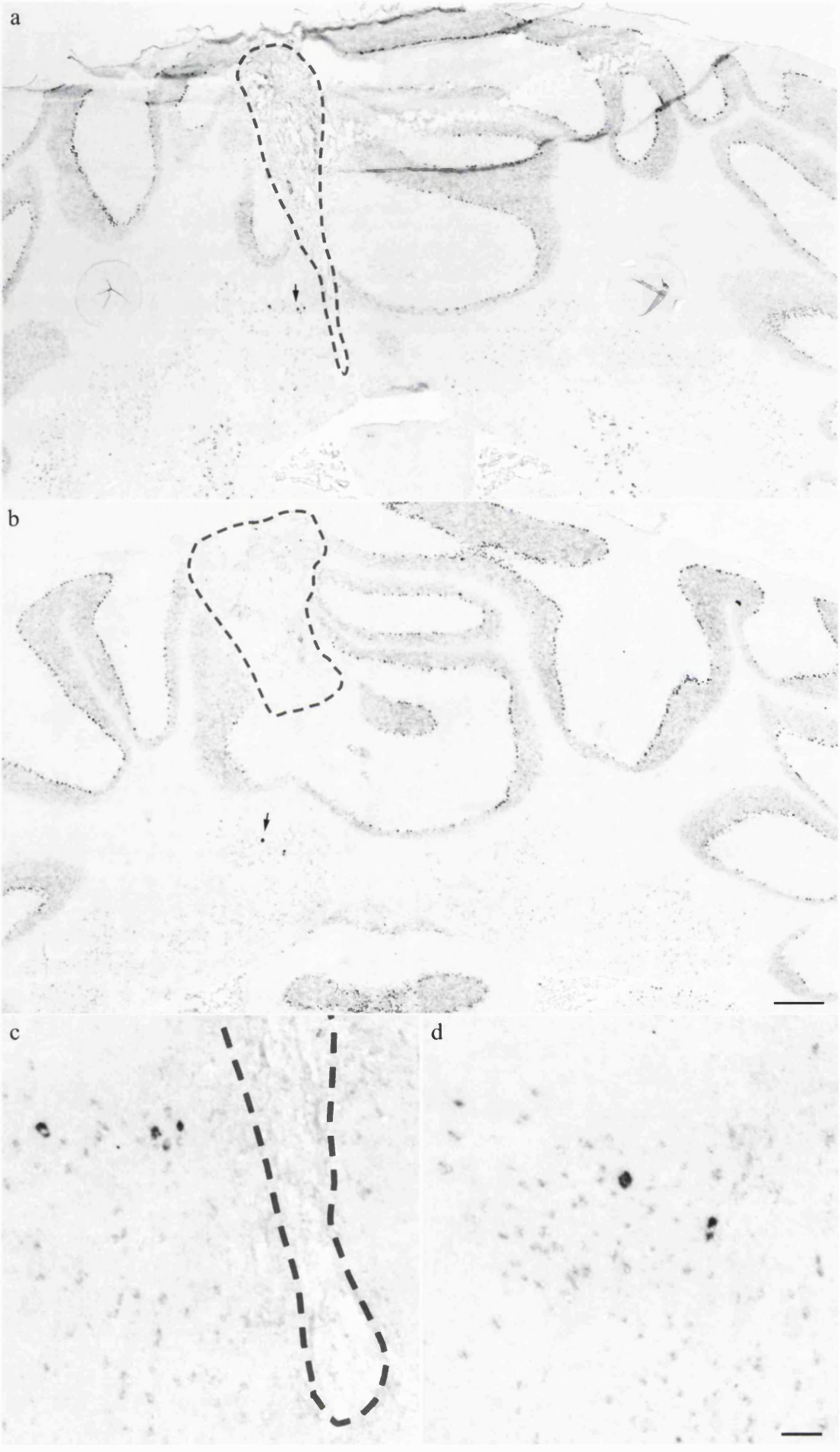
Bar 500μm

FIGURE 6.9

Fig. 6.10. Expression of *FKBP12* in the cerebellum 4 weeks (*a*,*c*) and 6 weeks (*b*,*d*) after implantation of a tibial nerve graft. At both time points, several neurons near the graft in the ipsilateral medial cerebellar nucleus can be seen to have upregulated *FKBP12* (indicated by arrows). *FKBP12*-positive neurons are shown at higher magnification in *c* and *d*.

Bars 500μm (*a*,*b*); 100μm (*c*,*d*).

FIGURE 6.10



Figs. 6.11, 6.12. Retrograde labelling with CTB applied to the distal graft and ISH for *FKBP12* on adjacent serial sections of cerebellum 4 weeks after graft implantation. Each column shows a set of three consecutive sections, the central section having been reacted by immunohistochemistry for CTB (centre panels) and so identifying neurons which have regenerated axons into the graft. Of those CTB-positive neurons which are also identifiable on the adjacent sections processed for ISH, all but one have upregulated *FKBP12* mRNA. A total of four sets of sections are shown, taken from 3 animals.

Bars 100μm.

FIGURE 6.11

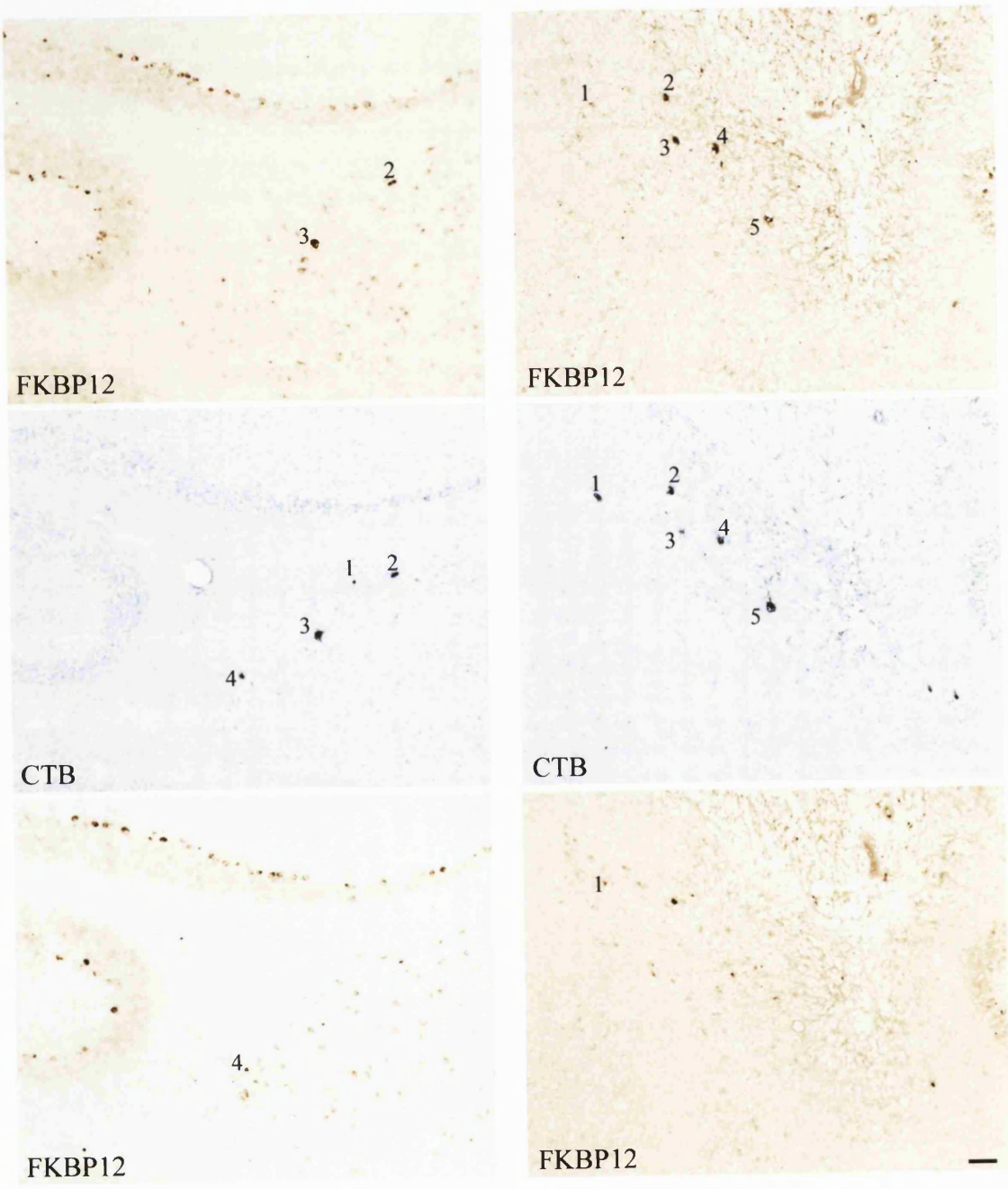


FIGURE 6.12

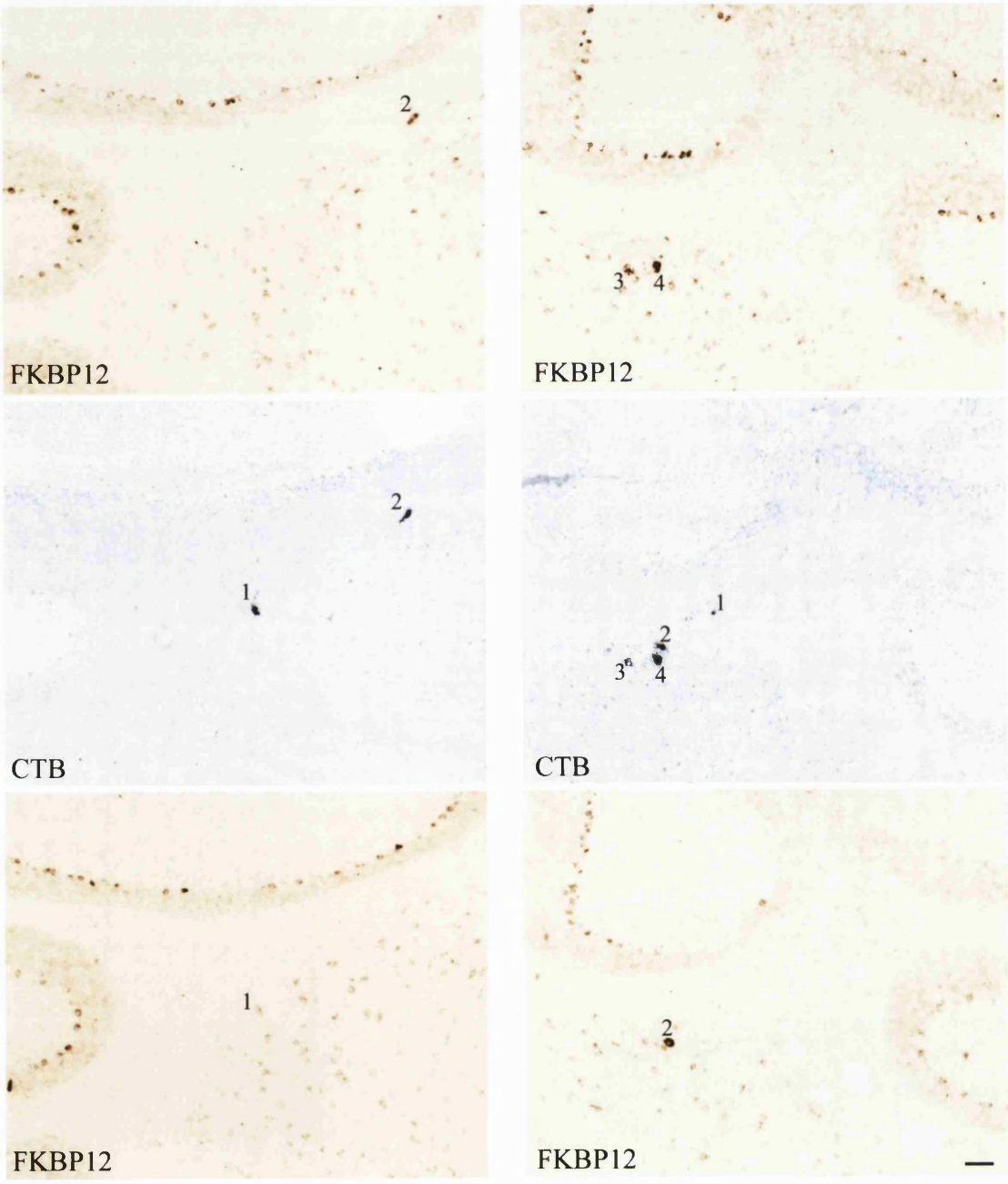
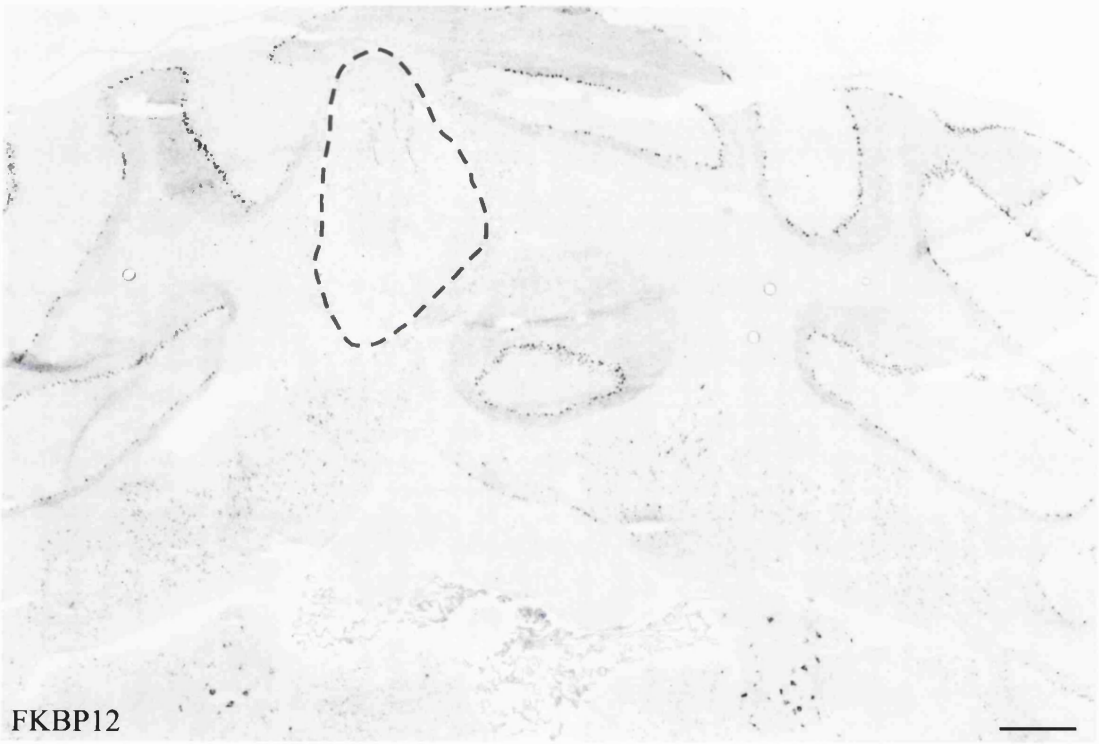


Fig. 6.13. Expression of *FKBP12* in the cerebellum 2 weeks after implantation of a tibial nerve graft (outlined) in which the cells have been killed by freeze-thaw cycles. No upregulation of *FKBP12* is seen in the cerebellar deep nuclei or elsewhere.

Bar 500μm.

FIGURE 6.13



Chapter 7

Effects of overexpression GAP-43 in dorsal root ganglion neurons of transgenic mice on sprouting and regeneration of axons after dorsal column injury or dorsal rhizotomy

7.1 Introduction

The strength of the cell body response to axotomy is a primary determinant of the vigour of subsequent axonal regeneration. This is most clearly observed following injury to the central axon branch of DRG neurons, in either the dorsal roots or the ascending dorsal columns of the spinal cord. Injury to these axons alone results in little cell body reaction and only slow regeneration in the dorsal root, poor invasion of a peripheral nerve graft attached to injured roots or inserted into the spinal cord and little sprouting in the spinal cord proximal to the site of an injury of the dorsal columns (Richardson and Issa, 1984; Richardson and Verge, 1987; Greenberg and Lasek, 1988; Oblinger and Lasek, 1988; Schreyer and Skene, 1993; Jenkins et al., 1993b; Chong et al., 1994a; Neumann and Woolf, 1999). An accompanying lesion of the nerve containing the peripheral axon branch of the same neurons improves regeneration of the central branch and provokes a strong cell body response. A major component of the cell body response is increased expression of GAP-43. This protein is consistently upregulated during regeneration and there is substantial evidence to indicate that it has a major function in the growth cone and may promote axon growth (Skene, 1989; Benowitz and Routtenberg, 1997; Oestreicher et al., 1997).

The aim of this study was to determine if GAP-43 expression alone would be sufficient to increase the regenerative powers of the central axons of DRG neurons, and thus whether it might partly mimic the cell body response without the need for a peripheral nerve injury. To this end, transgenic mice were studied which overexpress GAP-43 in the DRG neurons. The wt3 strain as described by Aigner et al. (1997) was used. These animals express avian GAP-43 under the control of a modified Thy-1 promoter, which drives neuron-specific transcription commencing post-natally. Strong avian *GAP-43* mRNA expression is found in DRG neurons of all sizes (Aigner et al. 1997; Mason et al. 2000).

Transgenic and non-transgenic mice underwent one of three procedures: transection of the dorsal columns at thoracic level; transection of the dorsal columns at lumbar level; or transection and re-anastomosis of the left L4-L6 dorsal roots. Subsequently all animals received an injection of cholera-toxin-B/horseradish peroxidase conjugate (CT-HRP) into the left sciatic nerve to transganglionically label axons in the spinal cord and the dorsal

root, and regeneration was assessed in each model. Animal usage and survival times are shown in Table 2.5 and for convenience are repeated in Table 7.1.

Surgical procedure	Genotype	Survival time	Orientation of sections	*n*
Thoracic dorsal column injury	Transgenic	2.5-3 weeks	Horizontal	3
	Transgenic	2.5-3 weeks	Parasagittal	2
	Non-transgenic	2.5-3 weeks	Horizontal	1
	Non-transgenic	2.5-3 weeks	Parasagittal	2
Lumbar dorsal column injury	Transgenic	3-4 weeks	Horizontal	4
	Transgenic	3-4 weeks	Parasagittal	7
	Non-transgenic	3-4 weeks	Horizontal	3
	Non-transgenic	3-4 weeks	Parasagittal	3
L4/L5 dorsal root injury	Transgenic	3 months	Horizontal	4
	Non-transgenic	3 months	Horizontal	2

Table 7.1 Survival times, processing and animal numbers used in studies on transgenic mice overexpressing GAP-43.

7.2 Results

i. Dorsal column lesions

A total of 25 mice received lesions of the dorsal columns and the ascending collaterals of primary sensory afferent fibres were transganglionically labelled with CT-HRP injected into the sciatic nerve. The lesion site and the dorsal column nuclei were removed 2.5-3.5 weeks later and processed for visualisation of the tracer.

None of the animals included in this study showed any labelling in the dorsal column nuclei, indicating that all ascending dorsal column axons were severed by the injury.

ii. Gross morphology of the lesions

Characteristic changes were observed in the gross morphology of the spinal cord at the lesion site (see Figs. 7.1, 7.2, 7.6, 7.8, 7.9, 7.10, 7.11). These changes were similar when lesions were performed at the thoracic or the lumbar level, and were most obvious in animals which were processed by taking parasagittal (rather than horizontal) sections. Typically, the surface of the cord at the lesion site was raised i.e. projected dorsally from the normal plane of the dorsal surface of the cord. The grey matter at the midline and the underlying ventral white matter both appear to have expanded around the lesion. The dorsal columns immediately caudal to the lesion site appeared to be displaced away from the lesion centre, such that the cut end of this fibre tract would be apposed to the dorsal surface of the cord as well as to the lesion itself (most easily seen in Figs. 7.1, 7.2). This observation was also supported by the behaviour of transganglionically labelled ascending dorsal column axons; approaching the lesion these were aligned at an angle to the horizontal, running towards the dorsal surface of the cord. The lesion site was typically discrete and had not extended greatly along the rostrocaudal axis. Little cavitation was seen in most animals. As well as the formation of scar tissue within the lesion (presumably astrogliotic scar tissue), there was also in all animals a large mass of tissue on the dorsal surface of the cord, presumably formed by meningeal cells and other cells of fibroblastic origin.

iii. Thoracic dorsal column lesions

4 transgenic and 3 non-transgenic wt3 mice received lesions of the dorsal columns of the spinal cord at the thoracic level and were allowed to survive 2.5-3 weeks. In neither transgenic nor non-transgenic animals did the ascending dorsal column axons show any great invasion of the lesion (Figs. 7.1-7.5). In non-transgenic animals the ascending dorsal column axons appeared to extend up to the caudal boundary of the lesion and also superficially as far as the dorsal surface of the cord and the boundary of the fibroblastic scar (Figs. 7.2, 7.4, 7.5*c*, *d*). In contrast, in transgenic animals axons appeared to die back from the lesion to a greater degree. Axons failed to extend to the caudal boundary of the lesion, and end bulbs were found over an area of greater caudal extent. Axons also failed to extend superficially to the dorsal surface of the cord and were either not found in

superficial horizontal sections or found in small numbers (Figs. 7.1, 7.3, 7.5*a,b*). No axon growth was seen into the fibroblastic scar on the dorsal surface of the cord in either transgenic or non-transgenic animals (Figs. 7.1, 7.2, 7.5*c,d*).

iv. Lumbar dorsal column lesions

11 transgenic and 3 non-transgenic wt3 mice received lesions of the dorsal columns at L2-L3 and were allowed to survive 3-3.5 weeks. Five transgenic animals and one non-transgenic animal were processed by taking horizontal sections. The remaining six transgenic and two non-transgenic animals were processed by taking parasagittal sections.

No consistent difference could be shown between transgenic and non-transgenic animals. In most transgenic animals, dorsal column axons did not consistently invade the lesion to a greater degree than was observed in non-transgenic animals, as demonstrated by the two examples shown in Fig. 7.6. However, one transgenic animal showed axonal labelling within the lesion and rostral to it (Fig. 7.7). Some of this labelling was found in the medial part of the cord; this was continuous with more extensive labelling in the lateral part of the cord, rostral and caudal to the lesion and ipsilateral to the tracer injection. Laterally, labelled axons were found as far as 3.25mm rostral to the lesion. Some of the labelled axons within the lesion appeared to originate from the severed dorsal columns, giving the appearance of remarkable growth into and beyond the lesion by dorsal column axons. However, this result was not replicated and no similar pattern of labelling was seen in any other animal.

In two further transgenic animals (processed by taking parasagittal sections), but not in non-transgenic animals, there appeared to be some axon growth within the lesion or distal to it (Fig. 7.8). In these animals the lesions were at the L3 level. It was not possible to determine whether these axons were extensions of dorsal column axons or extensions of their collaterals in the grey matter (although note they were found in positions which would indicate they were not merely intact collaterals).

In 3 further transgenic animals processed by taking parasagittal sections there appeared to be extensive invasion of the fibroblastic scar tissue overlying the lesion on the dorsal

surface of the spinal cord (Figs. 7.9, 7.10) which was not seen in non-transgenic animals (Fig. 7.11 *a,b,d,e*). However, the remaining transgenic animal processed by taking parasagittal sections failed to show extensive growth into the scar or the lesion (Fig. 7.11 *c,f*). Assessment of invasion of the dorsal scar tissue was not possible in animals processed by taking horizontal sections.

v. Dorsal root injuries

In four transgenic and two non-transgenic wt3 mice, L4-L6 dorsal roots were severed and re-anastomosed with 10-O sutures. Three months later, primary sensory afferent fibres were transganglionically labelled with CT-HRP injected into the sciatic nerve. The lumbar spinal cord and the dorsal column nuclei were removed and processed for visualisation of the tracer.

None of the animals included in this study showed any labelling in the dorsal coumn nuclei, showing that there were no axons spared by the lesion.

No difference was observed between transgenic and non-transgenic animals. The number of axons reaching the spinal cord appeared to be similar (Figs.7.12-7.16). In both transgenic and non-transgenic animals, of the small number of axons reaching the dorsal root entry zone (DREZ), a significant proportion crossed it and entered the spinal cord. Thus in the transgenic animal shown in Fig. 7.12, 5-6 axons are seen to approach the cord and two of these enter the grey matter of the dorsal horn. In the transgenic animal shown in Fig. 7.13, a single axon or axon fascicle crosses the DREZ and grows approximately 500μm into the grey matter. In the remaining two transgenic animals, all axons approaching the DREZ terminate in the dorsal root (Fig. 7.14).

Similar behaviour was observed in the non-transgenic animals, shown in Figs. 7.15-7.16. In each animal, small numbers of axons approach the DREZ and one axon crosses into the grey matter.

vi. Immunohistochemistry

To confirm the presence of transgenic GAP-43 protein in the central processes of primary sensory neurons, immunohistochemistry was performed on two unoperated transgenic mice, using the avian GAP-43 specific monoclonal antibody 5F10. Immunoreactivity was found in the ascending dorsal column axons and in their collaterals in the grey matter at the lumbar level (Fig. 7.17). No reaction product was seen on nearby sections processed using a procedure identical except for the omission of the primary antibody (not shown).

Fig. 7.1. Transganglionic labelling of dorsal column axons in a transgenic wt3 mouse, 21 days after a thoracic dorsal column injury (parasagittal section). Note that the labelled axons fail to approach either the lesion site itself or the dorsal surface of the cord. End bulbs can be observed approximately 450-1100µm from the lesion centre (dashed lines).

R:	Rostral	D:	Dorsal
C:	Caudal	V:	Ventral

Bars 250µm (top), 50µm (bottom).

FIGURE 7.1

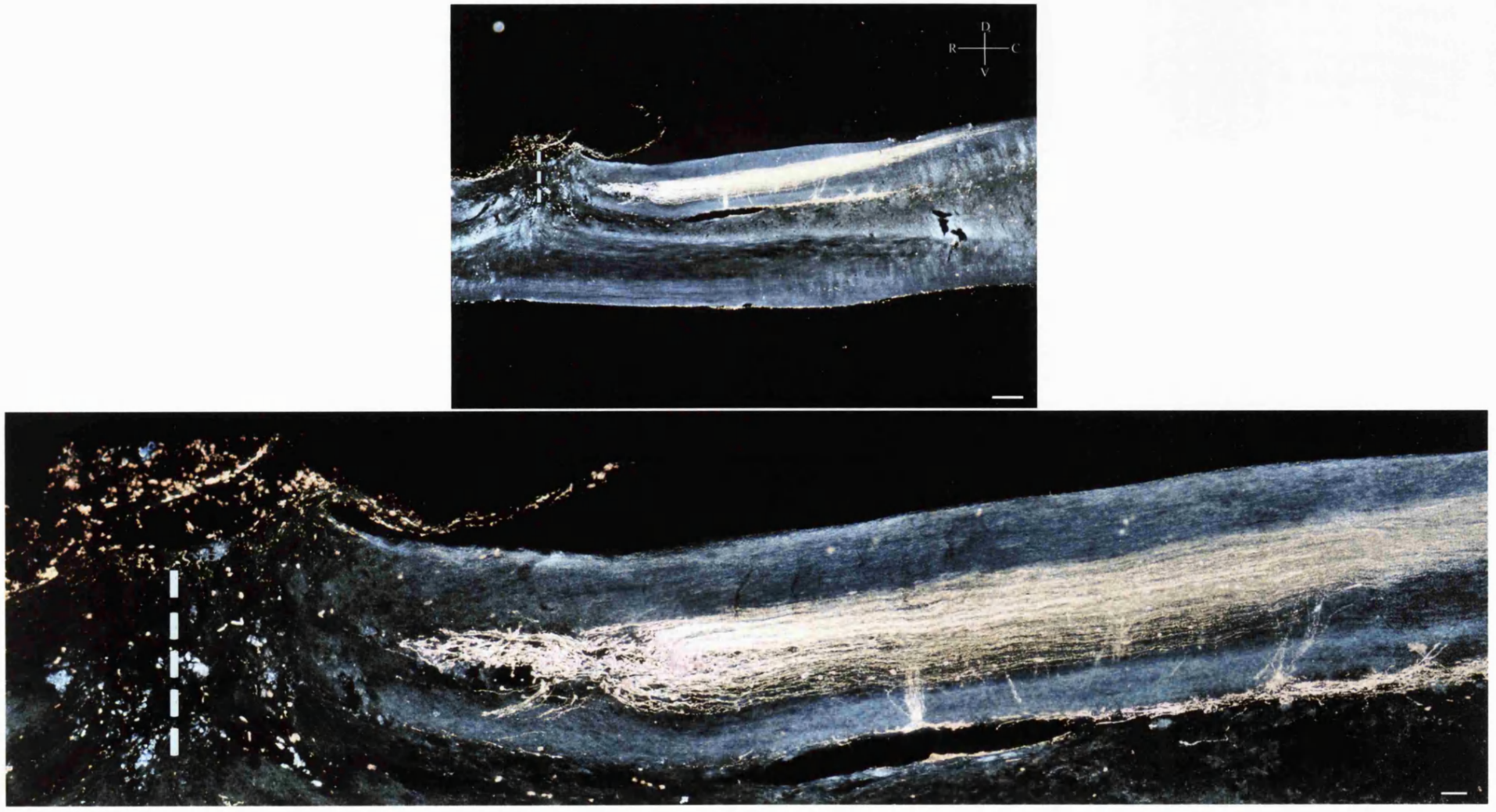


Fig. 7.2. Transganglionic labelling of dorsal column axons in a non-transgenic wt3 mouse 21 days after a thoracic dorsal column injury (parasagittal section). In *a* and the enlarged area of this panel shown in *b*, labelled axons approach within 150μm horizontally of the lesion centre (indicated by the blue vertical dashed line), but at their most rostral extent become more superficial, meeting the dorsal surface of the spinal cord (white dashed line). The boundary of spinal cord and scar tissue on another section is shown in *c* and is indicated by the dashed line. It can be seen that labelled dorsal column axons make a limited invasion of the scar tissue overlying the spinal cord, the maximum distance of ingrowth visible being about 175μm.

R: Rostral D: Dorsal

C: Caudal V: Ventral

Bars 250μm (*a*), 50μm (*b*), 20μm (*c*).

FIGURE 7.2

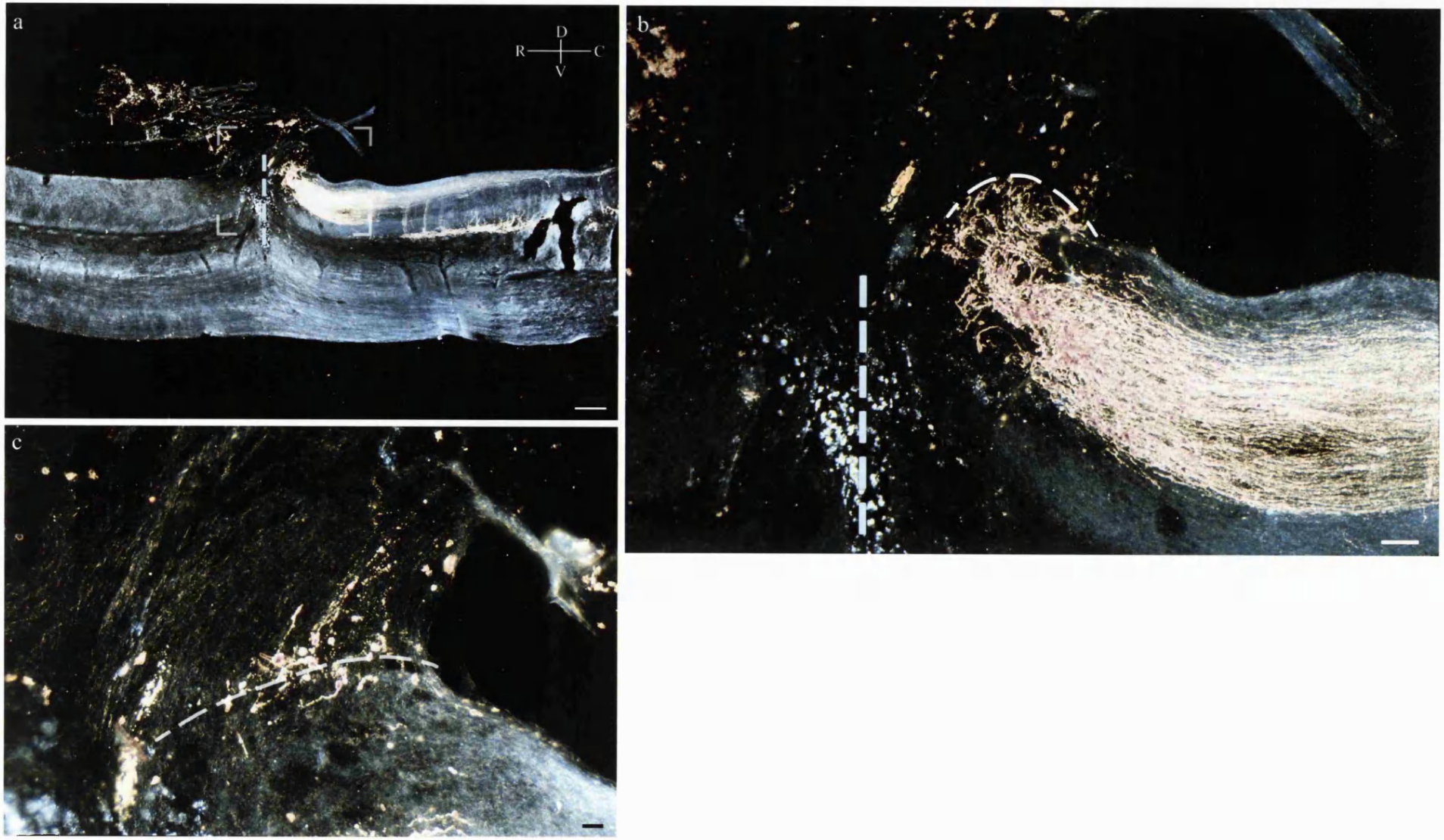


Fig. 7.3. Transganglionic labelling of dorsal column axons in a transgenic wt3 mouse 18 days after a thoracic dorsal column injury (horizontal section). Labelled dorsal column axons, to the left of the midline, fail to approach the lesion centre (dashed line) closer than approximately 350µm, and axonal end-bulbs can be seen up to 850µm away, in a pattern suggestive of retraction over this distance.

R: Rostral *l*: Left

C: Caudal *r*: Right

Bar 50µm.

FIGURE 7.3

Fig. 7.4. Transganglionic labelling of dorsal column axons in a non-transgenic wt3 mouse 18 days after a thoracic dorsal column injury (horizontal section). The framed areas in *a* and *b* are shown at higher magnification in *c* and *d*, and the position of the lesion is indicated by the vertical dashed lines. The majority of the labelled axons were found on the section shown in *a* and *c*. In *c* it can be seen that at this level the axons extend only to within 150µm of the lesion centre. However, close to the lesion axons and their end-bulbs are also to be found in the most superficial sections, one of which is seen in *b* and *d*. Axons reach within approximately 100µm of the lesion in this section.

R: Rostral *l*: Left

C: Caudal *r*: Right

Bars 100µm (*a*,*b*); 20µm (*c*,*d*).

FIGURE 7.4

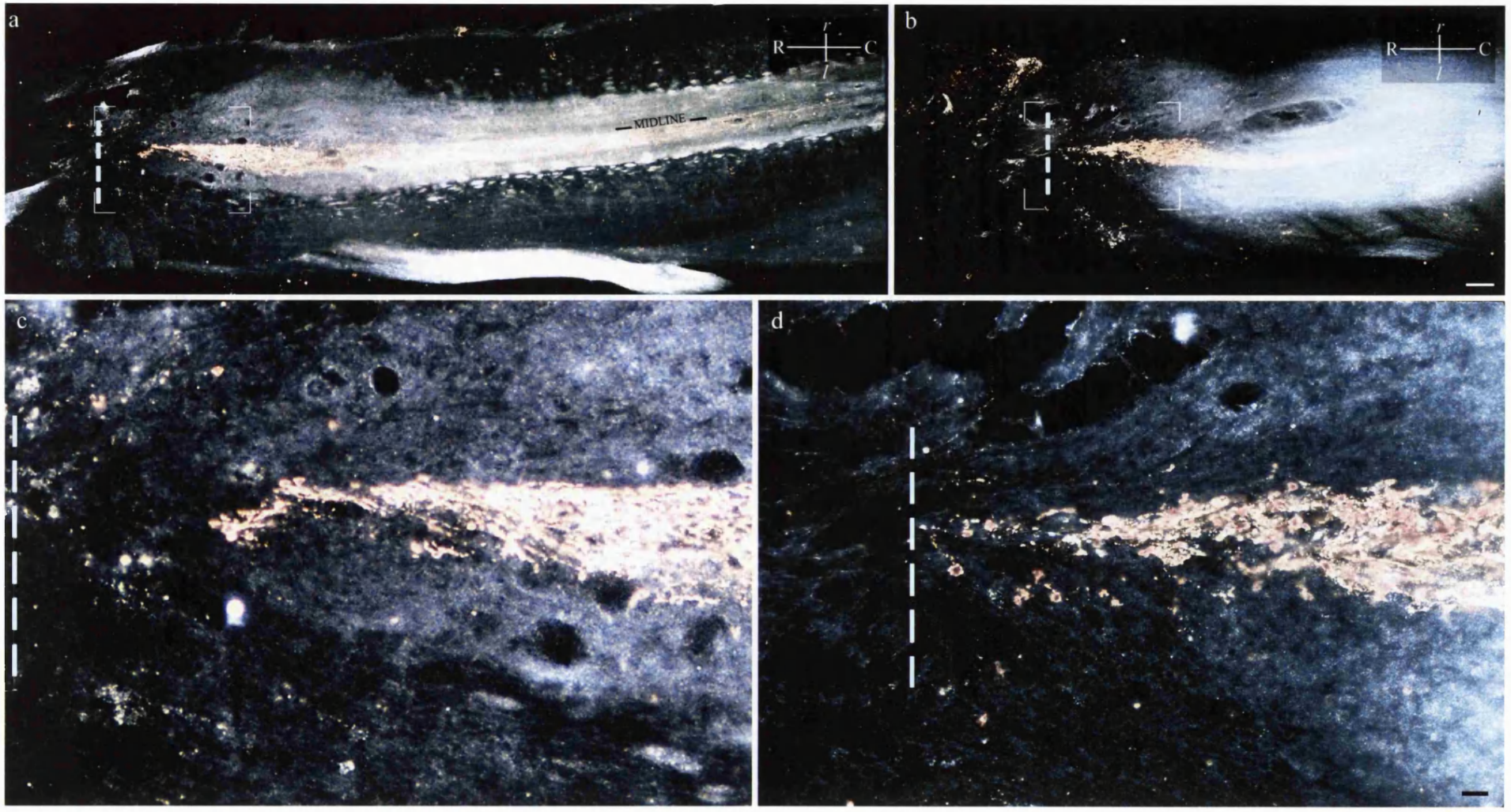


Fig. 7.5. Transganglionic labelling of dorsal column axons in a transgenic (*a,b)* and a non-transgenic (*c,d)* wt3 mouse following thoracic dorsal column injury. The framed areas in *a* and *b* are shown at higher magnification in *c* and *d*, and the position of the lesion is indicated by the vertical dashed lines. In the transgenic animal shown in *a* and *b* (horizontally sectioned; survival time 20 days), while a minority of axons approach the lesion, the majority of axon terminals are at least 400µm caudal to the lesion. In the non-transgenic animal shown in *c* and *d* (parasagittally sectioned; survival time 21 days), the majority of axons approach within 150µm horizontally of the lesion centre; again these axons become more superficial at the lesion site and approach the dorsal surface of the cord, although in this case there is no outgrowth into the overlying scar tissue.

R:	Rostral	*l*:	Left	D:	Dorsal
C:	Caudal	*r*:	Right	V:	Ventral

Bars 100µm (*a*), 50µm (*b*), 250µm (*c*), 20µm (*d*).

FIGURE 7.5

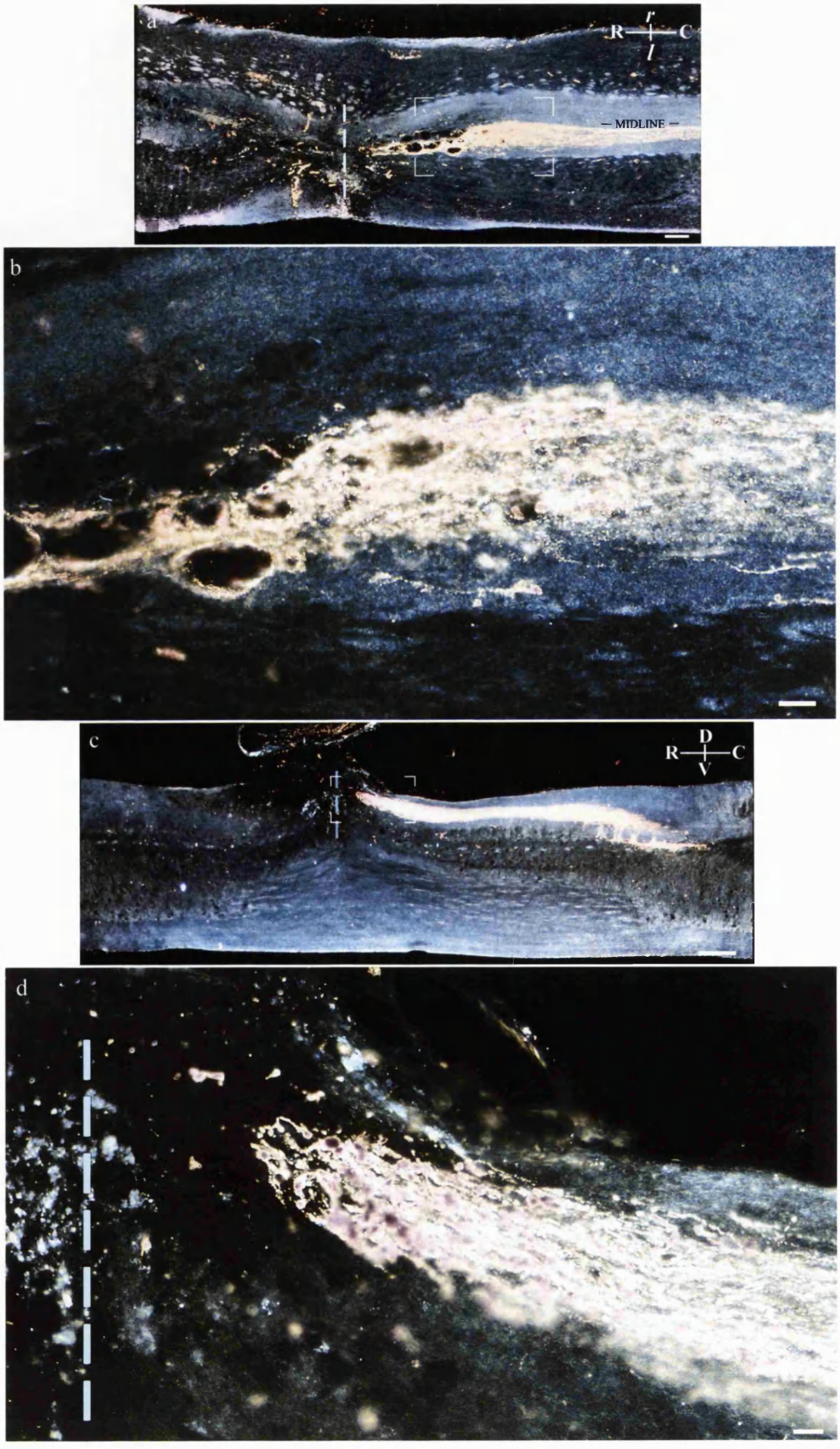


Fig. 7.6. Transganglionic labelling of dorsal column axons in a transgenic (*a,b*) and a non-transgenic (*c,d*) wt3 mouse 24 days after a lumbar dorsal column injury. The framed areas in *a* and *c* are shown at higher magnification in *b* and *d*. In both cases many axons terminate next to both the lesion site (dashed lines) and the scar tissue overlying the spinal cord, but no axon growth into either tissue is apparent.

R:	Rostral	D:	Dorsal
C:	Caudal	V:	Ventral

Bars 250μm (*a,c*); 50μm (*b,d*).

FIGURE 7.6

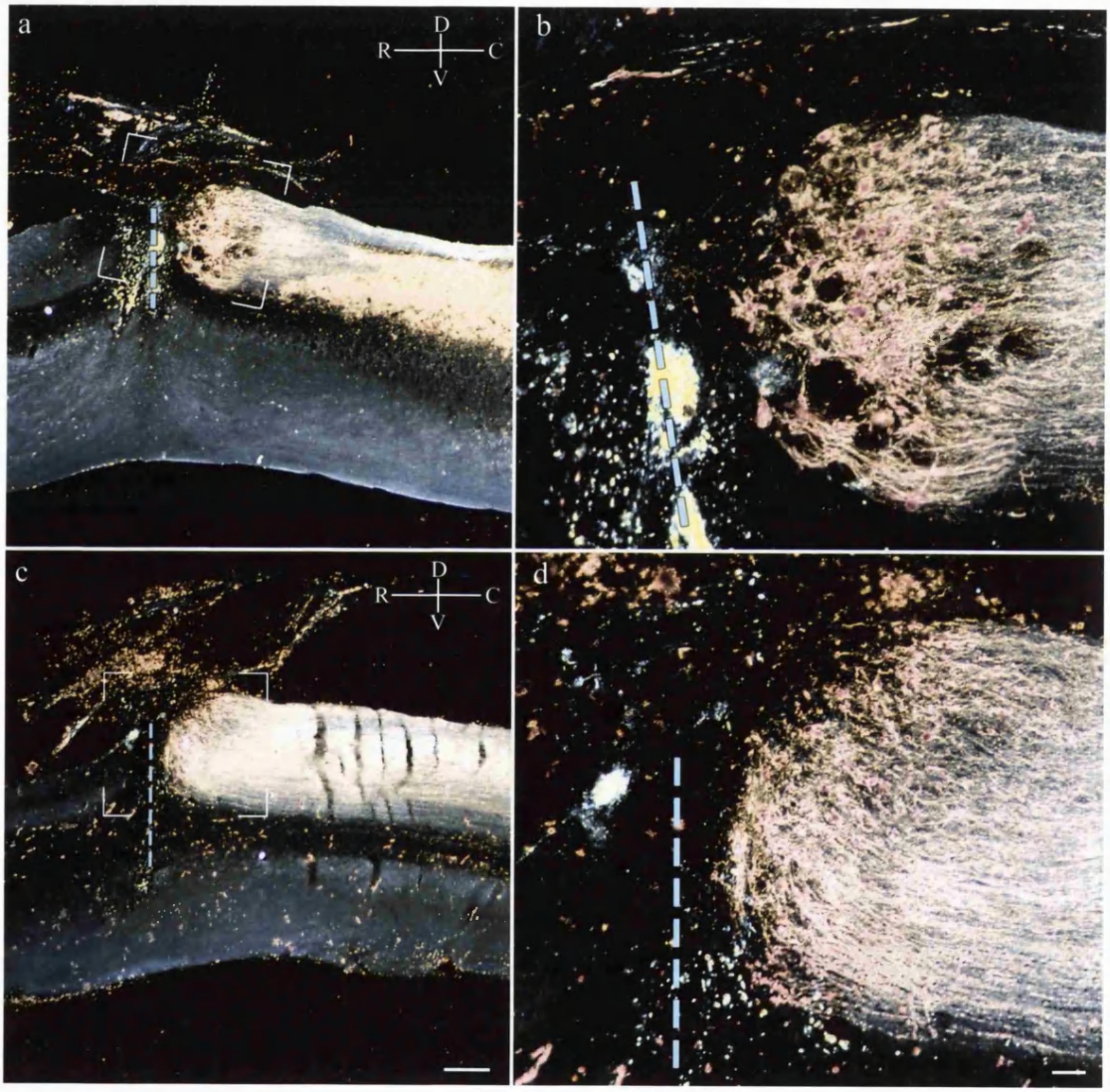


Fig. 7.7. Transganglionic labelling of dorsal column axons in a transgenic wt3 mouse 24days after a lumbar dorsal column injury. The framed area in *a* is shown at higher magnification in *b*, and the position of the lesion is shown by the blue arrows. In this animal an unusual pattern of labelling is seen in and around the lesion. In *a* and *b*, labelled axons appear to extend from the dorsal columns into the lesion and into the lateral part of the cord. Axons extend rostrally to the lesion in the lateral part of the cord and can also be seen on the incoming dorsal roots. On a nearby section, shown in *c*, an axon varicosity (white arrow) is visible within a dorsal root, approximately 3mm rostral to the lesion.

R:	Rostral	*l*:	Left
C:	Caudal	*r*:	Right

Bars 250μm (*a*,*b*), 20μm (*c*).

FIGURE 7.7

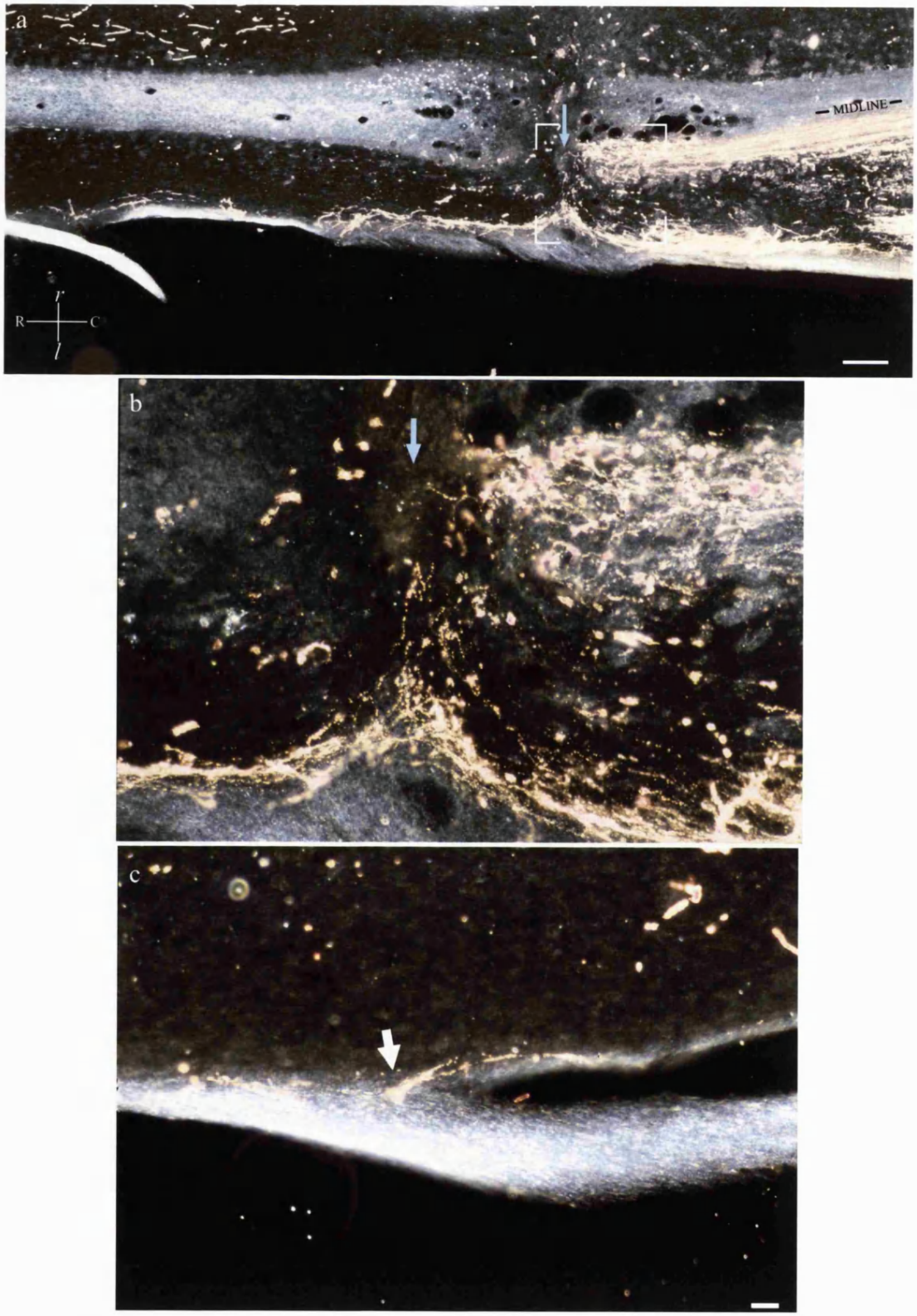


Fig. 7.8. Transganglionic labelling of dorsal column axons and their collaterals in two transgenic wt3 mice 22 days after lumbar dorsal column injury. The lesion site of one animal is indicated by the blue arrows in *a* and at higher magnification in *b*. A few axons can be seen within the lesion itself (white arrow in *b*), and appear to extend further ventrally than the surrounding axon collaterals terminating in the grey matter. In the animal shown in *c* and *d*, the lesion centre is indicated by the arrows in *c* and by the dashed line in *d*. Axons are seen a short distance rostral to the lesion in the region corresponding to the white matter of the dorsal columns. Note that while innervation of the grey matter is seen rostral to the lesion, no evidence was found for sparing of dorsal column axons in this animal.

Bars 100μm (*a*,*c*); 50μm (*b*,*d*).

FIGURE 7.8

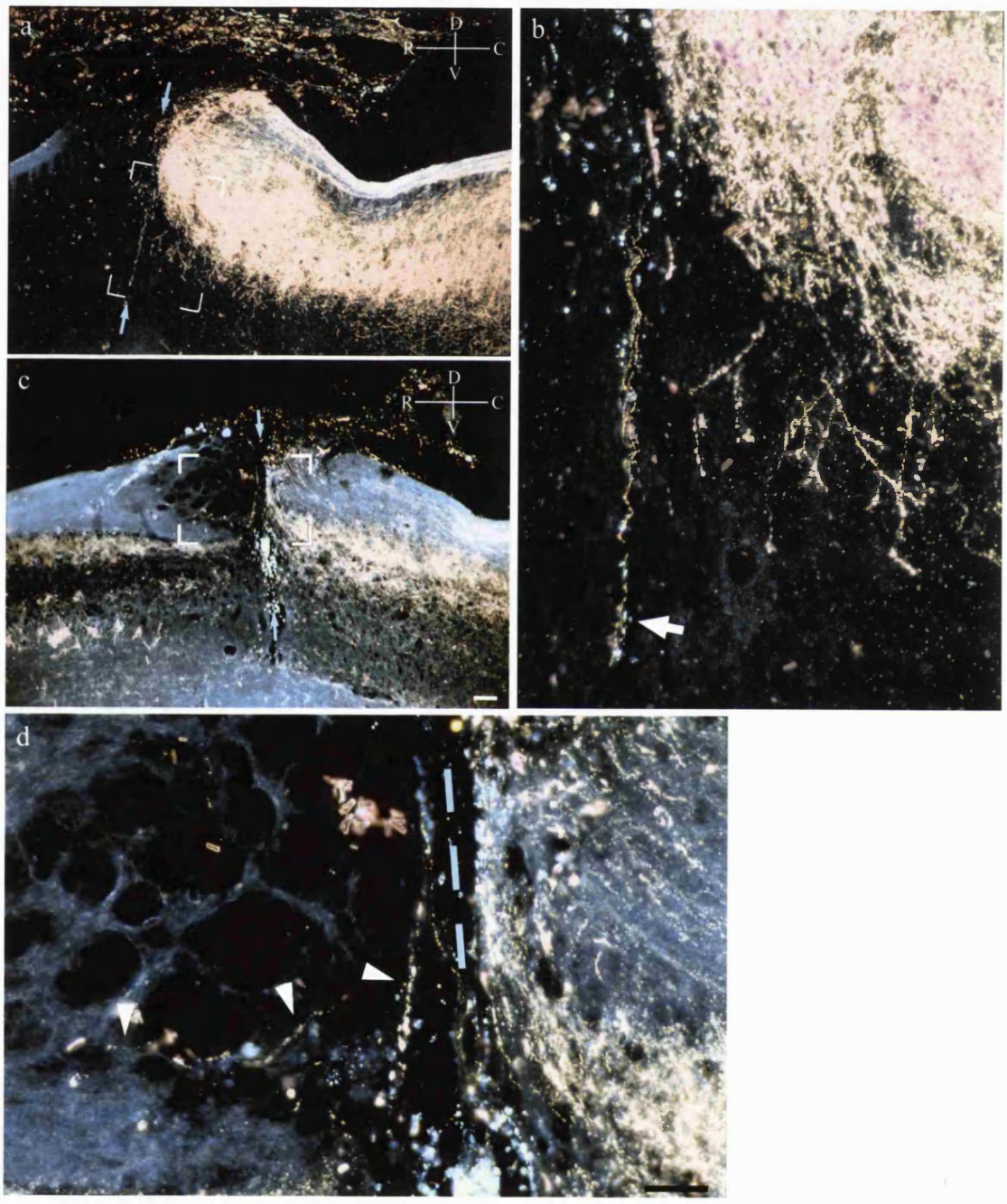


Fig. 7.9. Invasion of the scar overlying the spinal cord by dorsal column axons 28 days after a lumbar dorsal column injury in transgenic wt3 mice. Three sections from one animal are shown; framed areas in *a*, *c* and *e* are shown at higher magnification in *b*, *d* and *f* respectively and the centre of the lesion is indicated by the blue dashed lines. White dashed lines indicate the position of the boundary between the spinal cord and the overlying scar tissue. Extensive axon growth into the scar tissue on the dorsal surface of the cord can be seen in all three sections.

R:	Rostral	D:	Dorsal
C:	Caudal	V:	Ventral

Bars 250μm (*a*,*c*); 50μm (*b*,*d*,*f*); 100μm (*e*).

FIGURE 7.9

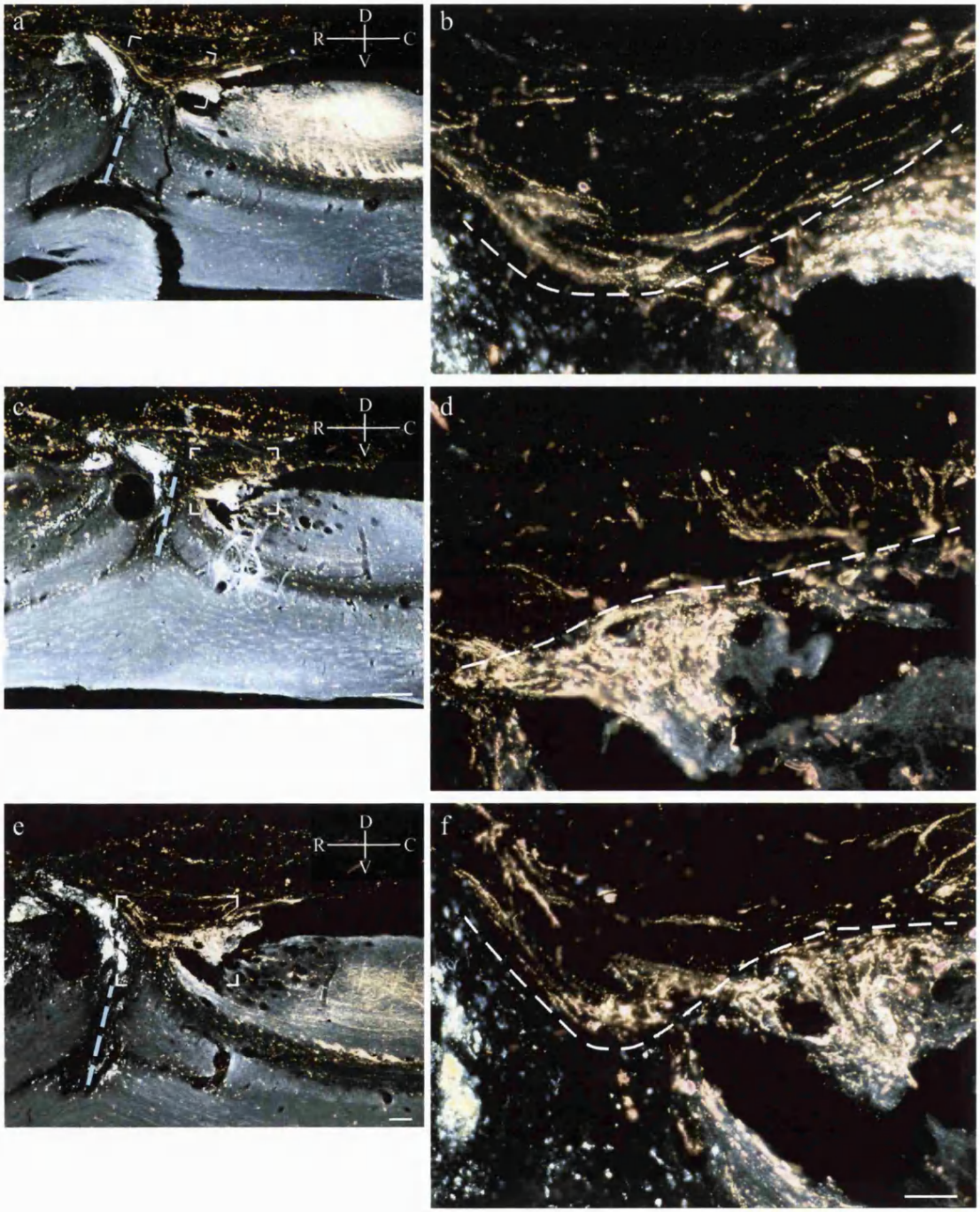


Fig. 7.10. Invasion of the scar overlying the spinal cord by dorsal column axons 24 days after a lumbar dorsal column injury in transgenic wt3 mice. The vertical blue dashed line indicates the centre of the lesion and the white dashed lines show the boundary of spinal cord and overlying scar tissue. In this animal a limited amount of growth into the scar tissue is apparent, and some growth around cavities in the tissue can be seen.

R:	Rostral	D:	Dorsal
C:	Caudal	V:	Ventral

Bars 250µm (top), 50µm (bottom).

FIGURE 7.10

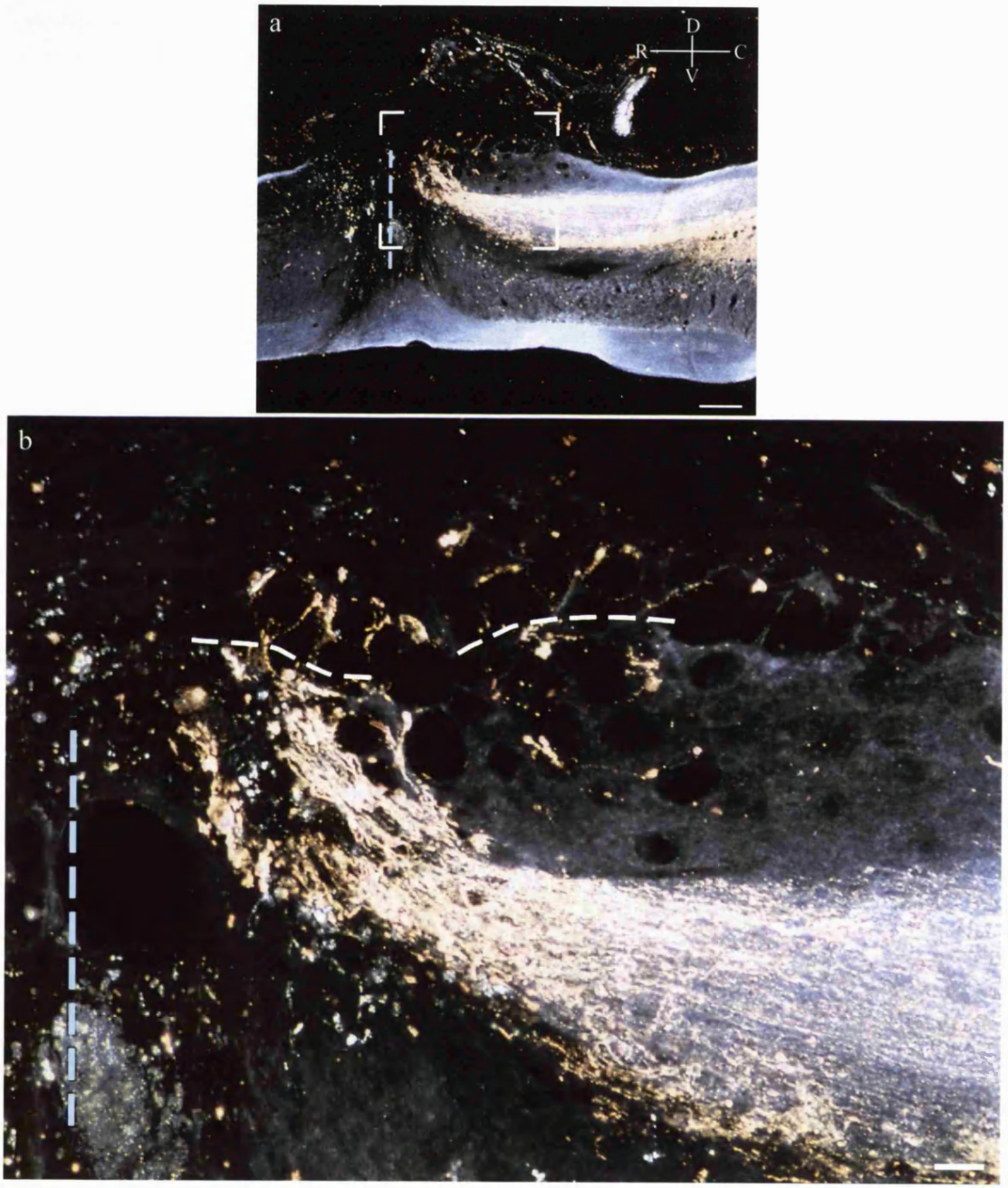


Fig. 7.11. Extent of invasion of the scar overlying the spinal cord by dorsal column axons 24 days a lumbar dorsal column injury in 2 non-transgenic wt3 mice (*a,b,d,e*) and 1 transgenic wt3 mouse (*c,f*). Framed areas of *a*, *b* and *c* are shown at higher magnification in *d*, *e* and *f* respectively. Lesion sites are indicated by the vertical blue dashed lines and the boundary of the overlying scar tissue is shown by the white dashed lines. In *a*, *b*, *d* and *e*, non-transgenic animals display little axon growth into the scar tissue on the dorsal surface of the spinal cord although a small amount of growth is seen in *b* and *e*. The transgenic animal shown in *c* and *f* also shows little or no growth into the scar tissue.

R:	Rostral	D:	Dorsal
C:	Caudal	V:	Ventral

Bars 250µm (*a-c*), 50µm (*d-f*).

FIGURE 7.11

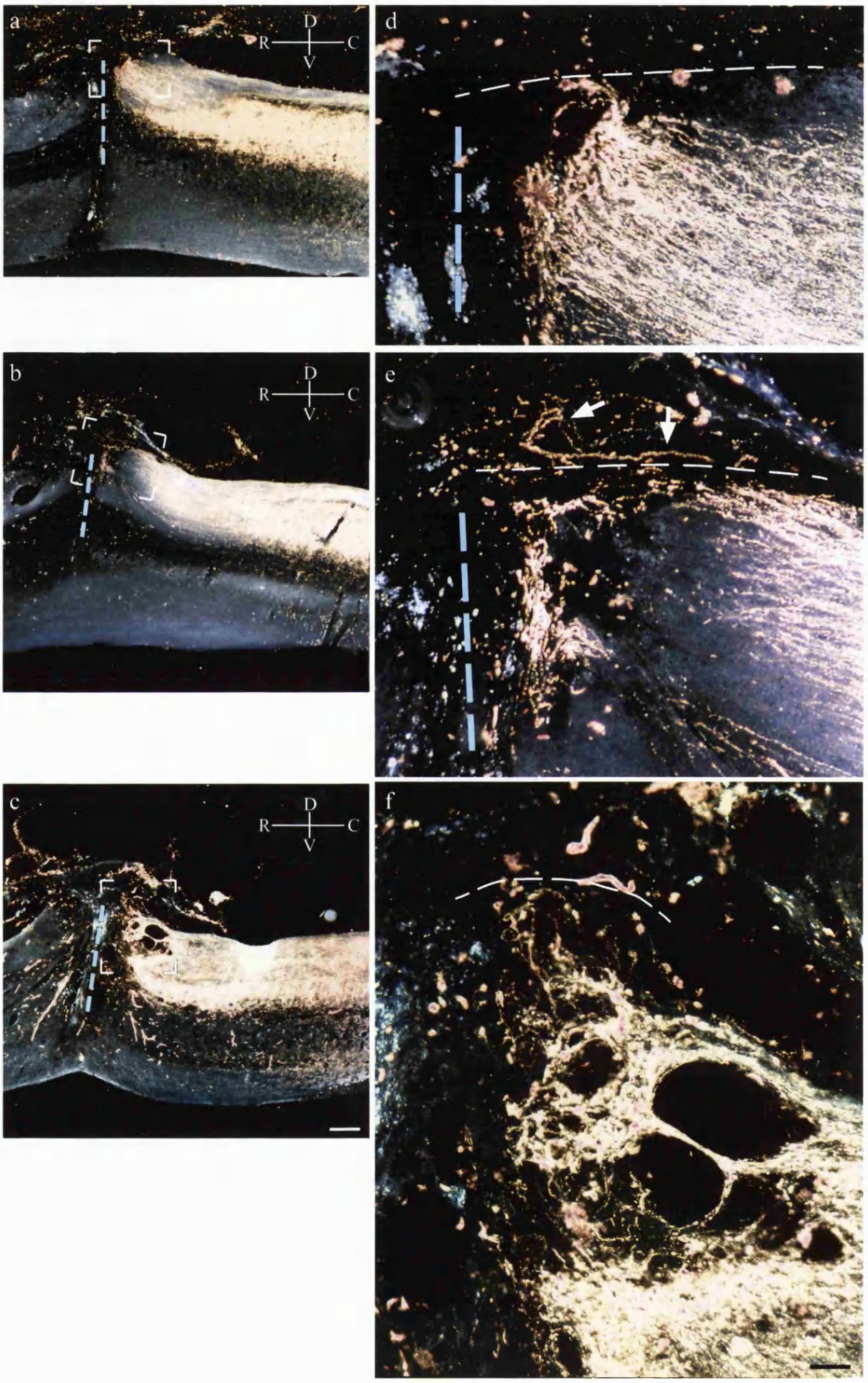


Fig. 7.12. Transganglionic labelling of dorsal root axons in a transgenic wt3 mouse 3 months after dorsal root injury and anastomosis. Few axons are seen to reach the spinal cord. Only 5-6 can be seen in the upper panel and one in the lower panel. However, of these, two axons appear to cross the dorsal root entry zone and grow a short distance within the grey matter (indicated by arrows).

R:	Rostral	*l*:	Left
C:	Caudal	*r*:	Right

Bar 20μm.

FIGURE 7.12

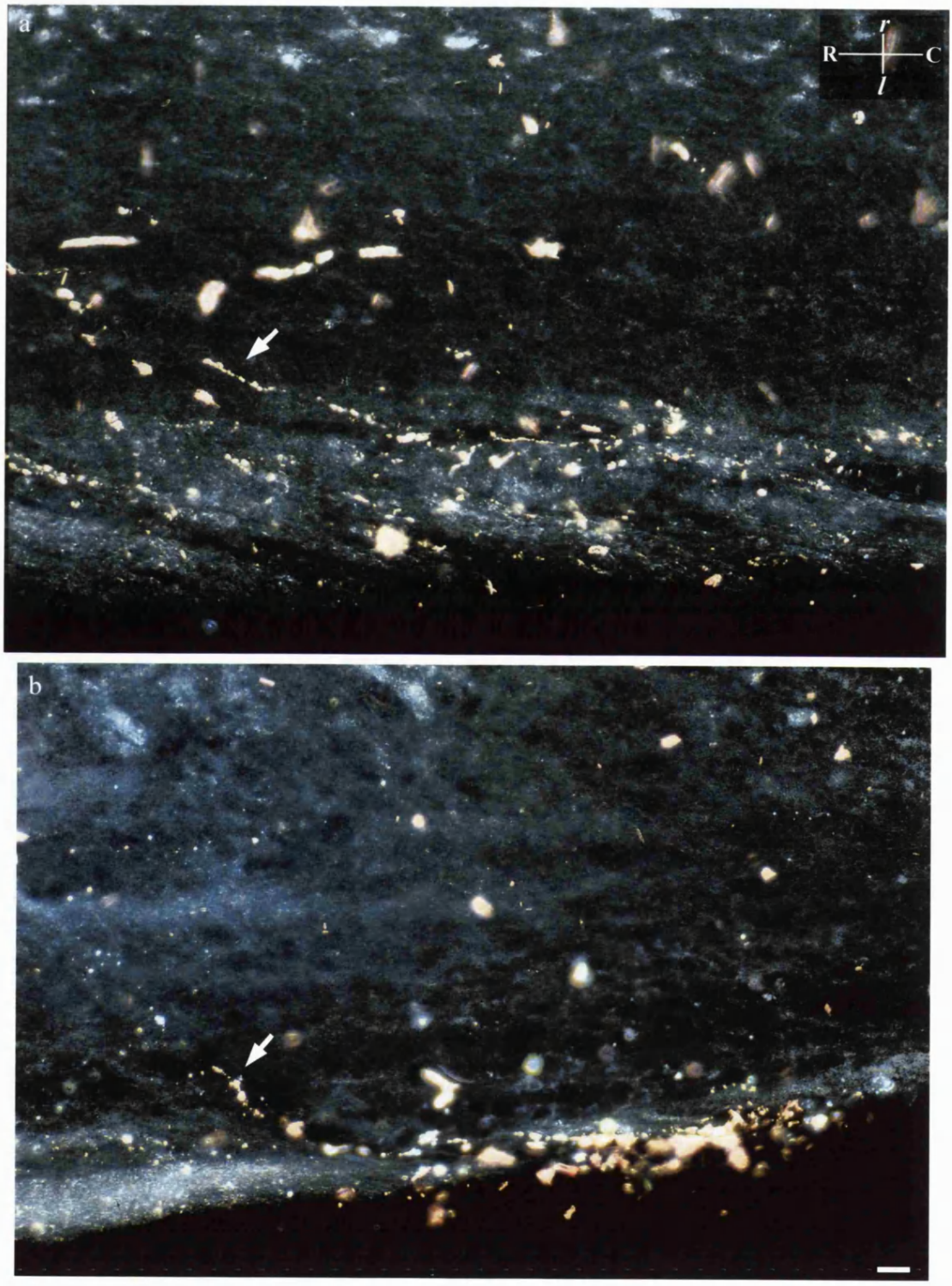


Fig. 7.13. Transganglionic labelling of dorsal root axons in a transgenic wt3 mouse 3 months after dorsal root injury and anastomosis. Three consecutive horizontal sections are presented in which a single axon can be seen to enter the grey matter of the dorsal horn. The bottom panel shows a composite drawing of all three sections. This axon appears to have grown across the dorsal root entry zone and approximately 500μm into the grey matter of the spinal cord. Key to drawing: W-white matter. G-grey matter.

R:	Rostral	*l*:	Left
C:	Caudal	*r*:	Right

Bars 50μm.

FIGURE 7.13

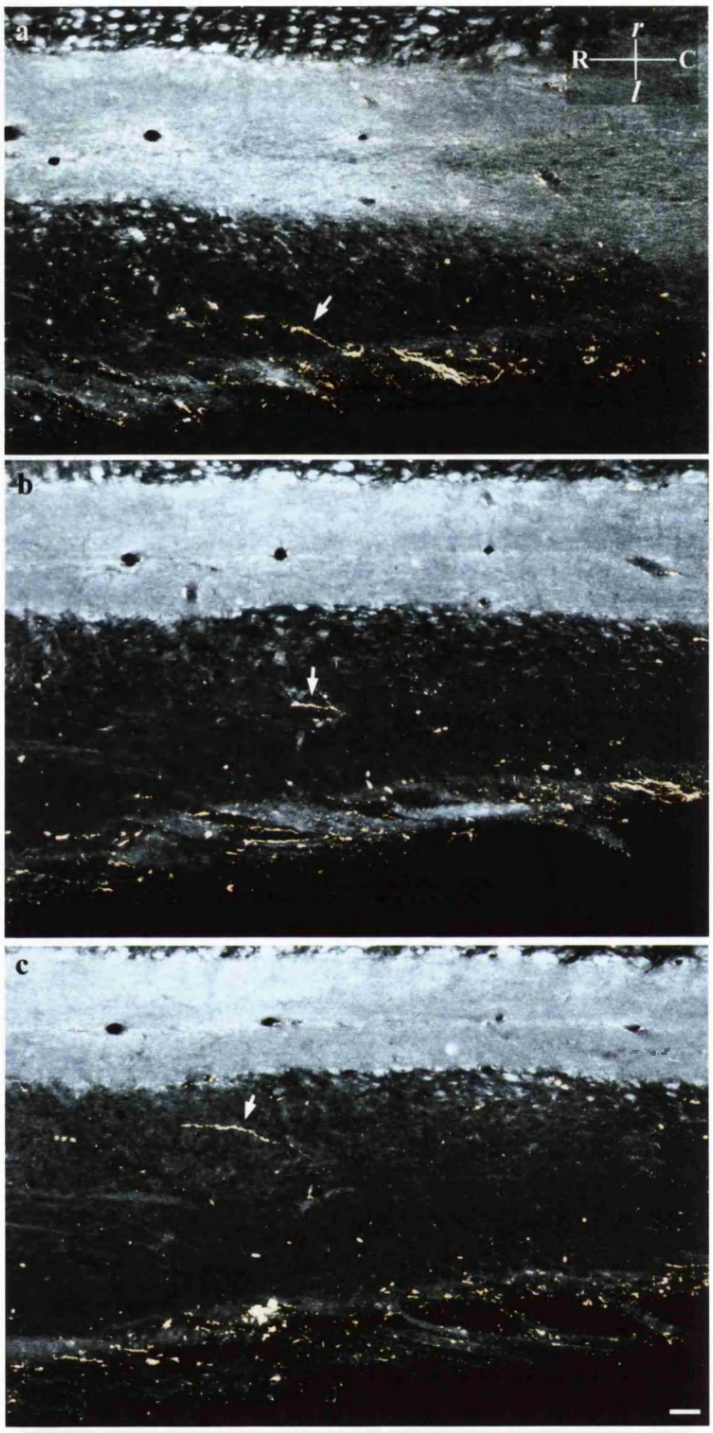


Fig. 7.14. Transganglionic labelling of dorsal root axons in a transgenic wt3 mouse 3 months after dorsal root injury and anastomosis. In this animal a few axons, indicated by arrows, are seen in the dorsal root only. None enter the dorsal horn and in the lower two panels end bulbs can also be seen in the dorsal roots.

R:	Rostral	*l*:	Left
C:	Caudal	*r*:	Right

Bar 20μm.

FIGURE 7.14

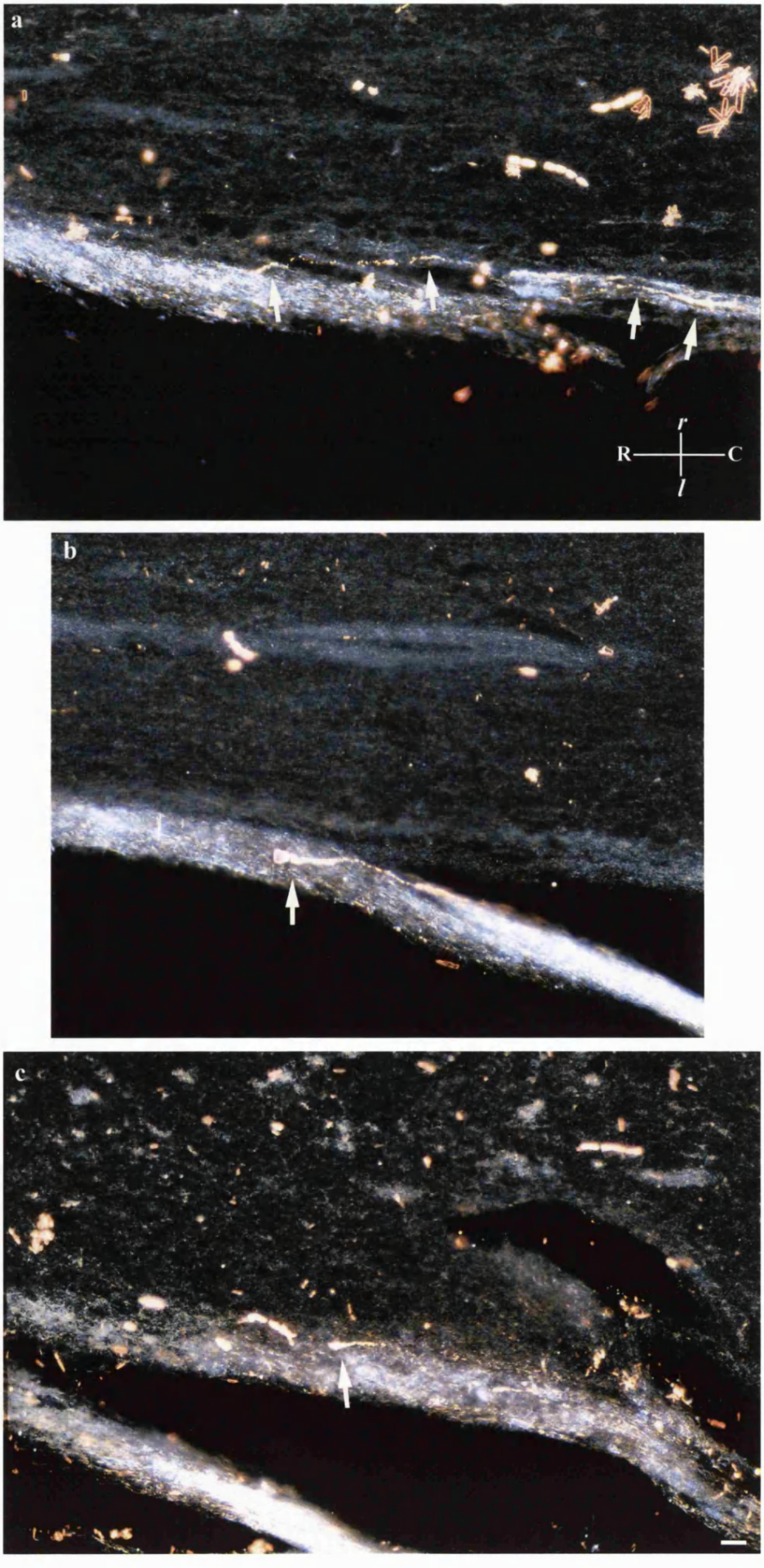


Fig. 7.15. Transganglionic labelling of dorsal root axons in a non-transgenic wt3 mouse 3 months after dorsal root injury and anastomosis. Only 2-3 axons are seen in the dorsal root approaching the spinal cord, but in the lower panel one enters the dorsal horn, and apparently is associated with a blood vessel.

R:	Rostral	*l*:	Left
C:	Caudal	*r*:	Right

Bar 25μm.

FIGURE 7.15

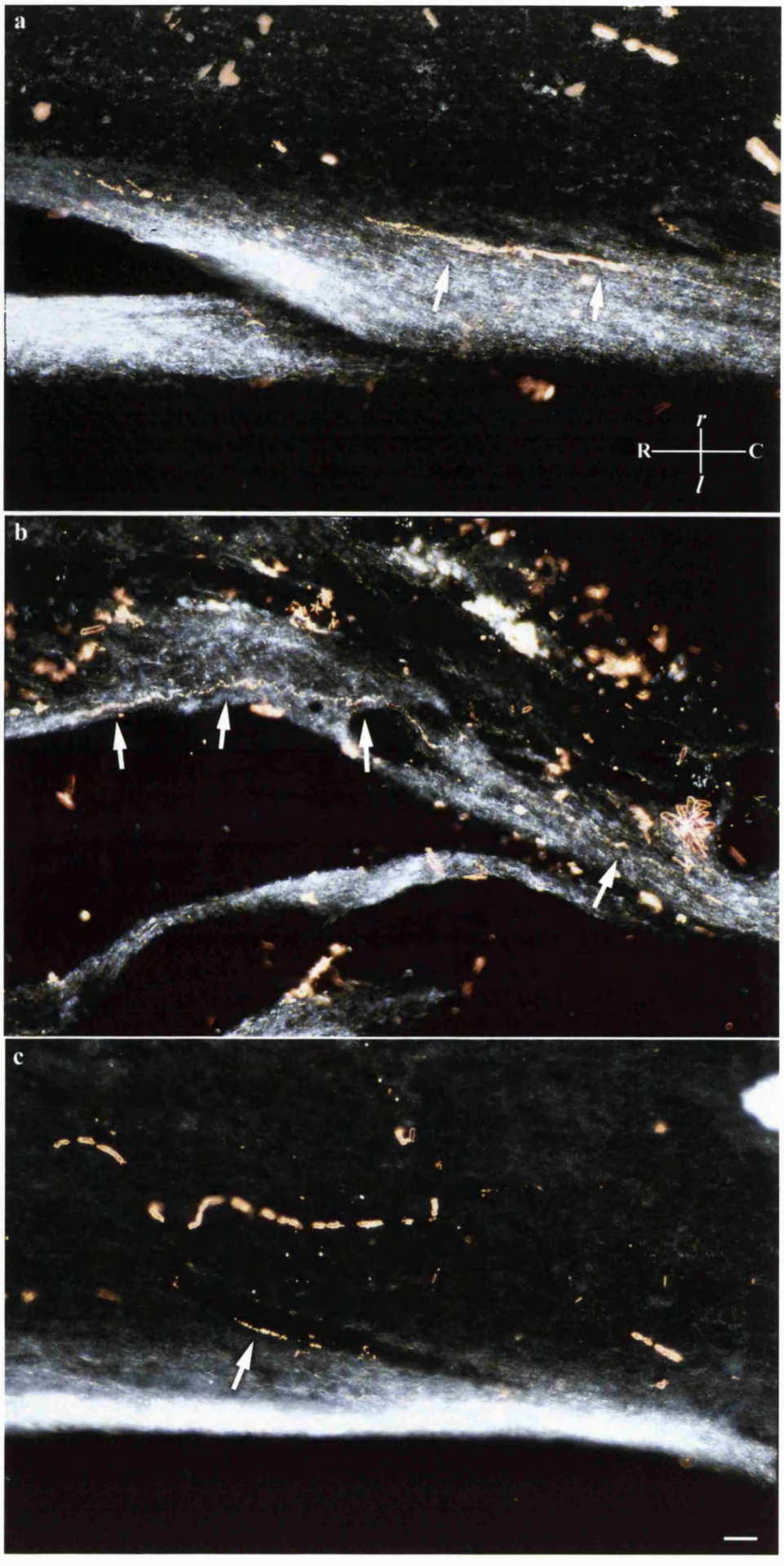


Fig. 7.16. Transganglionic labelling of dorsal root axons in a non-transgenic wt3 mouse 3 months after dorsal root injury and anastomosis. Several axons are seen to approach the grey matter of the spinal cord (indicated by filled arrows). One axon crosses the dorsal root entry zone and is seen within the grey matter (indicated by the arrow outline).

R:	Rostral	*l*:	Left
C:	Caudal	*r*:	Right

Bar 50μm.

FIGURE 7.16

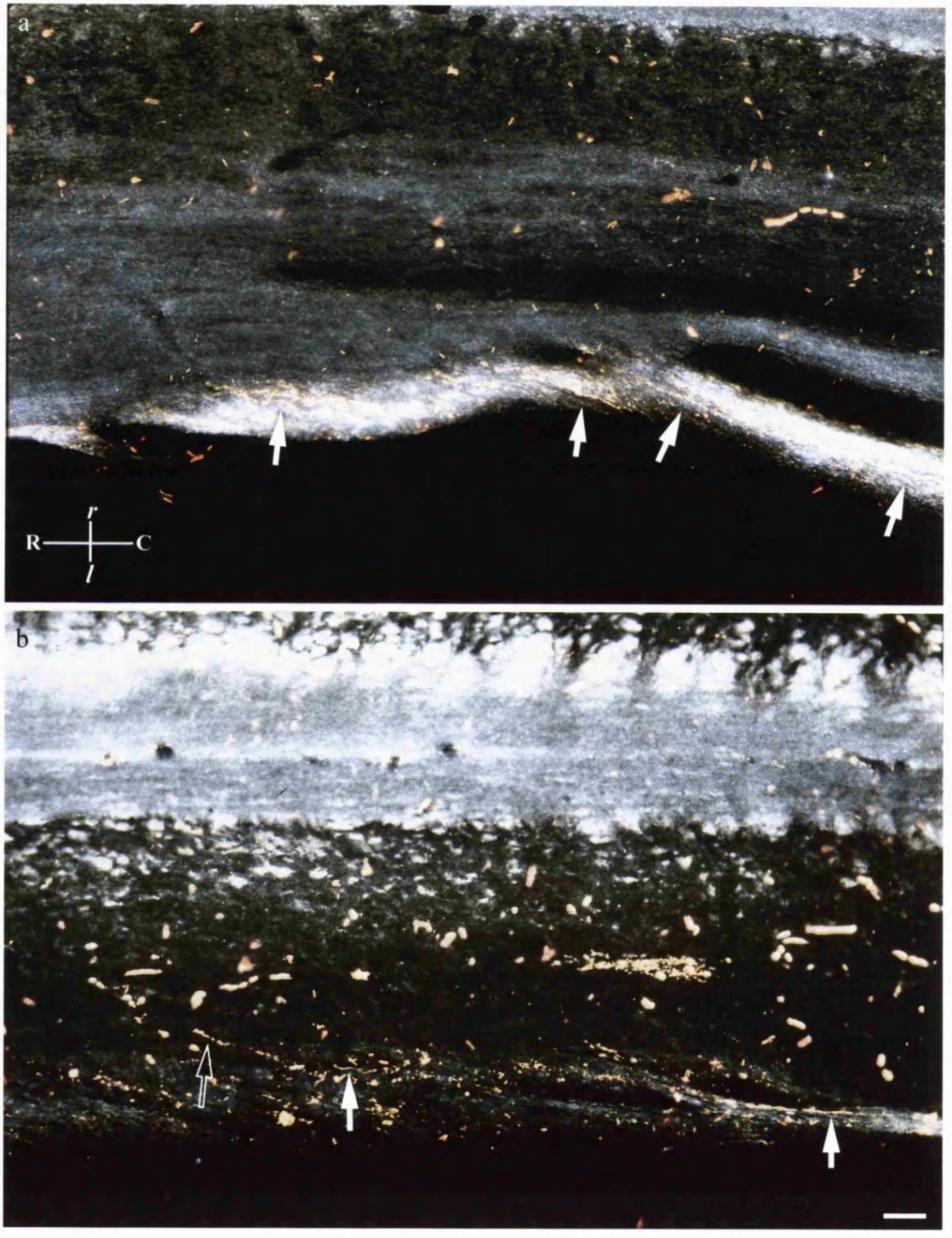
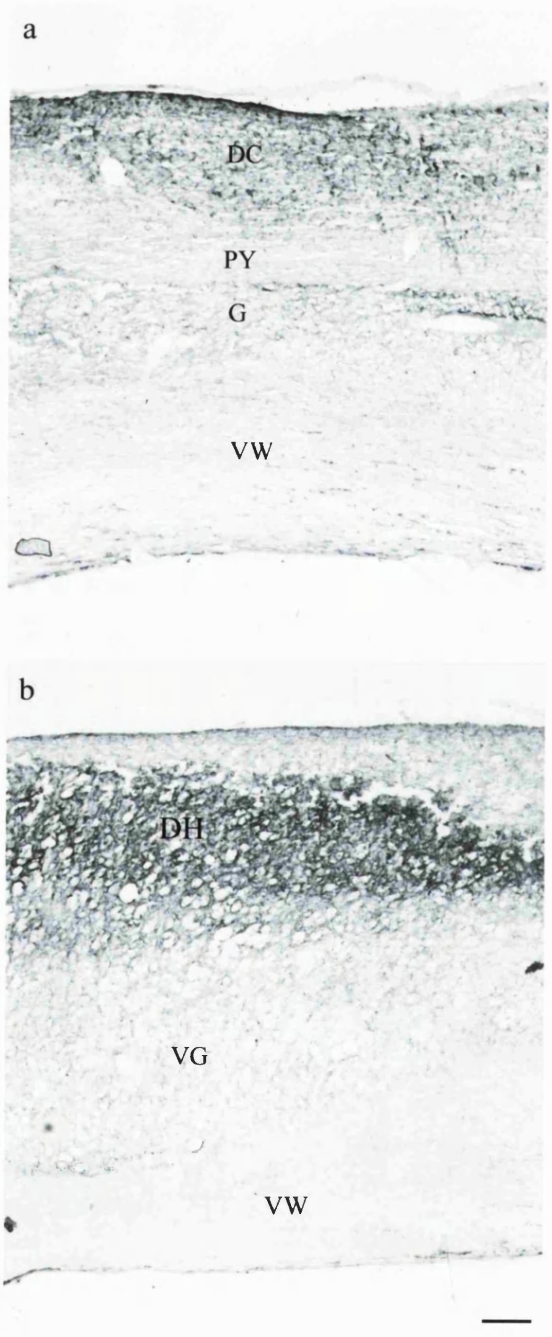


Fig. 7.17. Immunohistochemistry for avian GAP-43 with monoclonal antibody 5F10, on sections of spinal cord of a transgenic wt3 mouse. In the midline parasagittal section of L1 spinal cord shown in *a*, immunoreactivity can be seen in the ascending dorsal columns (DC), but not in the pyramidal tract (PY) immediately below. Some immunoreactivity can also be seen in restricted parts of the grey matter (G) and the ventral white matter (VW). In the parasagittal section of mid-lumbar cord shown in *b*, immunoreactivity can be seen in the dorsal horn (DH) which is extensively innervated by DRG neurons, but not in the ventral grey matter (VG) or ventral white matter (VW).

Bar 250μm.

FIGURE 7.17



Chapter 8

The expression of molecules associated with axon injury and regeneration in corticospinal neurons and rubrospinal neurons after axotomy

8.1 Introduction

The corticospinal tract and the rubrospinal tract are two major motor pathways descending in the spinal cord of rodents. The purpose of the experiments presented here was to investigate changes in expression of growth-associated genes in corticospinal and rubrospinal neurons following axotomy. The expression of mRNAs for c-Jun, ATF3, GAP-43, CAP-23, SCG10, L1, CHL1 and Krox-24 was examined by in situ hybridisation in three models.

Previous reports indicate that corticospinal neurons do not upregulate GAP-43 protein following axotomy. Reh et al. (1987) could not detect any increase in the anterograde transport of GAP-43 in the corticospinal tracts following axotomy 2-3 mm rostral to the pyramidal decussation and Tetzlaff and co-workers reported observations that only a very proximal axotomy caused upregulation of *GAP-43* mRNA by these cells, but did not publish full data for these experiments (Tetzlaff and Giehl, 1991; Tetzlaff et al., 1994). Thus, while the upregulation of *GAP-43* by corticospinal neurons would not be expected to follow cervical cord injury, it is possible that the expression of other growth-associated molecules might be upregulated, as changes in gene expression observed after axotomy do vary. For example, despite the fact that Purkinje cells do not regenerate into peripheral nerve grafts or upregulate GAP-43 after axotomy, they upregulate *c-jun*, the neurotrophin receptor p75 (Vaudano et al., 1998; Chaisuksunt et al., 2000a) and *CAP-23* (see Chapter 5 and Zagrebelsky et al., 1998) after a proximal axotomy. Gene expression was, therefore, examined in the motor cortex following cervical injury of the corticospinal tract. Since it is possible that axotomy at this level is simply too distal to provoke any response from cortical neurons, the corticospinal neurons in other animals were subject to a very proximal axotomy.

Rubrospinal neurons have previously been reported to upregulate c-Jun and *GAP-43* following injury to the spinal cord at the cervical, but not thoracic, level (Jenkins et al., 1993a; Fernandes et al., 1999).This correlates well with their ability to regenerate axons into nerve grafts implanted into the cervical cord but not when implanted into the thoracic cord (Richardson et al., 1984). However, the effects of cervical axotomy on the expression of many other growth- and axotomy- associated genes, including *ATF3*, *CAP-23*, *SCG10*,

L1, *CHL1* and *krox-24* have not previously been investigated. The rubrospinal tract was therefore cut at the C3 segment and the expression of these genes, and of *c-jun* and *GAP-43*, monitored in the red nucleus.

8.2 Results

i. Retrograde labelling of corticospinal neurons

In order to identify corticospinal neurons on sections processed for ISH, these neurons were retrogradely labelled by injection of CTB into the cervical spinal cord. Tracer was injected into dorsal funiculus of the spinal cord approximately 1mm above the lesion. This produced bilateral labelling of large numbers of neurons in layer V of the neocortex (Fig. 8.1*a*), visible 1 day and 7 days after injury. Corticospinal neurons in animals which received an intracortical knife lesion (to produce a very proximal axotomy) were retrogradely labelled by CTB injection into the C3 segment of the cord 24 hours before the lesion was made, to allow time for retrograde transport of the tracer. Retrogradely labelled neurons were also visible in the cortex in these animals (Fig. 8.1*b*), including the cortex superficial to the lesion (i.e. it was present in axotomised cells). In all cases corticospinal neurons were found in the areas designated forelimb (FL) and hindlimb (HL) by Paxinos and Watson (1986), but some were also found in areas designated as frontal and parietal cortex. The observed distribution was in agreement with the results of Miller (1987).

ii. Gene expression in the intact cortex

Expression of *c-jun*, *ATF3*, *GAP-43*, *CAP-23*, *SCG10*, *L1*, *CHL1*, and *krox-24* was examined in the neocortex of 2 unoperated rats, with particular attention being paid to those areas where corticospinal neurons are located (Figs. 8.2-8.9, lower panels). All the mRNAs examined were expressed in the neocortex, and were detectable in layer V neurons. In each case positive cells could be identified which appeared to be pyramidal neurons.

Comparison of signal strengths suggest that *GAP43*, *CAP-23* and *SCG10* were expressed at relatively high levels throughout the cerebral cortex. The levels of *L1* and *CHL1* mRNA

expression by neurons appeared to be lower, but similar to each other. Of the mRNAs for transcription factors, *krox-24* was most strongly expressed, followed by *c-jun*, and lastly *ATF3*. Expression of *ATF3* was very low, but detectable, and appeared to be expressed in a uniform manner by neurons in all layers.

In the area corresponding to FL and HL, layer V neurons showed stronger expression of *GAP-43*, *CAP-23*, *L1* and *CHL1* than neurons of other layers. *C-jun* mRNA expression in layer V neurons was approximately equal to, or slightly higher than, that found in other layers. *Krox-24* was found in many layer V pyramidal neurons in this region. Expression of all these molecules was higher in layer V neurons of the frontal cortex (where some corticospinal neurons were also found) adjacent and medial to FL and HL cortex. Expression of all the molecules except *krox-24* was fairly uniform in the other layers of cortex, with some variation: stronger expression of *c-jun* was found in layer II than in other layers (except layer V); no or almost no *GAP-43*, *L1* or *CHL1* was found in layer IV; and most cells in layers II and VI did not express *CHL1*, but a subpopulation expressed relatively high levels. Outside frontal areas, *krox-24* expression was highest in layers IV and VI; this was particularly visible in parietal cortex.

iii. Gene expression in corticospinal neurons after intracortical axotomy

In three animals, corticospinal neurons were retrogradely labelled, and 1 day later the animals received a knife lesion of the motor cortex angled to cut immediately under cortical layer V and parallel with it. Animals survived a further seven days. Subsequent histological analysis revealed that the lesions were correctly placed (Figs. 8.2-8.9, upper panels).

Alternate consecutive serial sections were processed for ISH and CTB immunohistochemistry. Many neurons immediately superficial to the lesion had upregulated mRNAs for GAP-43, SCG10, L1, CHL1, c-Jun and ATF3. Upregulation was apparent when neurons above the lesion were compared both to neurons in the intact animal (Figs. 8.2-8.7, lower panels) and neurons on the contralateral side of the operated animal (not shown). No change could be seen in *CAP-23* expression (Fig. 8.8), but *krox-24* appeared to be down-regulated in most neurons in layers II, III, and V to a low level, although it continued to be expressed by some layer V neurons (Fig. 8.9).

Adjacent sections processed for CTB immunohistochemistry demonstrated the presence of retrogradely labelled corticospinal neurons in the tissue examined. Further analysis allowed the identification of corticospinal neurons which were similarly positioned to cells on sections processed for ISH, and it is very likely that in most cases these are the same neurons. Many of the neurons which had upregulated *GAP-43*, *SCG10*, *L1*, *CHL1*, *c-jun* and *ATF3* appeared also to be present and retrogradely labelled on an adjacent section (Figs. 8.10, 8.11). Identified corticospinal neurons can thus be seen to have upregulated all five molecules, a result which is particularly striking when compared to corticospinal neurons in animals which have received cervical lesions of the corticospinal tract (compare Figs. 8.10 and 8.11 with Figs. 8.12-8.16).

Only those neurons within a limited distance of the lesion showed clear upregulation of *c-jun*, *GAP-43*, *SCG10*, *L1* and *CHL1*, detected by ISH. More distant corticospinal neurons had not upregulated the mRNA in question. This data is presented for one typical animal in Table 8.1. In this animal, corticospinal neurons could be identified up to a maximum distance of 500μm from the lesion. (Distances were measured on individual sections i.e. within the plane of sectioning). *ATF3* was upregulated in corticospinal neurons in all sections, while *CHL1* was upregulated consistently only if within 300μm of the lesion, although some neurons between 300-375μm also showed upregulation. *C-jun*, *GAP-43*, *SCG10* and *L1* were upregulated consistently by corticospinal neurons between 350 and 480μm from the lesion, as shown in Table 8.1.

iv. Gene expression in corticospinal neurons after axotomy in the cervical spinal cord

Gene expression was examined in the motor cortex 1 day and 7 days after a lesion to the corticospinal tract in the C3 segment of the cervical spinal cord, which was accompanied by an injection of tracer into the cord immediately rostral to the lesion. These time points were thought to represent a likely time-window for changes in gene expression representing a response to axotomy to occur. Alternate sections were processed for ISH and CTB immunohistochemistry. Patterns of expression of the growth-associated genes appeared to be the same as that found in the unoperated rat for each molecule. Analysis of adjacent sections allowed the identification of retrogradely labelled corticospinal neurons

Molecule	m RNA upregulated following proximal axotomy	Greatest distance from lesion of corticospinal neurons† showing mRNA upregulation
C-JUN	✓	350μm
ATF3	✓	500μm
GAP-43	✓	350μm
SCG10	✓	480μm
L1	✓	400μm
CHL1	✓	375μm*
CAP-23	✘	-
KROX-24	✘	-

†As identified by retrograde labelling on an adjacent serial section.

*CHL1 was consistently upregulated only when the distance from the lesion was below 300μm.

Table 8.1. Summary of the effects of intracortical axotomy on the expression of growth associated molecules by corticospinal neurons. All distances were measured within the plane of sectioning. Note that retrogradely corticospinal neurons were identifiable beyond the distance shown in the third column for c-Jun, GAP-43, SCG10, L1 or CHL1 but had not upregulated the mRNA in question.

---

in similar positions to cells showing ISH signal, which are very likely to be the same cells. At 1 day and 7 days after cervical spinal cord lesion, corticospinal neurons identified in this way expressed *GAP-43*, *CAP-23*, *SCG10*, *L1* and *CHL1* (Figs. 8.12-8.14). No difference in mRNA expression could be clearly demonstrated for any of these five molecules in corticospinal neurons between 1 day and 7 days post-lesion (Figs. 8.12-8.14). Care was taken to select neurons in approximately equivalent positions in the neocortex, but it should be noted that the variation in expression found in pyramidal neurons for most of these molecules along the mediolateral axis (see above) would make it difficult to detect small changes reliably.

No changes could be observed in *c-jun*, *ATF3* or *krox-24* in layer V pyramidal neurons as a whole following cervical axotomy. However, *c-jun*, *ATF3* and *krox-24* were not expressed at high enough levels in layer V pyramidal neurons to allow reliable comparison

between identified corticospinal neurons and cells on the sections processed for ISH (but note that a subset of layer V pyramidal neurons expressed significant levels of *krox-24*).

v. Retrograde labelling of rubrospinal neurons

Three animals received a lesion of the left lateral funiculus at C3, and in order to confirm the presence of rubrospinal neurons and to identify them on sections processed for ISH, CTB retrograde tracer was injected into the dorsal part of lateral funiculus immediately above the lesion. Animals were allowed to survive for 7 days and sections of midbrain were processed for CTB immunohistochemistry. Most retrogradely labelled neurons were found in the contralateral red nucleus. Small numbers of labelled neurons were also found in the ipsilateral red nucleus, the Edinger-Westphal nucleus and scattered through the deep mesencephalic tegmentum (Fig. 8.15). The observed distribution of retrogradely labelled neurons is in accord with previous findings on the projections from the brain to the dorsolateral funiculus of the spinal cord (Basbaum and Fields, 1979).

vi. Gene expression in intact and axotomised rubrospinal neurons

Alternate serial sections of midbrain were processed for ISH or CTB immunohistochemistry. Expression of mRNAs for c-Jun, ATF3, GAP-43, CAP-23, SCG10, L1, CHL1 and Krox-24 was examined in the red nuclei ipsilateral and contralateral to the lesion. The contralateral nucleus (containing axotomised neurons) was localised using retrograde labelling data from adjacent sections and the ipsilateral nucleus was assumed to be symmetrically positioned on the opposite side of the midbrain.

Neurons in the ipsilateral (uninjured) red nucleus expressed *c-jun*, *GAP-43*, *CAP-23*, *SCG10*, *L1*, *CHL1* and *krox-24*, but *ATF3* mRNA was undetectable (Figs. 8.16-8.19). ISH signals from riboprobes for *c-jun*, *CAP-23*, *CHL1* and *krox-24* were all low, while those for *GAP-43*, *SCG10*, and *L1* were all higher relatively, being easily detectable.

C-jun, *ATF3* and *L1* were clearly upregulated in rubral neurons contralateral to the lesion (Fig. 8.16), when compared with neurons in the ipsilateral red nucleus. Adjacent sections revealed large numbers of retrogradely labelled rubrospinal neurons. While *c-jun*

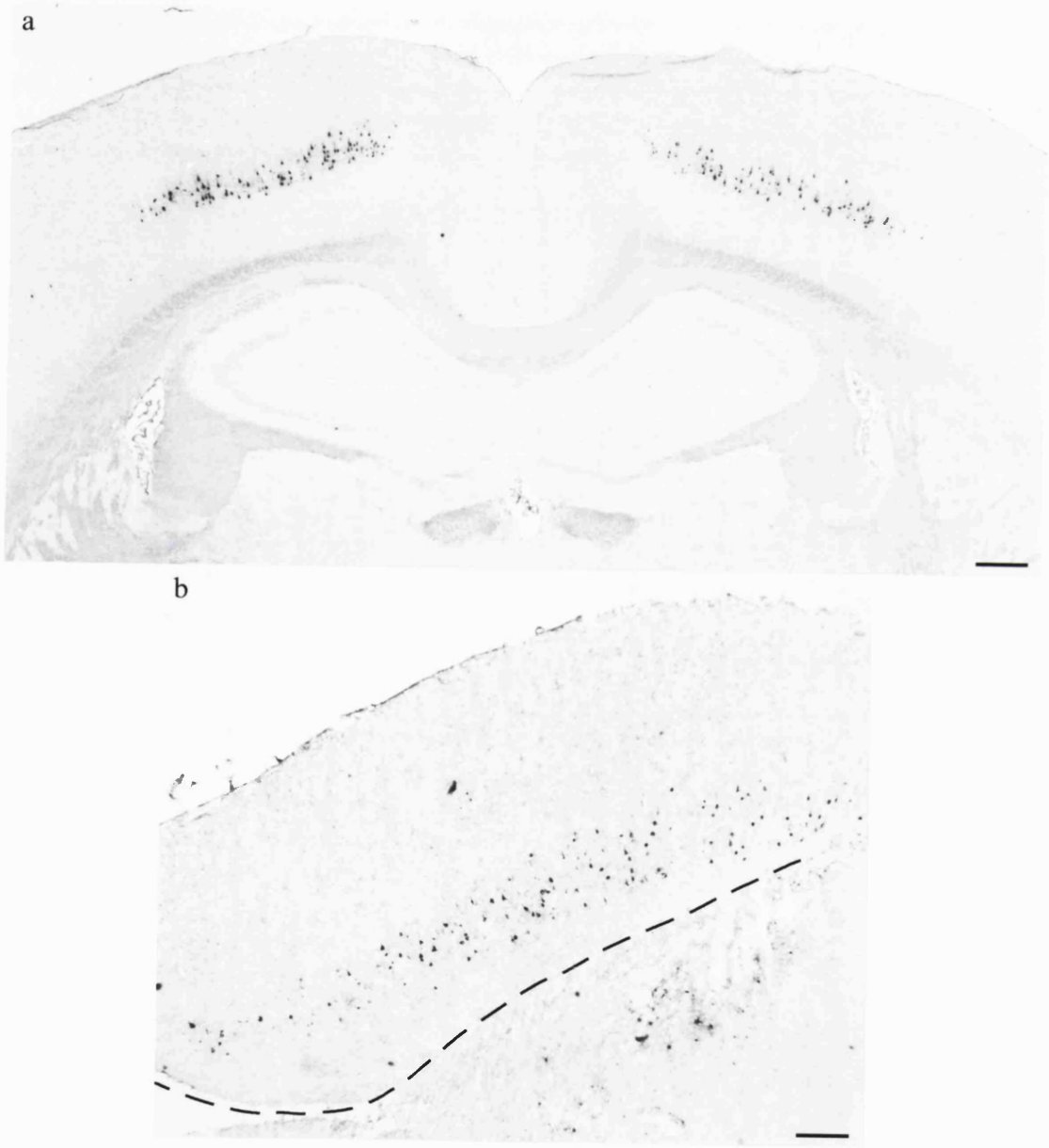
upregulation was seen in almost as many neurons, fewer neurons had upregulated *ATF3* and *L1* expression than were retrogradely labelled. No difference in gene expression was seen in levels of *GAP-43*, *CAP-23*, *SCG10*, *CHL1* or *krox-24* (Figs. 8.17-8.19) despite the clear presence of retrogradely labelled rubrospinal neurons indicated by CTB immunohistochemistry on adjacent sections (Figs. 8.17-8.19, insets).

A careful analysis was performed of adjacent serial sections processed either for CTB immunohistochemistry or for ISH with riboprobes for *c-jun*, *ATF3* or *L1* mRNA. Many of those neurons which were seen to have upregulated one of these mRNAs on those sections processed for ISH could be identified on an adjacent section and were retrogradely labelled from the spinal cord (Fig. 8.20).

Fig. 8.1. Retrograde labelling of corticospinal neurons after injection of CTB into the cervical spinal cord. The animal shown in *a* received a CTB injection and a lesion of the corticospinal tract, both in cervical spinal cord, 7 days previously. Many labelled neurons can be seen in layer V of neocortex on both sides. The animal shown in *b* received a CTB injection into the cervical spinal cord, followed 24 hours later by a knife lesion of the cortex to proximally axotomise corticospinal neurons. Seven days after the lesion, retrogradely labelled neurons are still visible above the lesion although they are reduced in size compared to the animal shown in *a*. The dashed line indicates the dorsal boundary of the lesion.

Bars 500μm (*a*), 250 μm (*b*).

FIGURE 8.1



Figs. 8.2-8.7. Expression of *c-jun*, *ATF3*, *GAP-43*, *SCG10*, *L1* and *CHL1* in neocortex 7 days after proximal axotomy caused by a knife lesion (upper panels) and in an unoperated control animal (lower panels). On each section shown in the upper panels the position of the lesion is shown by a dashed line marking its dorsal extent. All six mRNAs are expressed in the cortex of the control animal, including layer V where corticospinal neurons are situated. In the lesioned animal all six mRNAs are upregulated in many neocortical neurons and most, if not all, of these are found in layer V.

Bars 250μm.

FIGURE 8.2

FIGURE 8.3

FIGURE 8.4

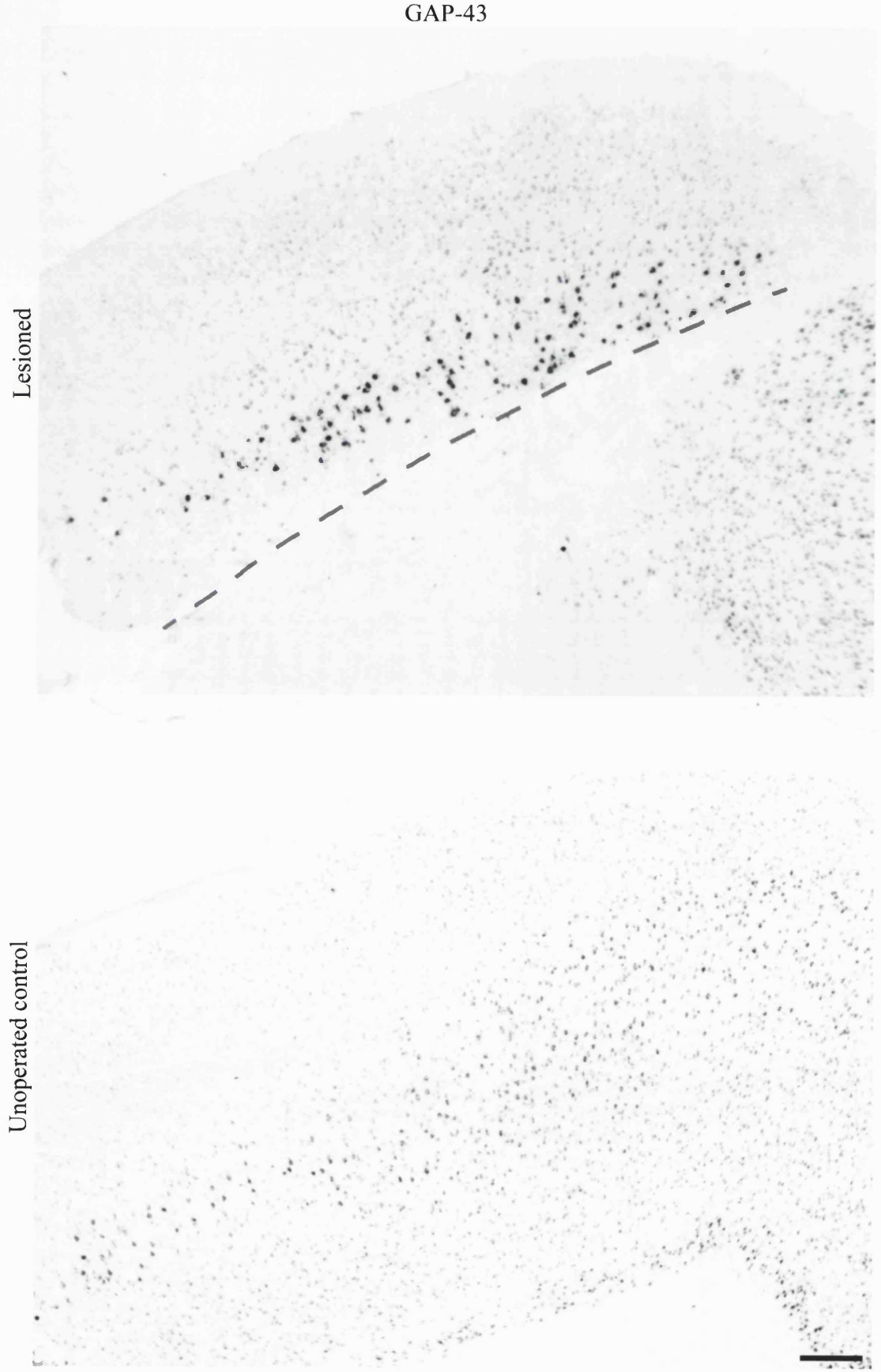


FIGURE 8.5

FIGURE 8.6

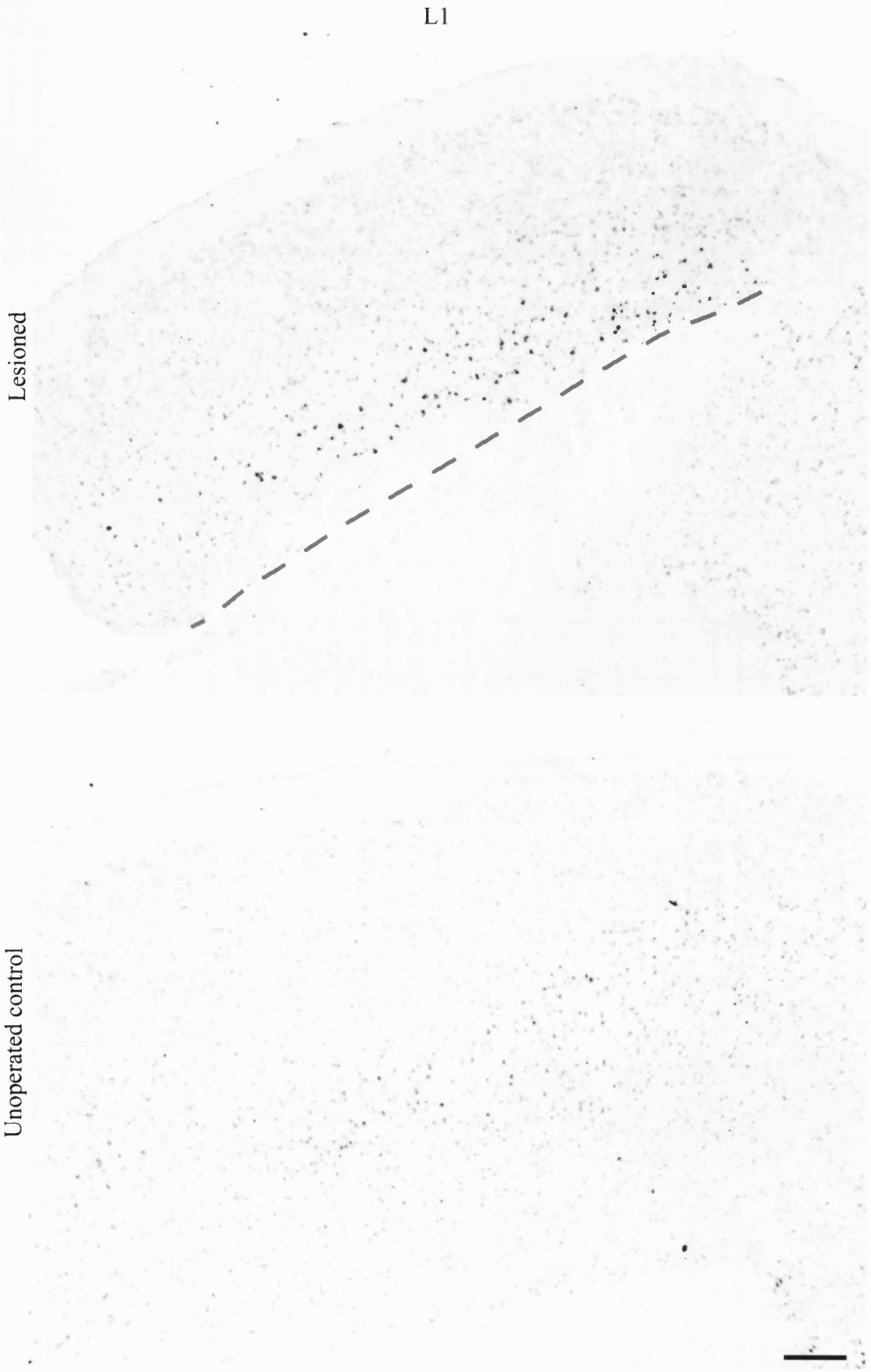


FIGURE 8.7

Fig. 8.8, 8.9. Expression of *CAP-23* and *krox-24* in neocortex 7 days after proximal axotomy caused by a knife lesion (upper panels) and in an unoperated control animal (lower panels). On each section shown in the upper panels the position of the lesion is shown by a dashed line marking its dorsal extent. Both mRNAs are expressed in the cortex of the control animal, including layer V where corticospinal neurons are situated. In the lesioned animal, *CAP-23* expression appears to be unchanged from the control animal, and *krox-24* expression has decreased in many neocortical neurons above the lesion.

Bars 250μm.

FIGURE 8.8

CAP-23

Figs. 8.10, 8.11. ISH for *c-jun*, *ATF3*, *GAP-43*, *SCG10*, *L1*, *CHL1* and *CAP-23* and retrograde labelling of corticospinal neurons 7 days after proximal axotomy. For each molecule except *CAP-23*, strong expression of the mRNA can be seen in several neurons (left panel of each pair). On the adjacent section, which was processed for CTB immunohistochemistry (right panel of each pair), retrogradely labelled corticospinal neurons are visible in similar positions and are very likely to be the same neurons as those seen in the left panel. These neurons are identified by arrowheads on both sections. The increases in mRNA expression may also be verified by comparing hybridisation signals with those shown in Figs. 8.12-8.14. *CAP-23* expression is unchanged from that seen in pyramidal neurons in the intact animal, or from that seen after a cervical spinal cord injury (compare with Fig. 8.12).

Bars 20μm.

FIGURE 8.9

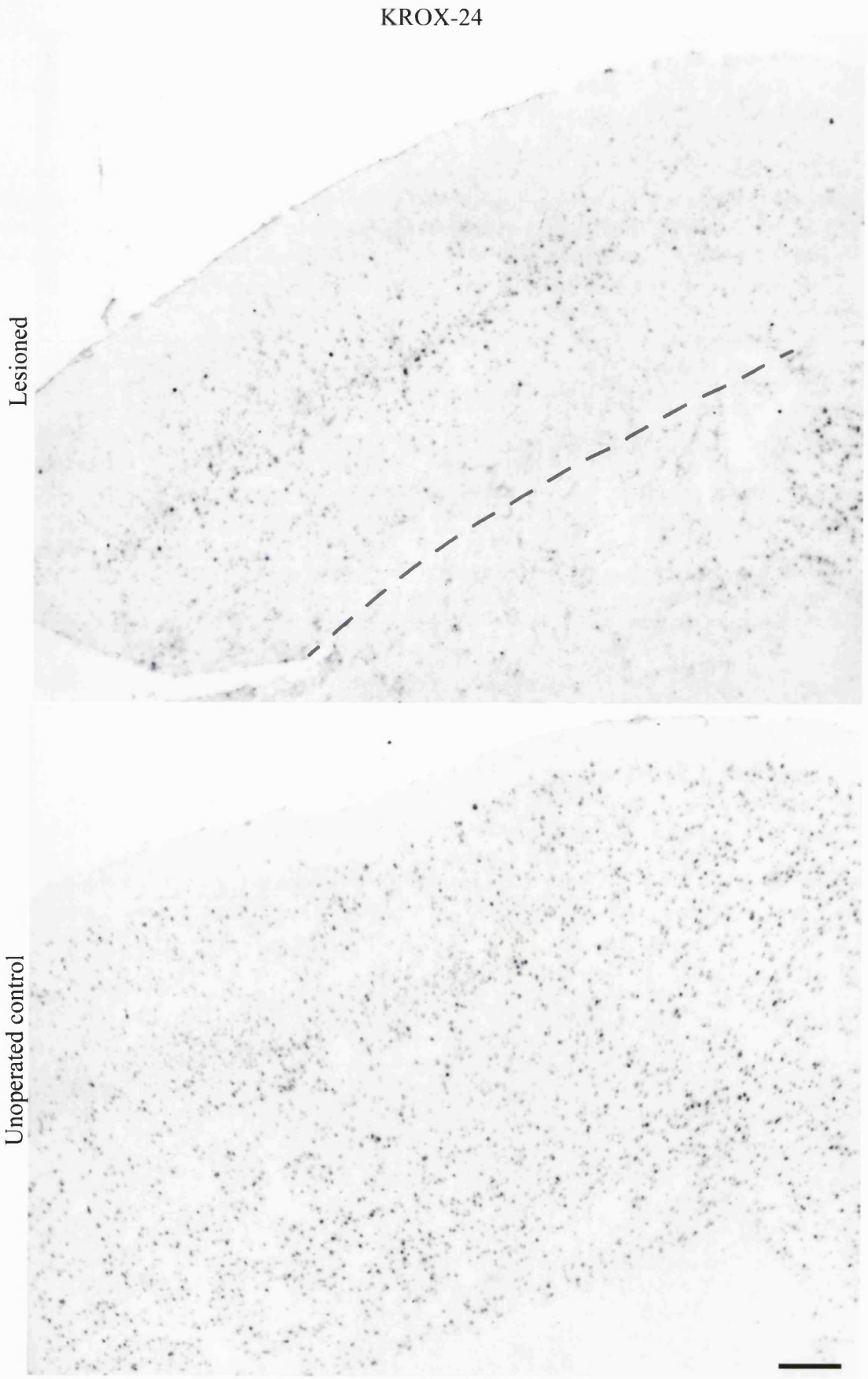


FIGURE 8.10

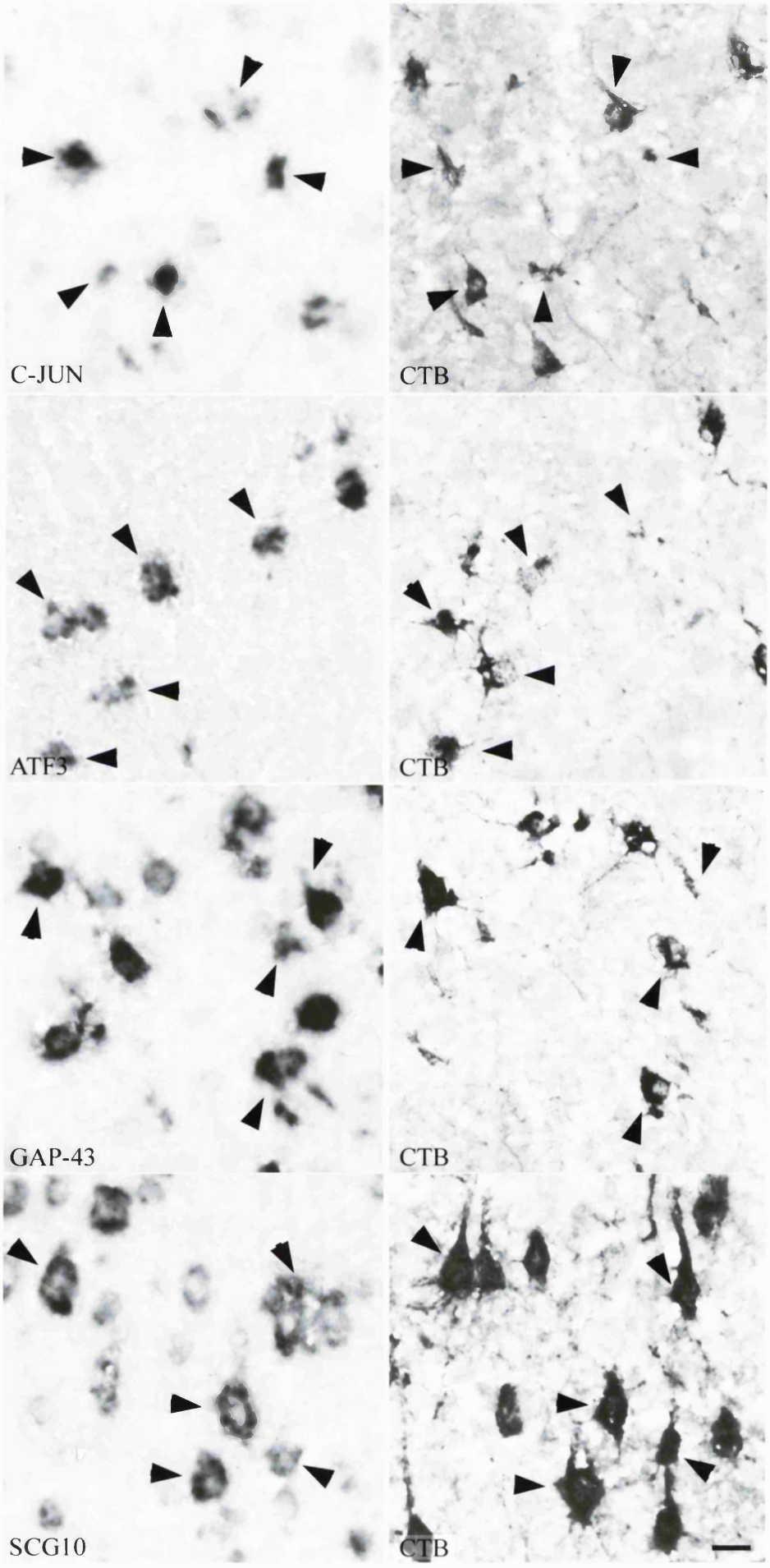
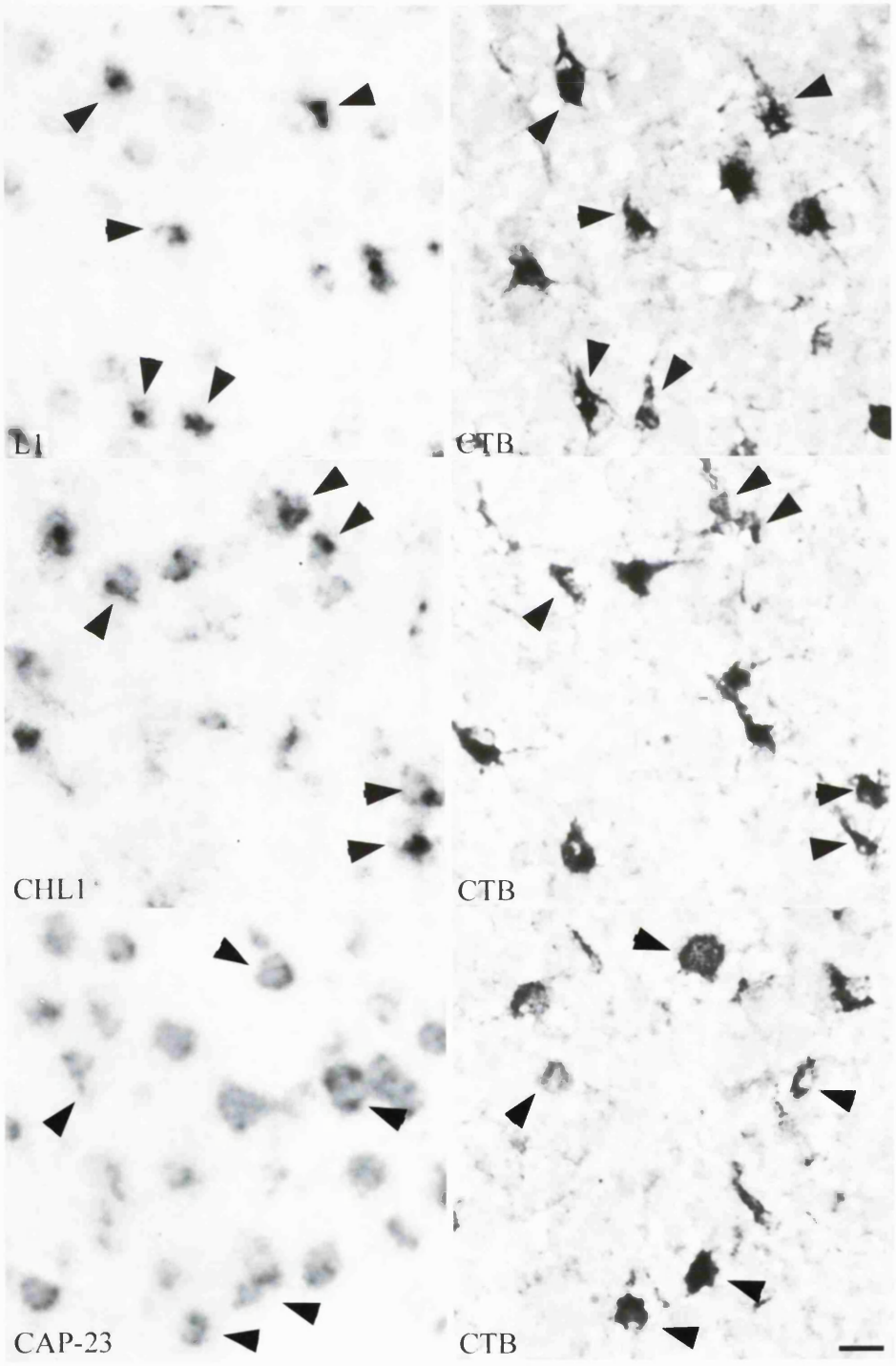


FIGURE 8.11



Figs. 8.12-8.14. ISH for *GAP-43*, *CAP-23*, *SCG10*, *L1* and *CHL1* in corticospinal neurons identified by retrograde labelling, 1 day and 7 days after axotomy in the cervical spinal cord. For each molecule, several pyramidal neurons are visible which display a hybridisation signal (left panel of each pair). On the adjacent section, which was processed for CTB immunohistochemistry (right panel of each pair), retrogradely labelled corticospinal neurons are visible in similar positions and are very likely to be the same neurons as those seen in the left panel. These neurons are identified by arrowheads on both sections. No difference can be observed in expression of any of these mRNAs between 1 day and 7 days post-lesion.

Bars 20μm.

FIGURE 8.12

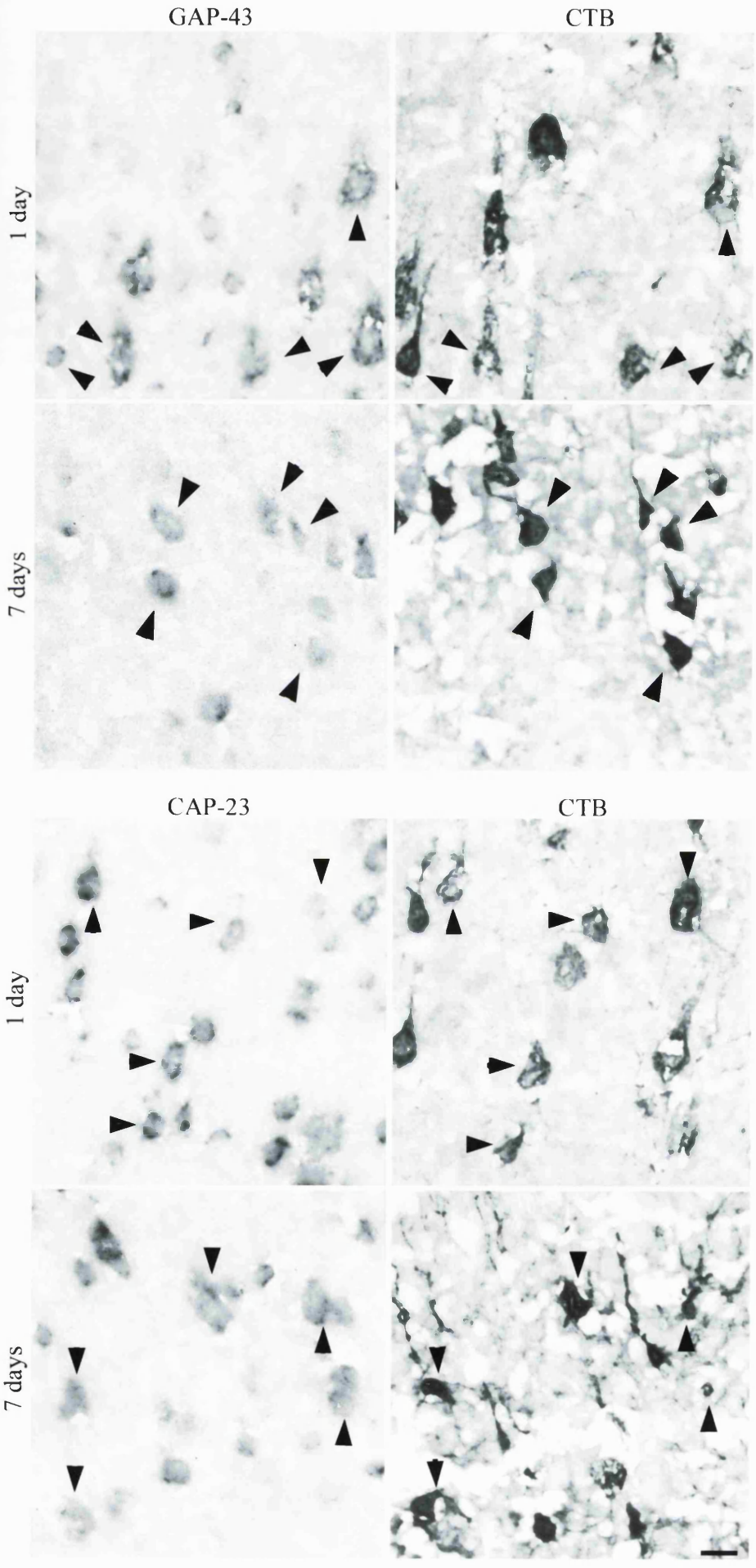


FIGURE 8.13

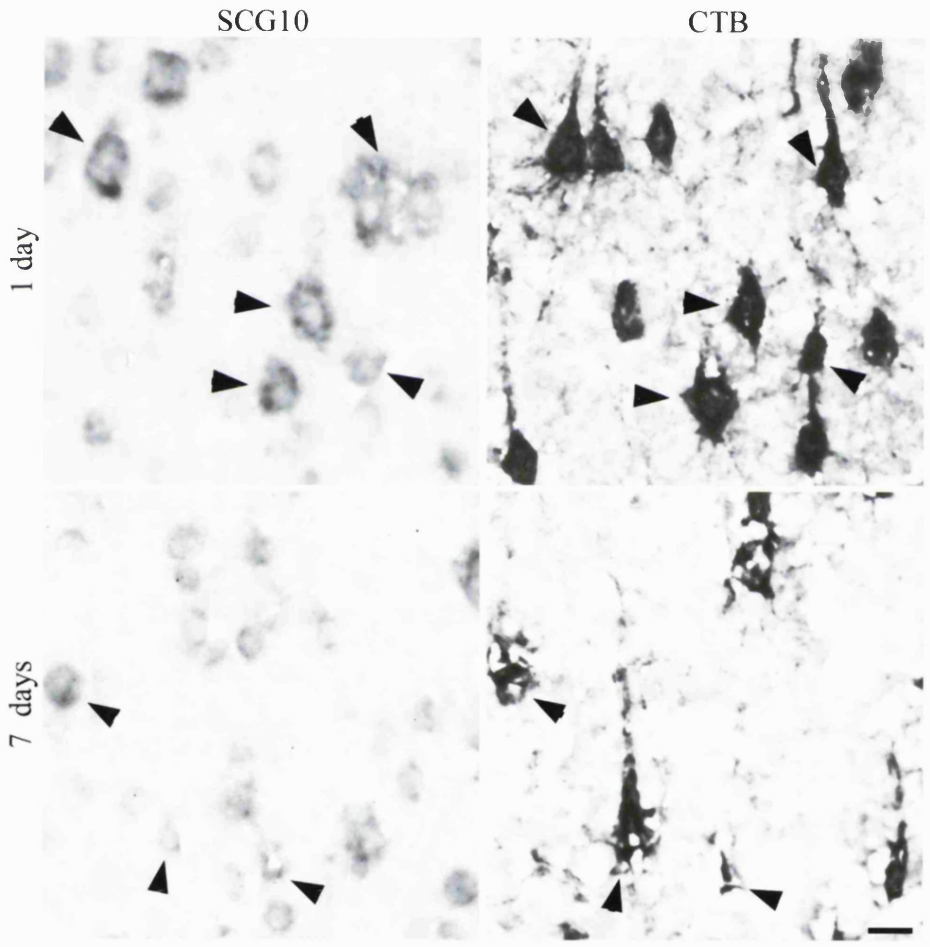


FIGURE 8.14

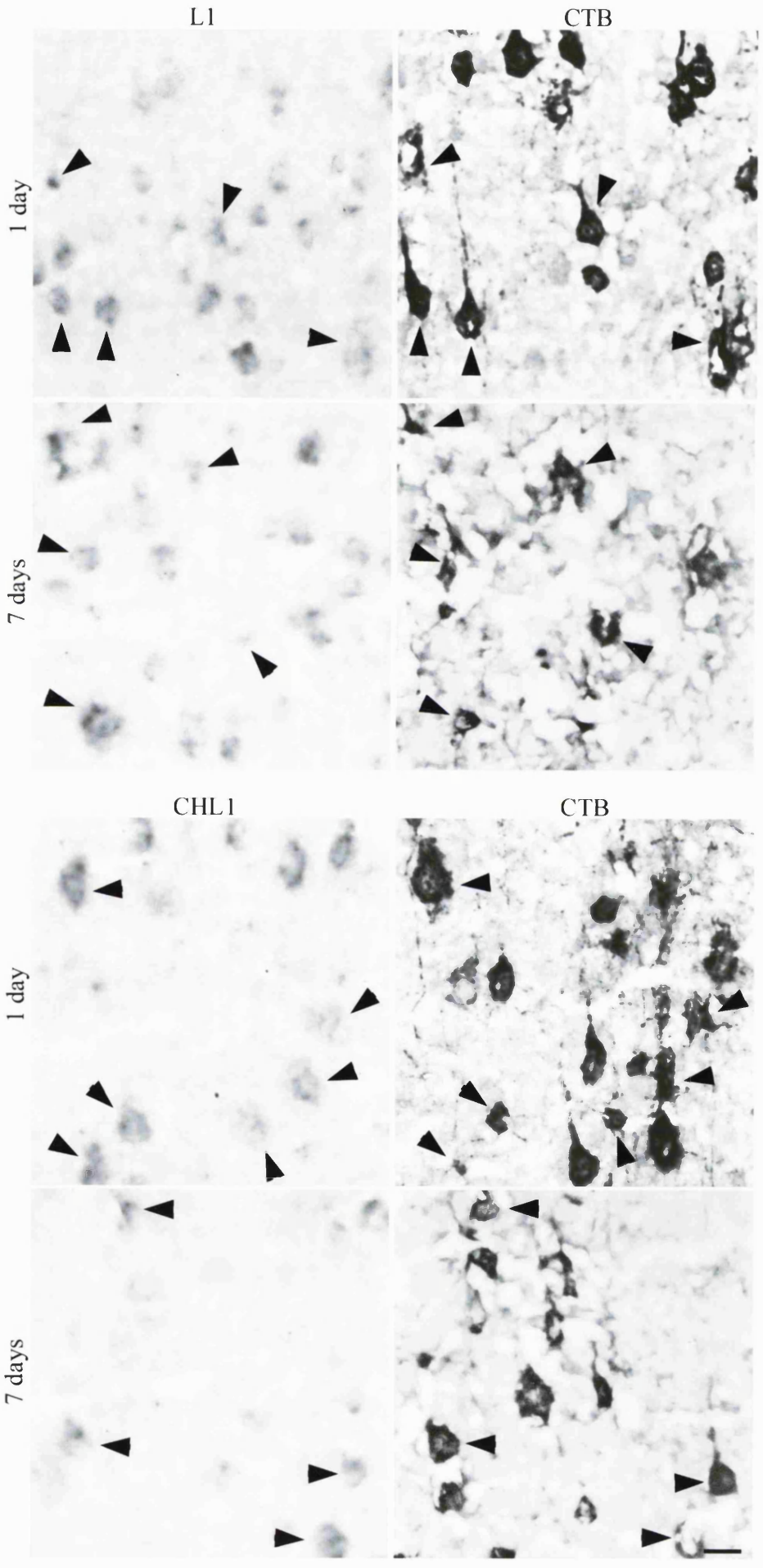


Fig. 8.15. Retrograde labelling of neurons in the red nucleus and elsewhere after injection of CTB into the dorsal lateral funiculus of the spinal cord. CTB immunohistochemistry identifies neurons in the midbrain which project to this part of the cord. Most are found in the contralateral red nucleus (R; right) but some are also found in the ipsilateral red nucleus (R; left), the Edinger-Westphal nucleus (EW) and scattered through the deep mesencephalic tegmentum (DMT). SNpc: substantia nigra pars compacta.

Bar 250μm.

FIGURE 8.15

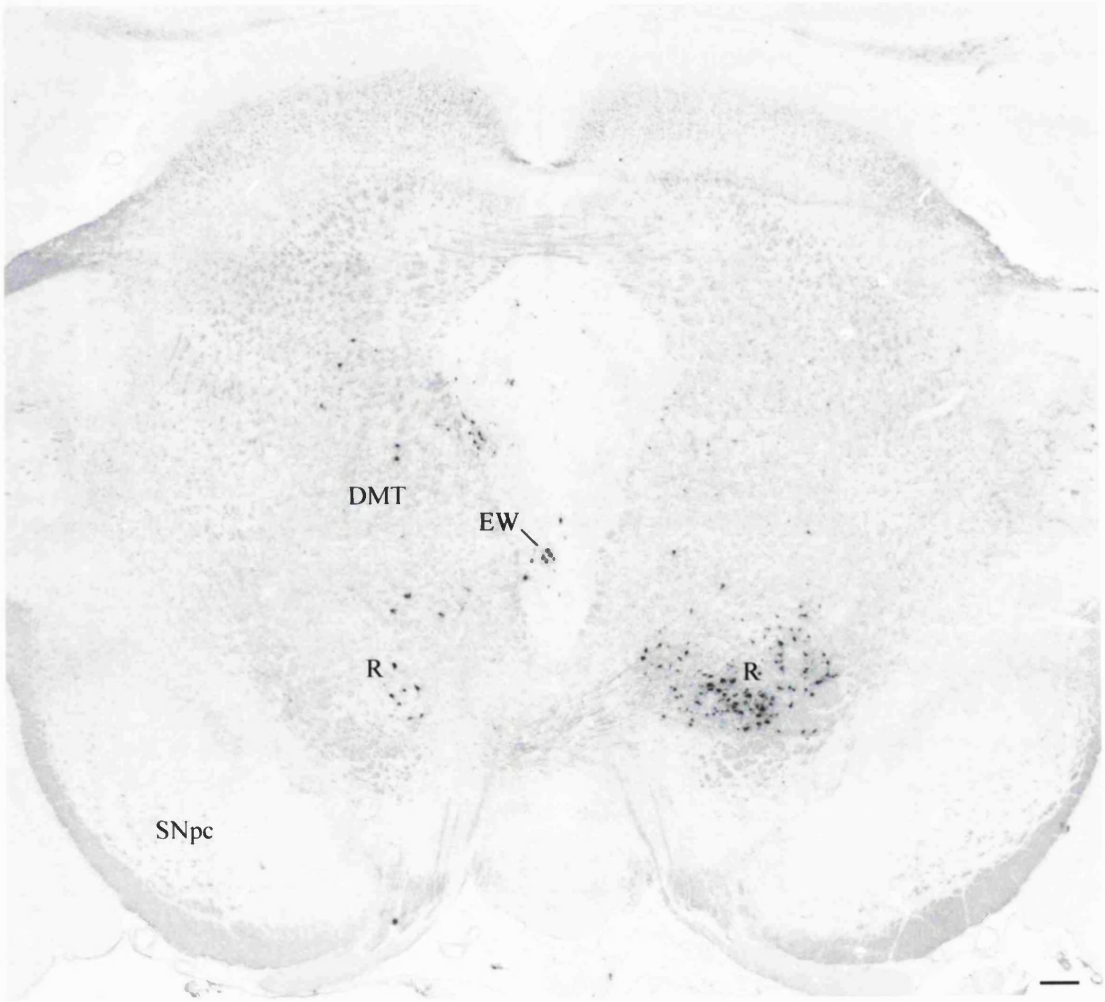
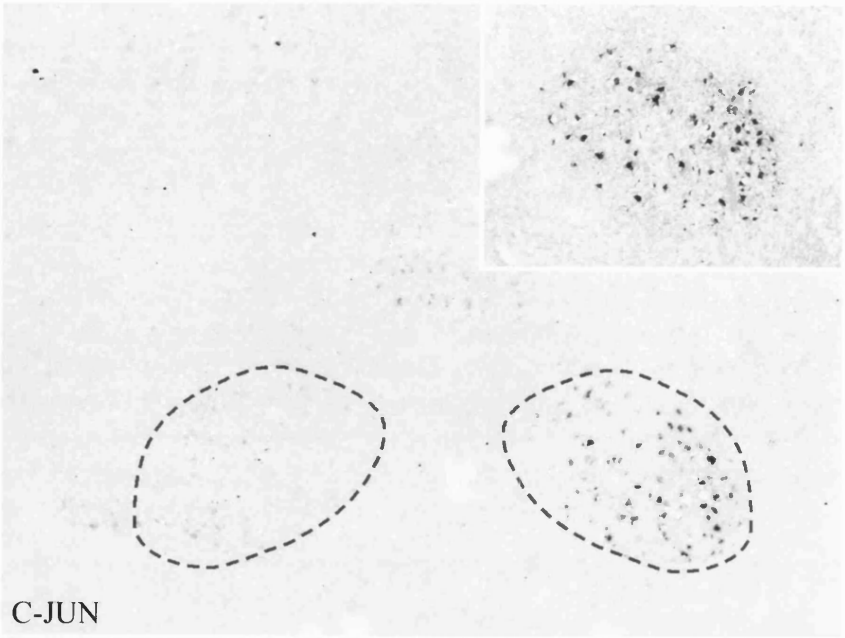
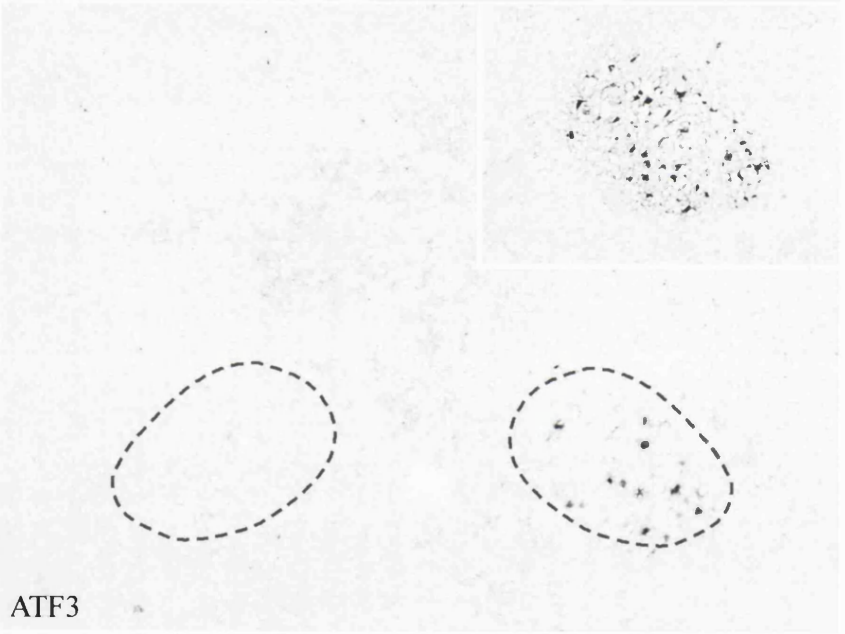


Fig. 8.16. Expression of *c-jun*, *ATF3* and *L1* in the red nuclei 7 days after unilateral transection of the lateral funiculus of the cervical spinal cord. In the main panels, ISH reveals that each of these mRNAs is upregulated in neurons of the contralateral red nucleus (outlined; right-hand side of sections) as compared to the ipsilateral red nucleus (outlined, left side). The insets show retrograde labelling of rubrospinal neurons with CTB on an adjacent section. Outlines of the contralateral red nucleus are based on the retrograde labelling data; the ipsilateral nucleus is assumed to be symmetrically opposite the midline.

Bar 250μm.

FIGURE 8.16





Figs. 8.17-8.19. Expression of *GAP-43*, *SCG10*, *CAP-23*, *CHL1* and *krox-24* in the red nuclei 7 days after unilateral transection of the lateral funiculus of the cervical spinal cord. As determined by ISH shown in the main panels, no difference can be seen in expression of each mRNA between the contralateral red nucleus (outlined; right-hand side of sections) as compared to the ipsilateral red nucleus (outlined, left side). The insets show retrograde labelling of rubrospinal neurons with CTB on an adjacent section. Outlines of the contralateral red nucleus are based on the retrograde labelling data; the ipsilateral nucleus is assumed to be symmetrically opposite the midline.

Bars 250μm.

FIGURE 8.17

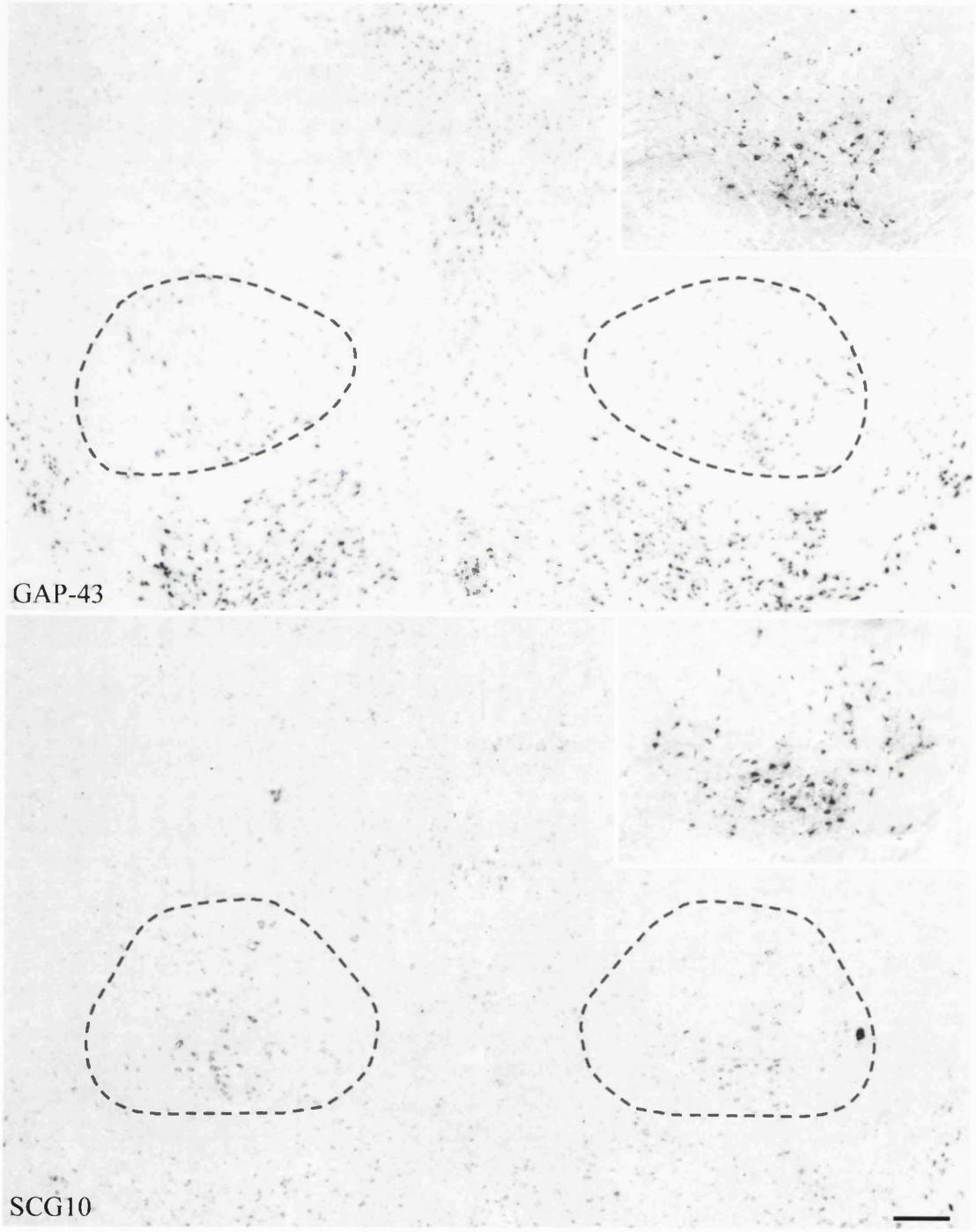


FIGURE 8.18

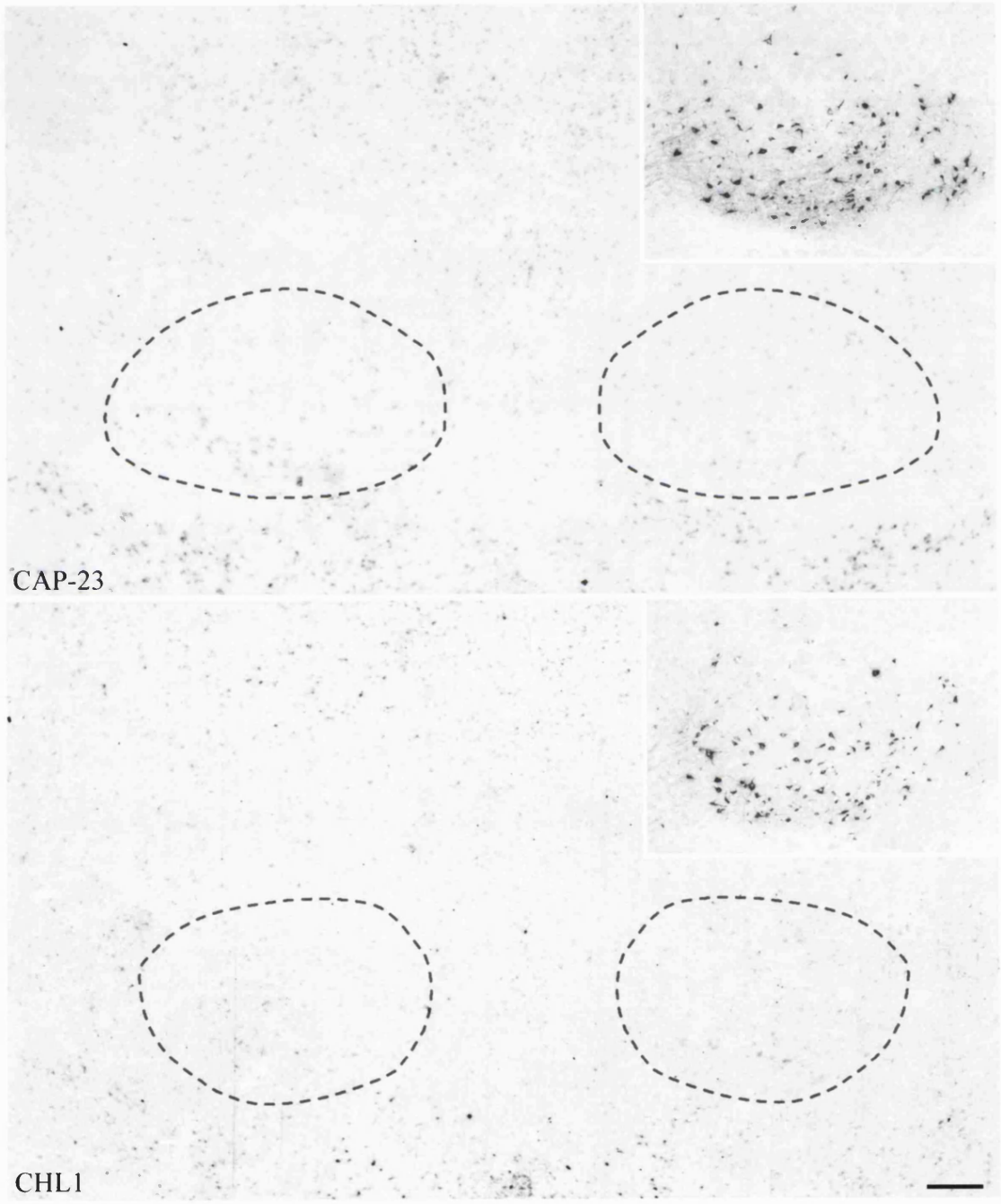


FIGURE 8.19

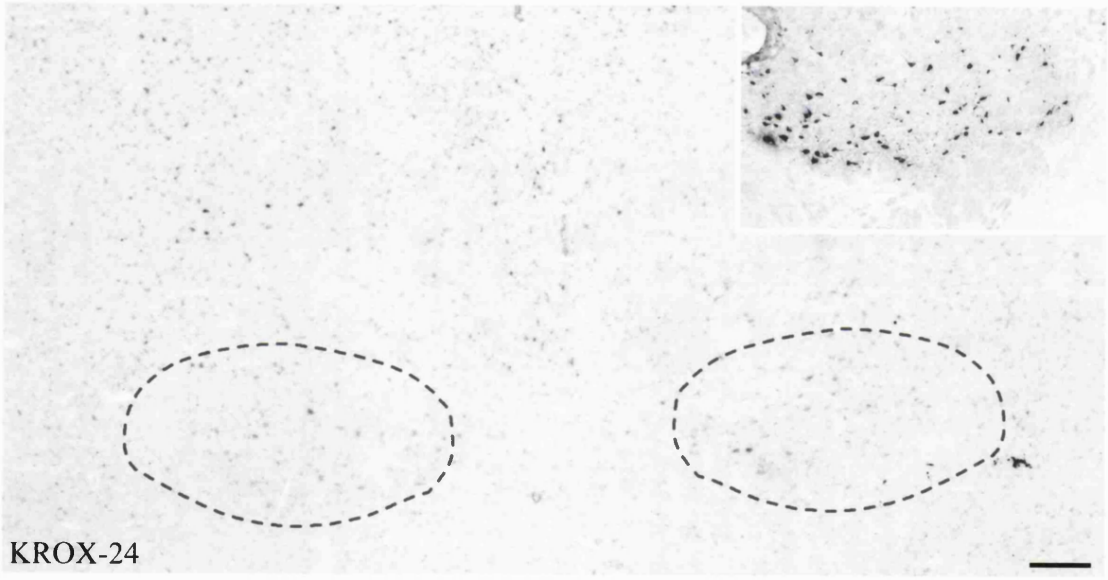
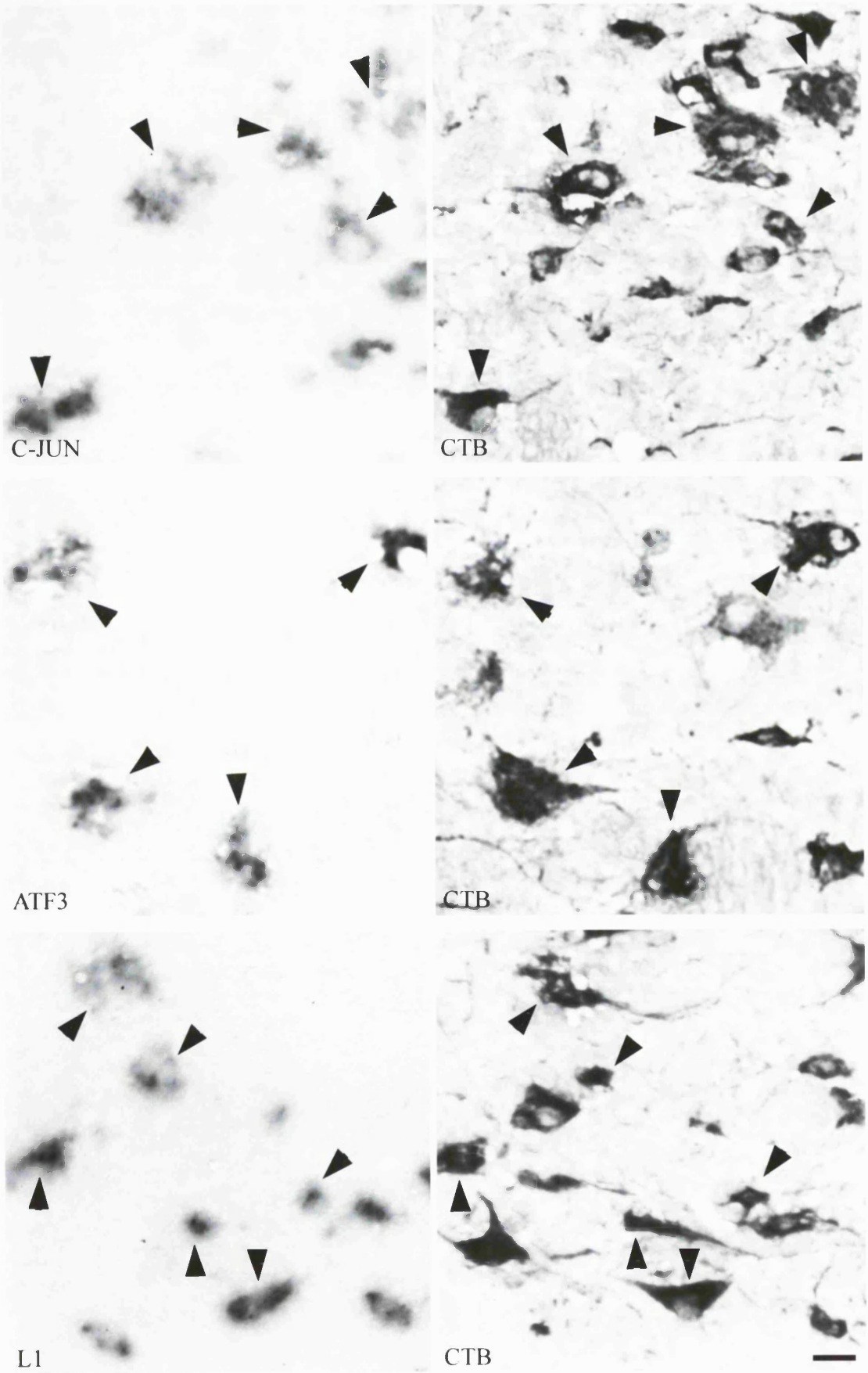


Fig. 8.20. ISH for *c-jun*, *ATF3* and *L1* in rubrospinal neurons identified by retrograde labelling 7 days after axotomy in the cervical spinal cord. For each molecule, strong expression of the mRNA can be seen in several neurons (left panel of each pair). Some of these are identifiable on an adjacent section processed for CTB immunohistochemistry (right panel of each pair), and are immunopositive, indicating they are retrogradely labelled rubrospinal neurons. These neurons are identified by arrowheads on both sections.

Bar 20μm.

FIGURE 8.20



Chapter 9

Discussion

9.1 Summary of results

In this study several aspects of the neuronal response to axotomy have been examined. Firstly, gene expression was studied in axotomised neurons which successfully regenerated their axons, and compared with gene expression in neurons which were unable to do so and in uninjured neurons. Messenger RNAs for the putative growth-associated molecules SCG10, CAP-23 and FKBP12 were all shown to be upregulated during axonal regeneration and appear to be co-regulated with the mRNA for the well-known growth-associated molecule GAP-43. Both *SCG10* and *CAP-23* were upregulated by DRG neurons and motor neurons with axons in the sciatic nerve during peripheral nerve regeneration. All three candidate molecules were shown to be upregulated by intrinsic CNS neurons regenerating axons into peripheral nerve grafts, whereas not even a transient upregulation was seen in populations of neurons which are unable to regenerate axons into grafts. Thus, implantation of a peripheral nerve graft into the thalamus resulted in the regeneration into the grafts of axons from neurons in the thalamic reticular nucleus (TRN) and a concomitant upregulation of *SCG10*, *CAP-23* and *FKBP12* mRNAs in neurons of this nucleus. Very few neurons in the dorsal thalamus regenerated their axons or upregulated these molecules. Similarly, implantation of a graft into the cerebellum elicited axonal regeneration by neurons of the cerebellar deep nuclei, and these neurons also upregulated *SCG10*, *CAP-23* and *FKBP12* mRNAs. Purkinje cells and other cells of the cerebellar cortex did not regenerate axons into grafts and most did not upregulate these molecules, except for a few proximally axotomised Purkinje cells which upregulated CAP-23.

Secondly, a study was made of the effect on regeneration of overexpression of GAP-43 in DRG neurons in transgenic mice. The central branches of DRG neurons show a limited response to axotomy, unless the cell-body is stimulated by an injury to the peripheral branch of its axon. The cell body response consists of the upregulation of a number of growth-associated molecules, including GAP-43. It also results in increased sprouting and regeneration of the injured central branches of these axons, in both the spinal cord following a dorsal column injury and in the dorsal roots after rhizotomy. To investigate the degree to which GAP-43 expression alone may boost sprouting and regeneration, transgenic wt3 mice, which overexpress GAP-43 in DRG neurons, and their non-transgenic

litter-mates, were given injuries to the ascending dorsal columns of the spinal cord, or to the lumbar dorsal roots, and the subsequent behaviour of the injured axons examined by transganglionic labelling from the sciatic nerve. Overexpression of GAP-43 did not consistently result in increased regeneration in dorsal column or dorsal root injuries, although in some animals which had received a dorsal column injury in the lumbar spinal cord an increase in sprouting out of the spinal cord was observed.

Thirdly, the response to axotomy of corticospinal and rubrospinal neurons was characterised in terms of the changes in expression of genes for a number of molecules known to be associated with neuronal injury and regeneration. The expression of *GAP-43*, *SCG10*, *CAP-23*, *L1*, *CHL1*, *c-jun*, *ATF3* and *krox-24* was examined in the axotomised cells. Corticospinal neurons failed to upregulate any molecule after an injury to the corticospinal tract at the cervical level, but upregulated *GAP-43*, *SCG10*, *L1*, *CHL1*, *c-jun* and *ATF3*, but not *CAP-23* or *krox-24* following very proximal axotomy caused by a slice lesion in the neocortex. Rubrospinal neurons upregulated only *L1*, *c-jun* and *ATF3* after an injury to the rubrospinal tract at the cervical level.

9.2 Expression of *SCG10*, *CAP-23* and *GAP-43* in intact and regenerating lumbar motor and sensory neurons

Following sciatic nerve injury, *SCG10*, *CAP-23* and *GAP-43* mRNAs were found to be upregulated in neurons in the L4 and L5 dorsal root ganglia, and in the motor neurons of the L4 and L5 segments of the lumbar spinal cord. This is the first evidence that *SCG10* and *CAP-23* mRNA levels are increased in regenerating neurons, and suggests therefore that SCG10 and CAP-23 proteins play a role in axonal regeneration. The upregulation of *GAP-43* is consistent with previous findings (Woolf et al., 1990; Chong et al., 1992; Schreyer and Skene, 1993) and served as a positive control to identify neurons mounting a regenerative response.

i. Time course of upregulation of SCG10, CAP-23 and GAP-43 in axotomised neurons

While the time course of upregulation was similar for both *SCG10* and *CAP-23* mRNAs, and was similar in motor and sensory neurons, detectable upregulation of *SCG10* and *CAP-23* mRNAs was slightly delayed relative to that of *GAP-43* mRNA. Differences in levels of *SCG10* and *CAP-23* between ipsilateral and contralateral neurons were visible at 3 days but not at 1 day, whereas upregulation of *GAP-43* was observed in both motor and sensory neurons at 1 day post-operation, in agreement with previous studies (Woolf et al., 1990; Chong et al., 1992). This might reflect a genuine delay in transcriptional increases of *SCG10* and *CAP-23*, but may also be due to the higher basal levels of expression of *SCG10* and *CAP-23* in motor and sensory neurons, which would mask a small relative increase. *GAP-43* is almost undetectable in motor and many sensory neurons, making small absolute increases more readily detectable. Therefore this apparent difference does not necessarily indicate a delay in the increase in transcription of *SCG10* and *CAP-23* mRNAs following axotomy.

ii. Co-expression of SCG10, CAP-23 and GAP-43 in axotomised neurons

Analysis of adjacent sections strongly suggested that all motor and DRG neurons which upregulated any of these molecules had upregulated all three. Combined with the similarities in regulation of expression described above, this provides strong evidence that *SCG10*, *CAP-23* and *GAP-43* mRNAs are co-regulated in motor and DRG neurons after axotomy. Such co-regulation is not always found with growth-associated molecules. For example, *L1* and *CHL1* are upregulated by many neurons regenerating their axons (Zhang et al., 1995; Zhang et al., 2000; Chaisuksunt et al., 2000a; Chaisuksunt et al., 2000b) but motor neurons and DRG neurons do not upregulate *L1*, and some sensory neurons also do not upregulate *CHL1* (Zhang et al., 2000). This may reflect a more universal role for the three molecules examined here in axon growth, by virtue of their functions in the growth-cone.

iii. Prolonged upregulation of SCG10, CAP-23 and GAP-43 in injured neurons after cut and ligation of the sciatic nerve

Cut and ligation injuries of the sciatic nerve resulted in continued elevated expression of *SCG10*, *CAP-23* and *GAP-43* by DRG and motor neurons. While expression of *SCG10*, *CAP-23* and *GAP-43* returned to basal levels when regeneration was completed, the continued production of these three mRNAs when target reinnervation was prevented suggests that connection of a neuron with its targets somehow regulates their expression. This is consistent with previous observations on the regulation of GAP-43 (Chong et al., 1994b) and other growth-associated molecules, such as *Tα1* tubulin mRNA (Miller et al., 1989) in injured neurons. It is likely that if target reinnervation is prevented, the program of gene expression for axonal regeneration continues and so, therefore, does the production of growth-associated molecules. Successful reconnection with targets and synaptogenesis is presumably signalled retrogradely to the neuronal cell body, perhaps switching the state of the neuron from a growth to a secretory phase (Greensmith and Vrbova, 1996).

iv. Expression of SCG10, CAP-23 and GAP-43 in axotomised neurons after dorsal rhizotomy

While injury to the dorsal roots results in significant transient upregulation of c-Jun (Jenkins et al., 1993a; Kenney and Kocsis, 1997) in DRG neurons, there is only a slight upregulation of *CHL1* (Zhang et al., 2000), and no increase in *GAP-43* is detectable unless the rhizotomy is very proximal (Chong et al., 1994a). Dorsal rhizotomy did not result in a detectable increase in *SCG10* or *CAP-23* in DRG. A small increase below the detection threshold cannot be ruled out but the essentially unchanged level is consistent with the minimal changes observed in other axonally transported growth-associated molecules.

v. Constitutive expression of SCG10, CAP-23 and GAP-43 in DRG neurons

In the intact dorsal root ganglion, *SCG10* was expressed by all neurons and *CAP-23* was expressed by most neurons with the exception of a subpopulation of large-diameter neurons, in which *CAP-23* mRNA was essentially undetectable. Frey et al.(2000) reported

that all DRG neurons expressed *CAP-23* mRNA. It is not clear what the reason may be for this discrepancy, other than the different method of in situ hybridisation used.

Of note was the finding that a population of small-diameter DRG neurons constitutively express high levels of *SCG10*, *CAP-23* and *GAP-43*. The purpose of this gene expression in uninjured neurons is not known but may reflect a role for growth-associated molecules in physiological plasticity. In addition, it may endow some neurons with the ability to regenerate axons rapidly following dorsal root injury. Andersen and Schreyer (1999) showed that some axons regenerate more quickly than the rest following dorsal root injury (without an additional peripheral lesion) and that the perikarya from which they originate are GAP-43 positive. The axons of GAP-43-positive neurons regenerated in injured dorsal roots at a rate comparable to that of other dorsal root axons in experiments when a conditioning peripheral lesion was also made (Richardson and Verge, 1987), inducing the increased production of GAP-43. However, the slower regeneration in the dorsal root of axons of GAP-43-negative neurons is also interesting; it may be that the low-levels of CAP-23 found in many DRG cells compensate for the absence of GAP-43 sufficiently to enable axon growth. If at least one of these molecules is required for axonal regeneration, large diameter DRG neurons which are negative for both CAP-23 and GAP-43 should not be able to regenerate their axons after dorsal rhizotomy.

It would also be interesting to determine if constitutive expression of *GAP-43*, *CAP-23* and *SCG10*, or combinations of these, correlates with established phenotypic markers of DRG neuron subtypes. It was previously shown that GAP-43 is made constitutively in trkA-positive cells (Verge et al., 1990), but those cells with particularly high levels of *GAP-43*, *CAP-23* and *SCG10* represent a distinct subpopulation of GAP-43-positive DRG neurons. The co-expression of these three mRNAs indicates that in addition to the apparent co-regulation of these three mRNAs during regeneration, they also appear to be partially co-regulated in intact neurons. Motor neurons and a subpopulation of DRG neurons contain low levels of c-Jun in the intact animal (Jenkins and Hunt, 1991), and while the roles of this transcription factor in neurons are complex and not completely understood, the co-expression of c-Jun with many growth-associated molecules during axon regeneration suggests it may be involved in regulating their expression. Therefore it would be of interest to know whether those DRG neurons which produce c-Jun constitutively also express

GAP-43, *CAP-23* and *SCG10*. If so, this would support a role for c-Jun in regulating expression of growth-associated molecules.

It was previously shown that CAP-23 protein was induced in intramuscular nerve of adult mice following a sciatic nerve injury (Caroni et al., 1997), where previously it was not detectable either in the nerve or at the neuromuscular junction. The present study showed that an increase in *CAP-23* mRNA accompanies this induction. However, *CAP-23* mRNA was also found at a detectable level in intact motor neurons, so it would be reasonable to expect some immunoreactivity in intramuscular nerve or, particularly given the reported association of CAP-23 with synaptic vesicles (Iino and Maekawa, 1999; Iino et al., 1999), at the neuromuscular junction. Possibly, CAP-23 protein may have been present at levels below those that could be detected by the techniques used by Caroni et al. (1997).

9.3 Expression of *SCG10*, *CAP-23*, *GAP-43 and FKBP12* in the thalamus and cerebellum, in the intact animal

i. SCG10, CAP-23, GAP-43 and FKBP12 expression in the intact thalamus

Some interesting observations were made concerning the distributions of these mRNAs in the intact thalamus. Of note was the overall similarity in the patterns of expression of *SCG10*, *CAP-23* and *GAP-43*, particularly as these showed marked variation between specific thalamic nuclei. The most obvious differences between the expression of *SCG10*, *CAP-23* and *GAP-43* were found in the TRN. The neuronal perikarya in this nucleus were devoid of *GAP-43*, and most were devoid of *CAP-23* (the exception being those in the most dorsal part of the nucleus, where it was detectable at a low level). In contrast, *SCG10* was expressed at a moderate level in neurons throughout the TRN, with a stronger signal being detected in the dorsal-most part, where the neurons also contained *CAP-23*. Most dorsal thalamic nuclei showed similar relative levels of *SCG10*, *CAP-23* and *GAP-43*, although there was variability in the strength of expression of these genes between nuclei. The broad similarities in distribution of these three mRNAs seems to indicate a greater requirement for growth-associated molecules in some neurons in the intact state and may also reflect co-regulation of these three molecules in the intact thalamus. Parallels may be drawn with the intact dorsal root ganglion, where a population of small diameter cells

contain high levels of these three mRNAs, and, as in the thalamus, all neurons show some basal expression of *SCG10*.

The finding, in the thalamus and in the lumbar DRG, that levels of these mRNAs are not only regulated similarly during axon regeneration but also to a more limited extent in the intact animal raises interesting questions about how their production is controlled, and supports the view that the functions of SCG10, CAP-23 and GAP-43 may be required in similar contexts. In further support of this, GAP-43 and CAP-23 have been shown to have partially overlapping and partially distinct functions (Frey et al., 2000).

FKBP12 mRNA levels showed much less variation throughout the various thalamic nuclei and did not correlate well with those of the other mRNAs, except that it too was absent in the TRN, in which respect *FKBP12* resembles *GAP-43* and, to an extent, *CAP-23*.

ii. SCG10, CAP-23, GAP-43 and FKBP12 expression in the intact cerebellum

Neurons of the cerebellar deep nuclei contained all of these mRNAs. Variation within these nuclei was observed only for *CAP-23*, where stronger expression was found in parts of the medial and lateral cerebellar nuclei. *SCG10* and *FKBP12* were expressed at relatively low levels in a uniform manner. *GAP-43* expression in these nuclei was previously shown to be very low or undetectable (Chaisuksunt et al., 2000a). Purkinje cells did not express *GAP-43*, but uniformly expressed high levels of *FKBP12*. More interesting was the distribution of *CAP-23*, which was found in some Purkinje cells in the paraflocculus and parts of the vermis, in particular lobule X (although an extensive survey of cerebellar cortex was not carried out). *SCG10* was found at moderate levels in all Purkinje cells, but stronger expression was found in some Purkinje cells, again in the paraflocculus. Curiously these did not appear to be in exactly the same place as those which contained *CAP-23*. These regions correspond to vestibulocerebellar cortex, but it is unclear what the functional significance is of this variation. As mentioned, constitutive production of growth-associated molecules may reflect the proposed role of such molecules in plasticity in the intact adult animal. However, there are few or no reports of plasticity of Purkinje cell fibres. Interestingly, Buffo et al. (2000) found that Purkinje cells in lobule X have a different morphology to those in other parts of the vermis, their axons ramifying

extensively in the granule layer to form a thick terminal network, whereas elsewhere they generally pass through this layer sending off only a few collaterals. It is not implausible that this may be connected to constitutive production of CAP-23, given this molecule's probable involvement in axonal sprouting and branching (Frey et al., 2000). If this is the case, one would also expect to find Purkinje cells with similar unusual morphology in the paraflocculus.

Such variation as was observed in expression of *GAP-43*, *SCG10* and *CAP-23* did not reveal any extensive correlation between levels of these three mRNAs in the cerebellum, although they are co-expressed in the deep nuclei and the granule cell layer.

9.4 Expression of *SCG10*, *CAP-23*, *GAP-43 and FKBP12* in the thalamus and cerebellum after implantation of a peripheral nerve graft

Following implantation of a peripheral nerve graft into the thalamus of the adult rat, neurons of the TRN regenerated axons into the graft. In this study, it was shown that following implantation of a peripheral nerve graft into the thalamus, many neurons in the TRN upregulated *SCG10*, *CAP-23*, *GAP-43* and *FKBP12* mRNAs. Similar results were found for all four molecules. Following implantation of a peripheral nerve graft into the cerebellum, neurons of the cerebellar deep nuclei regenerated axons into the grafts, in contrast to Purkinje cells and other neurons of the cerebellar cortex. This regeneration was accompanied by upregulation of *SCG10*, *CAP-23*, *GAP-43* and *FKBP12* mRNAs. Again, the results were similar for all four molecules. Three days after graft insertion, moderate upregulation was visible in neurons of the TRN or the medial cerebellar nucleus, typically in larger numbers of neurons than observed at later time points. At later time points (2-6 weeks) greater upregulation was visible but in a more restricted population.

These results confirm the status of *SCG10*, *CAP-23* and *FKBP12* as growth-associated molecules, and, combined with the observations of their upregulation during peripheral nerve regeneration (the present study; Lyons et al., 1995), firmly establish that they are consistently upregulated by neurons regenerating their axons and that upregulation of these three mRNAs, along with *GAP-43*, is a characteristic features of the regenerative cell body response to axotomy. The data presented here on *GAP-43* expression following nerve

grafting in the thalamus and cerebellum is consistent with previously published findings (Vaudano et al., 1995; Chaisuksunt et al., 2000a).

i. Retrograde labelling of neurons with axons in the grafts

Retrograde labelling from the distal graft showed that neurons in the TRN or the deep cerebellar nuclei had regenerated axons into the grafts placed in the thalamus or cerebellum respectively. The retrogradely labelled neurons, which had grown axons through the grafts, were found in the same areas of the nuclei as neurons showing increased mRNA levels of the molecules examined. In addition, analysis of adjacent sections processed for ISH and CTB immunohistochemistry allowed the identification of many neurons which were both retrogradely labelled and had upregulated one of these four molecules. While analysis of adjacent sections is not as accurate as double labelling on the same section, technical factors make this difficult if not impossible using the ISH technique employed here. None the less, a high degree of success was achieved in identifying retrogradely labelled neurons on adjacent sections, and in some cases all the retrogradely labelled neurons could be identified. It is reasonable to conclude, therefore, that the technique used to identify individual regenerating neurons in sections processed for ISH was sufficiently reliable for the requirements of this study.

It was not possible to show conclusively that those neurons which regenerated into the grafts were axotomised cells, rather than uninjured neurons which had sprouted collaterals into the grafts. However, in the thalamus, neurons which showed upregulation of *SCG10*, *CAP-23*, *GAP-43* and *FKBP12* mRNAs, and neurons which were retrogradely labelled were generally located in the part of the TRN which projects to or through the region of the thalamus containing the graft tip (i.e. rostrodorsal to it). Neurons of the TRN project to the dorsal thalamus generally in a mediocaudal direction (Ohara and Lieberman, 1985). In the cerebellum, all neurons which showed mRNA upregulation or retrograde labelling were located in the medial cerebellar nucleus, fairly near the graft and therefore likely to have been axotomised by graft implantation. Previous studies on nerve grafting in the cerebellum found regenerating neurons in the lateral and interposed, as well as the medial, cerebellar nuclei, but the position of such neurons in this study probably reflects the

consistently medial positioning of the grafts, and is consistent with their having been damaged by graft implantation.

ii. The short-term response of neurons in the TRN and medial cerebellar nucleus to axotomy

At three days after graft implantation into the thalamus or cerebellum, upregulation of *SCG10*, *CAP-23*, *GAP-43* and *FKBP12* was seen in large numbers of neurons in the TRN or medial cerebellar nucleus respectively. At the later time points of 2-6 weeks, the numbers of neurons showing strong signals had declined. Analysis of retrograde labelling and ISH at the later time points suggested that continued upregulation was confined to neurons which were retrogradely labelled and had therefore regenerated axons into the grafts; the numbers of neurons showing mRNA upregulation and retrograde labelling were similar, and retrograde labelling was found almost exclusively in neurons which also had upregulated one of the mRNAs. The higher number of neurons which had upregulated these molecules at the earlier time point may be interpreted as indicating that neurons in the TRN and cerebellar deep nuclei transiently upregulate *SCG10*, *CAP-23*, *GAP-43* and *FKBP12* after axotomy, and that this expression is prolonged only in those neurons which successfully regenerate axons into the peripheral nerve graft.

iii. Expression of SCG10, CAP-23, GAP-43 and FKBP12 in neurons which did not regenerate axons

Following the implantation of tibial nerve segments into the thalamus, most neurons in the dorsal thalamus did not upregulate any of the growth-associated molecules examined or regenerate into the grafts. A small number of these neurons were observed to upregulate *SCG10* and *GAP-43* two weeks after grafting, and in animals in which retrograde labelling was used, one animal showed retrograde labelling in a few dorsal thalamic neurons. All these were found close to the graft tip, and it is highly likely therefore that those few dorsal thalamic neurons showing increased levels of *SCG10* and *GAP-43* were regenerating axons into the graft. This is consistent with previous findings that small numbers of these neurons may sometimes regenerate into grafts if axotomised very close to their cell bodies (Morrow et al., 1993). In addition, previous studies have also indicated that some dorsal thalamic

neurons very close to a stab wound in the thalamus transiently upregulate *c-jun* and *GAP-43* (Vaudano et al., 1995; Vaudano et al., 1998), particularly in nuclei in the caudal and ventral parts of the dorsal thalamus. None of the animals examined at three days after graft insertion into the thalamus revealed any upregulation of the molecules examined in the dorsal thalamus.

Following graft implantation into the cerebellum, no upregulation of any of these molecules was observed in cerebellar neurons other than in the deep nuclei, with the exception of a few Purkinje cells close to the graft at three days post-surgery, which had upregulated *CAP-23*. Zagrebelsky et al. (1998) previously reported upregulation of CAP-23 protein in Purkinje cells close to a knife lesion of cerebellar white matter.

It appears that unless neurons of the dorsal thalamus and Purkinje cells are very proximally axotomised, not even a transient increase in upregulation of these growth-associated molecules occurs. With this exception, therefore, it can be stated that in the regions of the CNS examined in the present study, only regeneration-competent neurons respond to axotomy by upregulating growth-associated molecules. This supports the idea that it is intrinsic properties of neurons which determine the strength of the cell body response, the associated changes in gene expression, and their competence to regenerate axons. Presumably, since prolonged expression of the genes studied was limited to those regeneration-competent neurons which could be retrogradely labelled from the grafts, regenerating axons must detect signals from the grafts to maintain the expression of neuronal growth-associated genes.

iv. The long-term nature of the increased expression of SCG10, CAP-23, GAP-43 and FKBP12 in neurons regenerating axons into nerve grafts

All four molecules studied were upregulated in TRN neurons and deep cerebellar neurons for at least 6 weeks after graft insertion. Previous studies have shown that *GAP-43* expression continued at least 30 days, *c-jun* expression lasted at least 7-9 weeks and *L1* expression continued at least 10 weeks after grafting in the thalamus. The retrograde labelling data indicates that axons of nearly all neurons with upregulated gene expression had reached the end of the graft by 6 weeks, the longest time point examined. The

prolonged expression of genes for growth-associated molecules in regenerating neurons of the TRN and deep cerebellar nuclei most likely reflects the fact that the graft does not contain any suitable targets for innervation and so the neuronal program of regeneration continues. In terms of signals the neurons may receive, the nerve graft clearly supplies sufficient signals to maintain the axon-growth program, but not appropriate target-derived signals which might normally lead to its termination as in successful peripheral nerve regeneration. This situation is analogous to that seen in the peripheral nerve following a cut and ligation, when axons are prevented from reaching their targets. However, whereas axotomy alone appears to be sufficient to maintain the expression of growth-associated genes in peripheral neurons, axonal regeneration into grafts is necessary for prolonged expression by intrinsic CNS neurons.

v. Comparisons of mRNA levels

SCG10 mRNA showed by far the greatest increase in the TRN following grafting, relative both to its basal level and to the levels found in other neurons, such as dorsal thalamic neurons and cerebral cortical neurons. *SCG10* was upregulated to the degree that it was expressed significantly more strongly in regenerating neurons of the TRN than in any other neurons in the forebrain, and the increase was more marked even than that of *GAP-43*. *CAP-23* and *FKBP12* mRNAs showed a more modest upregulation, and were expressed at a level comparable to that seen in neurons of the adjacent dorsal thalamus.

While comparison cannot easily be made of absolute levels of different mRNAs identified with probes of differing length and base composition, it is possible to compare signals from the same probe on different tissues. Following implantation of a nerve graft in the cerebellum, *SCG10* levels in regenerating deep cerebellar neurons were comparable to those seen in regenerating TRN neurons, but were upregulated from a higher basal level. *CAP-23* expression was at least as high as that seen in the TRN, but again was increased from a higher basal level so the relative increase was smaller. *FKBP12* levels were similar in regenerating TRN and deep cerebellar neurons.

9.5 Neuronal cell bodies found in peripheral nerve grafts implanted in the cerebellum

An interesting finding of this study was that many of the nerve grafts implanted into the cerebellum contained ectopically located neuronal cell bodies. These were observed on sections processed for ISH for SCG10, CAP-23 and GAP-43 and those processed for CTB immunohistochemistry (i.e. for retrograde labelling).

Several features indicated that these cells were indeed neurons. They were relatively large, being 15-30 μm in diameter. They also contained high levels of *SCG10*, *CAP-23* and *GAP-43* mRNAs. All are neuronal molecules, and while GAP-43 is also known to be made by Schwann cells in certain circumstances (Woolf et al., 1992b) and there is evidence from the present study that they may also make CAP-23, SCG10 is thought to be neuron-specific in the adult animal (Stein et al., 1988b; Ozon et al., 1999). In animals where retrograde labelling was employed, these cells were labelled with CTB injected into the distal graft, indicating not only that they are neurons, but also that they had extended an axon into the distal graft. Neuronal-type morphology was also apparent when these cells were examined at high magnification.

Five out of 19, or 26% of living grafts implanted in the cerebellum contained such ectopic neurons, compared to none out of 18 grafts implanted in the thalamus and during previous experiments in this laboratory only one such ectopic neuron has been observed in several hundred grafts implanted in the thalamus. Neuronal cell bodies are sometimes found in peripheral nerve endoneurium, and these are regarded as displaced primary sensory or sympathetic ganglion neurons. However, these are rare, and the high frequency of their presence in cerebellar grafts combined with the differential occurrence of this phenomenon between thalamic and cerebellar implants suggests that there is some property of the cerebellum which causes this to happen. It is possible that unknown factors in the cerebellum stimulate precursor cells in the grafts to differentiate into neurons. Alternatively, it has been shown that ependymal cells which line the fourth ventricle are neural stem cells (Johansson et al., 1999), so it is possible that these were induced to migrate into the graft and differentiate into neurons. These cells normally proliferate after

injury and generate glial cells, and while the observations in this study require confirmation, it would be of note if factors in injured peripheral nerve induce neurogenesis.

9.6 Neurons that fail to regenerate axons into peripheral nerve grafts

i. The response of Purkinje cells to axotomy

Following implantation of a peripheral nerve graft in the cerebellum, small numbers of Purkinje cells near the graft upregulated *CAP-23* in one of three animals 3 days after grafting. CAP-23 upregulation in axotomised Purkinje cells has previously been reported (Zagrebelsky et al., 1998), and it is now clear that some Purkinje cells respond to a very proximal axotomy by upregulating a limited selection of growth-associated molecules. As well as upregulating CAP-23, increases in the expression of *c-jun* (Chaisuksunt et al., 2000a), NADPH diaphorase (Zagrebelsky et al., 1998) and p75 (Vaudano et al., 1998) have all been shown. However, Purkinje cell axons have never been observed to regenerate axons into grafts. Expression of these molecules alone is clearly not enough to promote regeneration. In addition, expression of GAP-43 in Purkinje cells in transgenic mice failed to promote regeneration into Schwann cell implants (Buffo et al., 1997) or peripheral nerve grafts (Y. Zhang, personal communication). In these animals, some Purkinje cells would be expected to express GAP-43, CAP-23, SCG10 and FKBP12, and a few proximally injured cells may also have expressed c-Jun as well as increased levels of CAP-23. It therefore seems that in this case expression of a variety of growth-associated molecules is insufficient to promote regeneration. This may be because the response of Purkinje cells to injury, which is minimal in magnitude and comparatively incomplete in terms of upregulation (as opposed to constitutive expression) of known growth-related genes, does not result in expression of other molecules which may also be required for axon growth.

Another possibility is that the failure of Purkinje cells and some other classes of neuron to regenerate into a graft may be due to their expression of receptors for specific pathfinding molecules which prevent them from entering peripheral nerve tissue. A possibility would be receptors for components of PNS myelin. However, predegenerate peripheral nerve grafts, in which myelin has largely been cleared, also fail to support ingrowth from Purkinje cells, as do Schwann cell implants, which do not contain myelin until they begin to ensheath

ingrowing axons (Buffo et al., 1997). Nonetheless, several potent inhibitory molecules, such as tenascin C (Martini et al., 1990), chondroitin sulphate proteoglycans (Zuo et al., 1998) and NG2 (Schneider et al., 2001), are present in peripheral nerve tissue and may be able to limit growth by Purkinje cell axons.

ii. Expression of growth-associated molecules in uninjured neurons

It is apparent that the ability to upregulate growth-associated molecules is probably in most cases a key factor in determining how able neurons are to regenerate axons into a nerve graft. However, it is interesting that little correlation can be found between the basal levels of expression of the molecules examined and the ability to regenerate axons into a peripheral nerve graft. Constitutive production of growth-associated molecules does not confer the ability to regenerate axons on most neurons. Neurons in the dorsal thalamus, cerebral cortex and hippocampus all express growth-associated genes constitutively at a moderate or high level but are poor at regeneration into grafts. Neurons of the TRN have the lowest basal levels of expression of *GAP-43* and *CAP-23*, but are the most able to regenerate their axons. In fact hippocampal neurons appear to have high levels of almost all growth-associated molecules so far examined but have very rarely been shown to regenerate axons into peripheral nerve grafts. In these regions there appears to be an inverse correlation between expression of these genes and regenerative competence, but this does not extend to the cerebellum. Here, the regeneration-competent deep cerebellar neurons all express *GAP-43* and *CAP-23* at similar levels to that seen in the dorsal thalamus, whereas most Purkinje cells express neither *GAP-43* or *CAP-23*. Elsewhere, neurons of the SNpc also express *GAP-43* constitutively, and regenerate axons into grafts without apparently upregulating this molecule (Woolhead et al., 1998), although they do show some upregulation of *c-jun* (Chaisuksunt, 1999). Similarly, no correlation can be found between constitutive production of the growth-associated molecules SCG10, FKBP12, L1 or CHL1 and the ability to regenerate axons into nerve grafts (Zhang et al., 1995; Chaisuksunt et al., 2000a; Chaisuksunt et al., 2000b; the present study).

iii. Growth-associated molecules, regeneration and plasticity

The growth-associated molecules examined here, in addition to participating in regeneration after axotomy, may have additional roles in plasticity. It is, therefore, interesting to consider the relationship between axon regeneration and plasticity in the adult CNS. Plasticity takes several forms, varying from changes in synaptic efficacy (reviewed by Paulsen and Sejnowski, 2000; Hansel et al., 2001; Winder and Schramm, 2001) through the generation or deletion of synapses (Klintsova and Greenough, 1999), to collateral sprouting over long distances (Darian-Smith and Gilbert, 1994). The involvement of axon growth in this last example, and possibly in other forms of plasticity, suggest that these processes may utilise essentially the same cellular mechanisms as axonal regeneration. The distribution of neurons with significant levels of GAP-43 within the adult mammalian brain led to the suggestion that it was involved in plasticity processes in the adult (Neve et al., 1988; Benowitz and Routtenberg, 1997). For example, in the neocortex of primates GAP-43 is much more strongly expressed in limbic and associational areas than in primary motor and sensory areas. It is also strongly expressed in the rat hippocampus, an area where various forms of synaptic plasticity, such as long-term potentiation, have been extensively studied. Many pathways to, from and within the hippocampus also show a marked capability for collateral sprouting following lesion-induced denervation (reviewed by Frotscher et al., 1997), suggesting they may be capable of morphological plasticity. Sprouting of hippocampal mossy fibres (dentate granule cell axons) follows kainic acid-induced seizures, and this is accompanied by GAP-43 upregulation in the granule cells (Bendotti et al., 1994; Cantallops and Routtenberg, 1996). This association with areas of plasticity in the adult and the continued production of GAP-43 throughout the period of synaptogenesis in development, after growth cones have contacted targets, suggests it may be important for synapse formation.

CAP-23 and *SCG10* showed a strikingly similar distribution to *GAP-43* in many parts of the rat brain (although with some important differences). The co-expression of these growth-associated genes implies that many of the molecules required for axon growth are required for plasticity-associated structural changes in the adult. There is evidence that SCG10 is also involved in plasticity-type changes; following unilateral ablation of the cortex, some corticostriatal neurons on the contralateral side sprout collaterals and form

replacement synapses. Upregulation of SCG10 in cortical layer V pyramidal neurons coincides with this process (McNeill et al., 1999). Mild upregulation of *SCG10* was also seen in the hippocampus following deafferentation by lesioning the entorhinal cortex, which results in compensatory sprouting by hippocampal neurons (Zarow and Finch, 1995). The expression pattern of CAP-23, along with the properties which it shares with GAP-43 (such as its effects on cell and nerve terminal morphology) imply it probably also has a role in plasticity in the adult.

However, while cortical output neurons and many neurons in the hippocampus show a high capability for structural changes, particularly in response to lesions which denervate their target regions, their cell body response to axotomy is muted and their capacity for axon regeneration into nerve grafts is very low. If these neurons are capable of axon elongation in some contexts, why are they not capable of it following axotomy, even if presented with a peripheral nerve graft? As discussed with reference to Purkinje cells, one reason may be that their growth-cones carry receptors for inhibitory molecules which prevent them entering peripheral nerve tissue. This aspect of the problem is highlighted by corticospinal neurons, which fail to regenerate into peripheral nerve grafts even if encouraged to grow significant distances by neurotrophic factors (Blits et al., 2000). While it has not been reported whether these treatments also result in upregulation of growth-associated molecules in corticospinal neurons, these neurons generally fail to respond to axotomy in the spinal cord and it seems likely that this is an equally important reason for their failure to regenerate. It is possible that in some neurons, control mechanisms over axonal growth, and growth-associated molecules, are geared towards plasticity-driven growth in response to synaptic activity or deafferentation, rather than towards regeneration in response to axotomy. If this is the case it may be that structural plasticity precludes axon regeneration. For example, some growth-cones may be programmed to navigate in grey matter and avoid any white matter and peripheral nervous tissue.

Certainly the relationship between regeneration and plasticity appears to be a close one. This is particularly clear in lower organisms such as *Aplysia californica*, where there is evidence that synaptic plasticity and regeneration are initiated by the same retrograde signals from the axon (Ambron et al., 1995; Gunstream et al., 1995). In *Aplysia* and other gastropods, the processes involved in regeneration of the nervous system have been

described as extreme examples of those seen during learning (Moffett, 1995). There is some evidence that the mechanisms regulating plasticity and regeneration may overlap in mammals; molecules in the environment which have been identified as promoting or inhibiting regeneration, such as neurotrophins and Nogo, may have functions in the adult which are involved in the regulation of plasticity. There is now considerable evidence that neurotrophins act to regulate activity-dependent synaptic plasticity, including long-term potentiation in the hippocampus (Thoenen, 2000; Lu and Gottschalk, 2000; Schinder and Poo, 2000), and following a unilateral lesion of the pyramidal tract, neutralisation of Nogo with the IN-1 antibody resulted in increased compensatory collateral sprouting of corticofugal fibres across the midline, resulting in contralateral innervation of the red nucleus and pons and regeneration of the injured axons into the ipsilateral spinal cord. Colchicine treatment, which blocks axonal transport, or injection of IN-1 antibody into the cerebellum caused upregulation of c-Jun and other molecules, and the effect of the latter treatment was to enable extensive sprouting of intact axons.

On the other hand, it should also be noted that there are neurons with axons capable of synaptic plasticity, structural plasticity and regeneration into peripheral nerve grafts or Schwann cell implants, namely those of the inferior olive, whose axons terminate as climbing fibres in the cerebellum (Bravin et al., 1997; Anderson et al., 1998; Strata and Rossi, 1998; Hansel et al., 2001). This indicates that a capability for various forms of plasticity is not necessarily mutually exclusive with the ability to respond to axotomy and regenerate the axon.

9.7 Functions of SCG10, CAP-23 and GAP-43 in the growth cone

The data presented here provide good but circumstantial evidence that CAP-23 and SCG10 are, like GAP-43, important for axon regeneration. CAP-23 is functionally similar to GAP-43, and these molecules have been implicated in the regulation of actin dynamics in the growth cone, while SCG10 is involved in microtubule dynamics. Both actin and microtubule dynamics are central to growth-cone function.

i. Cytoskeletal dynamics in the growth-cone

Observations of growth cones in vitro generally reveal a fan-like structure at the axon terminal consisting of a central domain and a peripheral, lamellar, domain (Bridgman et al., 1986). The cytoskeleton in the central domain consists largely of microtubules, while that of the peripheral domain is composed of radial actin filaments which are aligned with filopodia, and which are also cross-linked by actin networks (Lewis and Bridgman, 1992; Lin and Forscher, 1993). These actin components move in a retrograde direction, while polymerisation takes place at the leading edges. This retrograde motion is thought to drive growth-cone movement via controlled interaction of the actin cytoskeleton with the substrate (reviewed by Lin et al., 1994; Suter and Forscher, 2000).

The actin-based filopodia are highly motile themselves and scan the extracellular space for guidance cues (reviewed by Kater and Rehder, 1995). Filopodia are highly sensitive to such cues, as contact of a single filopodium with guidance cues is sufficient to change growth-cone behaviour (O'Connor et al., 1990). Microtubules are also involved in growth-cone remodelling during axon growth and pathfinding. For example, studies of growth-cone behaviour show that following filopodial interaction with targets, or with other axons and growth-cones, microtubule bundling and reorientation occurs. This is also seen during growth-cone turning away from an unfavourable substrate (Tanaka and Kirschner, 1995).

ii. Functions of GAP-43 and CAP-23

There is considerable evidence that GAP-43 functions by influencing actin dynamics in the growth-cone, although several mechanisms have been proposed by which it might act. CAP-23 appears to have a related function and both molecules may participate in regulation of the actin cytoskeleton in the peripheral domain of the growth cone. Two of the proposed mechanisms by which GAP-43, and by implication CAP-23, may act, clearly involve modulation of actin-regulatory molecules: regulation of the local availability of the intermediate messenger PIP_2 (Laux et al., 2000) and action via the rho-family GTPases (Aarts et al., 1998).

GAP-43 knockout mice develop a grossly normal nervous system with defects in axon routing (Strittmatter et al., 1995), but there has been no published report of neuroanatomical investigations in the CNS of CAP-23 knockouts. The phenotype of the GAP-43 and CAP-23 knockout mice (i.e. grossly normal development of the nervous system) does, however, suggest that these molecules are not essential for axon elongation but are important for pathfinding, particularly at crucial decision points. Such activity may be connected with the regulation of growth-cone morphology. GAP-43 knockout mice exhibit serious axonal pathfinding errors during development of the CNS, exemplified by misrouting of retinal ganglion cell axons at the optic chiasm, and defects in the innervation of terminal fields in the lateral geniculate nucleus (LGN) (Strittmatter et al., 1995; Sretavan and Kruger, 1998; Zhu and Julien, 1999). This suggests that growth-cone behaviour at these points reflects a particular requirement for GAP-43. Interestingly, the morphology of retinal ganglion cell growth-cones has been examined at the various stages of the journey from retina to tectum, and was found to vary considerably (Bovolenta and Mason, 1987; Godement et al., 1990). In the optic nerve and optic tract, growth-cones were compact and had few filopodia. However, the morphology of growth cones was different in both areas where pathfinding abnormalities have been observed in GAP-43 knockout mice. In the optic chiasm growth-cones increased in complexity, becoming wider, developing more filopodia and branching more, and as they approach the LGN the growth-cones and trailing axons developed expansions which also gave rise to filopodia. These morphological changes would be consistent with there being a need for the growth cone to sample the environment better for guidance cues and it seems plausible that GAP-43 may be required for this process; GAP-43 has profound effects on morphology when expressed in cultured cells and GAP-43-depleted neurons grown in vitro are deficient in induced spreading and branching (Aigner and Caroni, 1995). However, no study has been made of retinal ganglion cell growth-cone morphology in GAP-43 knockout mice. It is also possible that GAP-43 and CAP-23 may be involved in transducing signals in the filopodia.

iii. Regulation of GAP-43 and CAP-23 activity

If GAP-43 and CAP-23 do lie on a pathway between extracellular guidance cues and the cytoskeleton it is reasonable to suppose that their activity is regulated by signalling mechanisms activated by receptors. It is not fully established how this regulation occurs,

but in the case of growth-cone responses to NCAM, Meiri et al. (1998) have shown, using cultured neurons from GAP-43 knockout mice, that GAP-43 is required for NCAM promotion of neurite growth. They also elucidated a pathway from the extracellular receptor to GAP-43 phosphorylation. In their model, NCAM activates the FGF receptor, leading to arachidonic acid production, which in turn stimulates phosphorylation of GAP-43 by PKC. Both GAP-43 and CAP-23 are phosphorylated by protein kinase C but in most cases the pathways from receptors on the plasma membrane to these molecules have yet to be unravelled.

iv. Functions of SCG10

SCG10 may have a similar type of role to GAP-43 and CAP-23 in the central zone of the growth cone. Microtubules alternate between periods of steady growth and rapid depolymerisation, a process known as dynamic instability, first described by Mitchison and Kirschner (1984). The dynamic instability of microtubules has been observed in growth-cones, where single microtubules may transiently invade lamellipodia in the peripheral domain (Tanaka and Kirschner, 1995) and even filopodia (Gordon-Weeks, 1991; DiTella et al., 1994). Its importance for proper growth-cone function has been demonstrated using microtubule-stabilising pharmacological agents which inhibit growth-cone motility and turning behaviour. Thus, taxol, which inhibits microtubule depolymerisation, prevents axon growth in vitro when used at micromolar concentrations applied to the growth cone (Letourneau and Ressler, 1984; Bamburg et al., 1986). Taxol also prevented microtubule invasion of the peripheral domain (Tanaka et al., 1995). Lower (nanomolar) concentrations of taxol and vinblastine, which also inhibits dynamic instability, have been found to allow growth but to prevent turning of growth-cones in response to repulsive cues. Taxol prevented growth-cones moving on laminin from turning at a boundary with the less permissive tenascin-C (Williamson et al., 1996). Similarly, taxol and vinblastine prevented growth cones extending on fibronectin turning away from a boundary with CSPG (Challacombe et al., 1997). Typically growth cones stalled at the substrate boundary for as long as the drugs were applied. Clearly, microtubule instability is an important part of growth cone cytoskeletal dynamics, but regulation of microtubule dynamics is less well understood than that of actin dynamics. While MAPs, which are thought to promote microtubule assembly, have been well-characterised (Tucker, 1990), few molecules have

been identified which regulate microtubule disassembly. It appears that SCG10 and other stathmin family members fulfil this role.

v. Regulation of SCG10 activity

Actin filaments and microtubules overlap at the transitional zone between the central and peripheral zones of the growth cone. It has been shown that interference with actin dynamics also prevents rearrangements of microtubules (Lin and Forscher, 1993), suggesting that these respond to morphological changes which first occur in the peripheral zone. SCG10 is itself regulated by several kinases so it may be that these kinases transmit signals originating in the peripheral zone. Of particular interest is the finding that SCG10 has a phosphorylation site for the cAMP-dependent protein kinase PKA, which is likely to be phosphorylated in vivo (Antonsson et al., 1998). Phosphorylation of stathmin family proteins inhibits their microtubule depolymerising activity. Several experiments have shown that cAMP levels and PKA activity in the growth-cone can profoundly affect its behaviour, and in particular can determine whether the growth-cone finds certain extracellular molecules to be attractive or inhibitory guidance cues. Thus cultured embryonic *Xenopus* spinal neurons, which normally grow towards a source of BDNF or netrin-1 will grow away from them if a specific inhibitor of PKA or a competitive analogue of cAMP is added to the medium (Ming et al., 1997; Song et al., 1997). Conversely, these neurons are repelled by a gradient of a soluble fragment of the inhibitory myelin component MAG, but will grow towards it in the presence of another cAMP analogue which activates PKA (Song et al., 1998). Similarly, neurite growth of cultured postnatal cerebellar neurons and DRG neurons from older animals is inhibited by MAG. This inhibition was overcome by addition of a PKA-activating analogue of cAMP, and by prior exposure to neurotrophins, again by a PKA dependent mechanism (Cai et al., 1999). Lastly, rat DRG neurons switch their response to MAG at around P4, from finding it a growth-promoting substrate to an inhibitory one. This switch was shown to be due to a drop in endogenous cAMP levels in these neurons (Cai et al., 2001).

While these effects of PKA have not yet been localised within the growth cone, and they may be confined to the peripheral domain or filopodia only, it is also possible that regulation of SCG10 by PKA plays a role in the modulation of growth-cone responses.

PKA phosphorylation of SCG10 may be expected to lead to greater microtubule extension and microtubule invasion of the peripheral domain.

vi. SCG10, CAP-23, GAP-43 and calcium signalling

Lastly, GAP-43, CAP-23 and SCG10 may all potentially respond to Ca^{2+}-mediated signalling in the growth-cone, potentially allowing these three molecules to respond to the same signals. Ca^{2+} levels in the growth cone has been implicated in various sorts of growth-cone behaviours including turning responses to repellent cues and growth-cone collapse, and can affect the speed of neurite growth (see Song and Poo, 1999). Both GAP-43 and CAP-23 interact with calmodulin in a Ca^{2+}-dependent manner, while a possible pathway to SCG10 is via adenylate cyclase, which is stimulated by Ca^{2+}/CaM to produce cAMP, which would lead to PKA activation.

vii. Summary

GAP-43 and CAP-23 are likely to be involved in the regulation of the actin cytoskeleton in the growth cone in response to signals generated by receptors for guidance cues, while SCG10 may be involved in the regulation of microtubule dynamics in response to these signals. GAP-43 and CAP-23 seem to be capable of promoting axon growth, particularly in conjunction with each other (Caroni et al. 1997; Bomze et al., 2001). Overexpression of SCG10 in PC12 cells also led to faster neurite outgrowth (Riederer et al., 1997). If these molecules are involved in transmitting information from guidance signals to the cytoskeleton, these effects may be because their presence in the growth cone renders it more receptive to growth-promoting signals.

9.8 Functions of FKBP12

FKBP12 has been proposed to play a role in axon regeneration and to mediate neurotrophic actions of the immunosuppressant drug FK506 and its analogues. These drugs have been shown to accelerate peripheral nerve regeneration in vivo (Gold et al., 1995; Gold et al., 1997; Gold et al., 1998b; Jost et al., 2000; Lee et al., 2000), and to promote neurite outgrowth in vitro (Lyons et al., 1994; Steiner et al., 1997b), although the

mechanisms of these actions are not fully understood. While a considerable amount of data exists concerning these effects there exists some controversy in the literature, particularly concerning the in vitro effects of these drugs. The neurotrophic effects of both FK506 and several of the analagous molecules have been disputed (Harper et al., 1999; Becker et al., 2000; Parker et al., 2000). Gold et al. (1999) have reported that the neurotrophic actions of FK506 are not mediated in vivo by FKBP12 but rather via a different FK-binding protein, FKBP52, ultimately acting on the glucocorticoid receptor; however, in vivo experiments on this point are currently difficult because of the non-viability of the FKBP12 knockout mice (Shou et al., 1998). On the other hand, FKBP12 mRNA was shown to be upregulated by neurons supplying the sciatic and facial nerves following an injury, supporting the notion that FKBP12 plays a role in axon regeneration (Lyons et al., 1995).

The findings presented here support the view that FKBP12 has a role in axon growth, although it is far from clear what that role might be. It may or may not be connected with the effects of immunophilin ligands on neurite outgrowth in culture and on axonal regeneration in vivo. For example, these drugs may act instead on cells in the damaged nerve itself; FK506 accelerates Wallerian degeneration in vivo and promotes Schwann cell proliferation in vitro (Fansa et al., 1999). More than one mechanism may be at work and indeed some evidence for this was found in the first in vitro study to demonstrate a neurotrophic activity of FK506 (Lyons et al., 1994). Rapamycin and FK506 acted additively to promote neurite outgrowth in PC12 cells, but in cultured DRG neurons rapamycin blocked the neurotrophic effect of FK506.

i. FKBP12 as a component of multimeric receptors

What is known of the function of FKBP12 suggests that among other things it may be involved in calcium signalling. FKBP12 interacts with the intracellular calcium channels, the ryanodine receptor (RyR) and the inositol-1,4,5-triphosphate receptor (IP_3R). FKBP12 forms an integral part of the RyR complex (Collins, 1991), and it is dissociated from the complex by FK506. Without FKBP12 the calcium flux properties of the channel are altered such that it is less effective at both retaining calcium when closed and releasing it when open (Jayaraman et al., 1992; Brillantes et al., 1994). A similar situation is found for IP_3R, which is found on the endoplasmic reticulum and the plasma membrane. FKBP12 is again

associated with each subunit of this receptor, is dissociated by FK506 or rapamycin and improves the calcium flux properties of the receptor (Cameron et al., 1995).

FKBP12 also interacts with the transmembrane receptor for TGFβ family cytokines, and may function to inhibit spontaneous self-activation of this receptor (Wang et al., 1996; Chen et al., 1997). The type I subunit of TGFβ receptor (TβR-I) is responsible for signalling to downstream molecules, and is activated via phosphorylation by the type II subunit (TβR-II) when the two components are brought together by the ligand TGFβ. FKBP12 inhibits signalling by the TGFβ receptor at low concentrations of ligand and may prevent spontaneous activation of the receptor if the two subunits associate in the absence of ligand, and therefore serve to improve signalling reliability in this receptor.

As FKBP12 appears to improve receptor efficiency in several cases, it is possible that the interaction of FKBP12 with receptors may have a role to play in the process of growth-cone navigation. Clearly, many receptors for extracellular signals are present on the growth-cone. Alternatively, FKBP12 may be important for the correct functioning of proteins involved in intracellular signalling pathways which are used by the growth cone during pathfinding or in signal transduction processes in the cell body. For example, the yeast TOR protein, with which FKBP12 interacts when complexed with rapamycin, has effects on microtubule dynamics (Choi et al., 2000), although as with calcineurin this interaction most likely depends on the presence of the immunosuppressant drug.

ii. FKBP12 as a calcineurin-docking protein

In addition, when complexed with FK506, FKBP12 associates with and inhibits calcineurin, the Ca^{2+}- dependent phosphatase, which is known to dephosphorylate GAP-43 (Liu and Storm, 1989). FKBP12 has also been shown to interact with calcineurin as part of a ternary complex of these two molecules and IP_3R, leading to the suggestion that FKBP12 may function to anchor calcineurin to this receptor (Cameron et al., 1997). A calcineurin-docking function was also proposed for FKBP12 when complexed with the TGFβ receptor (Wang et al., 1996). Such a function is made more plausible by the high degree of colocalisation between FKBP12 and calcineurin (Dawson et al., 1994).

iii. FBKP12 and neurotrophin signalling

Promotion of neurite outgrowth by FK506 and rapamycin in PC12 cells and in cultured neurons was dependent on the presence of submaximal concentrations of NGF i.e. a level of NGF below that which produces the maximum amount of neurite outgrowth. This suggests that in PC12 cells FK506 and rapamycin act by increasing the sensitivity of these cells to NGF (Lyons et al., 1994). This effect might also be responsible for the increase in speed of regeneration observed in vivo, as Schwann cells in the distal stump of an injured nerve upregulate NGF (Heumann et al., 1987; Funakoshi et al., 1993). If neuronal FKBP12 does mediate the effects of FK506 and rapamycin some interaction of FKBP12 with signalling pathways activated by the neurotrophin receptors might be expected. However, this has not been demonstrated.

iv. Rotamase activity of FKBP12

FKBP12 appears to interact with a wide range of molecules. When first identified it was found to have peptidyl-prolyl isomerase or rotamase activity but it is not clear to what degree this activity is important to its cellular function. Rotamase activity is known to promote correct protein folding (reviewed by Schiene and Fischer, 2000) and may assist the facilitation of conformational changes in other proteins (Ng and Weis, 1998). Given the stable association of FKBP12 with RyR and IP_3R, it may stabilise these proteins in the correct conformation or facilitate conformational changes upon opening or closing of the receptor ion channel. However, mutated forms of FKBP12 which lack rotamase activity were shown to function as a component of RyR (Timerman et al., 1995) without affecting the calcium-flux properties of this receptor. While no specific role for the rotamase activity of FKBP12 has yet been identified, it has been demonstrated that rotamase activity can be physiologically important; the rotamase action of cyclophilin was shown to be important for collagen assembly (Steinmann et al., 1991; Compton et al., 1992; Bachinger et al., 1993) and folding of transferrin (Lodish and Kong, 1991).

Inhibition of the rotamase activity of FKBP12 does not appear to be important for the actions of the immunophilin ligands in immunosuppression. Several analogues of FK506 have been developed which bind FKBP12 and inhibit its rotamase activity but lack

immunosuppressive action (Bierer et al., 1990; Dumont et al., 1992; Ocain et al., 1993). Nor does it appear to be important in the promotion of axon growth, as no correlation could be found between inhibition of rotamase activity and neurite outgrowth assays across a wide range of molecules analogous to FK506 (Hamilton and Steiner, 1998). However, it is the only established enzymatic activity of FKBP12 and so it may be expected to be important in a physiological context.

v. FKBP12 knockout mice

FKBP12 knockout mice have been generated (Shou et al., 1998). Most of these animals die shortly after birth, because of cardiac dysfunction. The nervous system of these animals was not studied in detail, so it is not known whether FKBP12 deficiency results in any abnormalities in nervous sytem development, such as the pathfinding errors which were found in GAP-43 knockout mice (Strittmatter et al., 1995). The only study to examine neurite outgrowth by FKBP12 deficient neurons was that of Gold et al. (1999), in the context of the effect of FK506. They compared cultured embryonic hippocampal neurons from FKBP12-knockout and wild-type mice. No difference was observed with respect to speed of axon growth.

vi. Summary

The function of FKBP12 in axon regeneration is likely to be largely by interaction with other molecules to improve their functional properties, to facilitate the formation of protein complexes, or to facilitate conformational changes. The upregulation of FKBP12 by neurons regenerating their axons indicates some requirement for such activity and it would be interesting to know which molecules FKBP12 interact with during axonal regeneration.

9.9 Overexpression of GAP-43 in transgenic mice

Experiments were conducted to determine if neuronal GAP-43 expression in transgenic mice could to any degree promote regeneration of injured central branches of axons of DRG neurons, and thus mimic the cell body response to axotomy.

i. Lesion site morphology after dorsal column injuries

It is worth briefly commenting on the gross morphology of the lesions made in the dorsal columns of wt3 mice, as this was a little different to that previously described in the rat (e.g. Neumann and Woolf, 1999). The lesion sites examined in the present study showed a lesser tendency towards cyst formation. This is commonly the case in studies of mouse spinal cord injury (Ma et al., 2001), and may reflect less secondary cell death in the spinal cord, or an increase in fibrosis at the injury site. The tissue around the lesion showed an apparent tendency to expand, with the result that the dorsal part of the cord protruded at the lesion site, and the dorsal columns themselves appeared to become displaced as they approached the lesion. It might be fruitful to examine the alignment of glial processes caudal to the lesion to determine if this also changes in a similar fashion. Little comment has been made on this phenonemon previously, but it has implications for attempts to promote regeneration of dorsal column axons in laceration models (at least in mice), as the axon terminals do not seem to be well-placed to grow across the lesion after the changes described.

ii. Thoracic dorsal column injuries

Following thoracic dorsal column injury, GAP-43 expression in the injured axons did not appear to promote regeneration into or across the lesion, as might have been expected, since conditioning injuries which upregulate GAP-43 cause axons to grow into the lesion site (Bavetta et al., 1999; Neumann and Woolf, 1999). Instead, a noticeable increase in retraction of severed axons from the lesion was observed. Whereas dorsal column axons of non-transgenic animals generally approached the lesion itself and the adjacent dorsal surface of the spinal cord, but showed little growth into the lesion or into the scar tissue capping the lesion, in transgenic animals axons terminated a much greater distance caudal to the lesion and, probably as a consequence of this, were not seen to extend into the superficial parts of the dorsal white matter. Given the well-established growth-promoting properties of GAP-43 this result is somewhat surprising. GAP-43 expression has been shown to have significant positive morphological effects, causing the formation of neurites in non-neuronal cells in vitro and axonal sprouting in vivo (Zuber et al., 1989; Widmer and Caroni, 1993; Nielander et al., 1993; Verhaagen et al., 1994; Aigner et al., 1995). Clearly,

however, following thoracic dorsal column injury, GAP-43 expression alone does not mimic the cell body response, and instead of promoting regeneration, it has the opposite tendency. The role of GAP-43 in the growth-cone is still not well understood, but it interacts with signalling mechanisms in a variety of ways. Perhaps in this context one might speculate that increased amounts of GAP-43 in the growth-cones of these axons renders them more sensitive to inhibitory signals, such as those found in CNS myelin. GAP-43 function in growth-cones has been tested in vitro in the context of promotion of growth by NCAM (Meiri et al., 1998), but there are no reports of its effects on growth-cone responses to inhibitory molecules. An increase in growth-cone motility might result in increased retraction if only inhibitory molecules are present in the surrounding environment.

iii Lumbar dorsal column injuries

However, such explanations are difficult to reconcile with the results seen following dorsal column lesions in the lumbar spinal cord. In this model, GAP-43 overexpression did not result in increased retraction of dorsal column axons. Rather, a tendency towards increased axon growth was observed, albeit inconsistently. Dorsal column axons of non-transgenic animals showed a consistent pattern of labelling, similar to that seen in non-transgenic animals following thoracic-level lesions: axons approached the junction of the lesion and the dorsal surface of the cord, but showed little tendency towards growth into the lesion or into the scar tissue capping the lesion. In contrast, several transgenic animals showed extensive axon growth into the scar tissue overlying the lesion site.

One animal also showed labelling to suggest remarkable growth through and beyond the lesion. However, it cannot be stated with certainty that this is the case. Certainly the labelling pattern observed does not resemble that seen in any other animal, or the innervation pattern seen in an intact animal (not shown). Labelled axons were observed in the lateral grey matter and lateral white matter, where transganglionically labelled axons would normally not be found. They were also observed clearly within the lesion itself, and between the lesion centre and the labelling in the lateral cord. It is not clear whether this labelling is due to regeneration and as this result was not replicated in any of the other animals it cannot be considered significant. Rostral to the lesion the labelled axons were

predominantly lateral and restricted to the side ipsilateral to the injection of tracer. This argues against the possibility that axon regeneration has been observed, as it is not obvious why regenerating dorsal column axons would be restricted to growing to one side of the cord only. Because the animals received lesions in the medial part of the cord, it cannot be ruled out that these laterally placed axons were intact axons which had sprouted to form aberrant labelling patterns prior to lesioning; spontaneous sprouting at the neuromuscular junction was observed in these GAP-43-overexpressing transgenic mice (Aigner et al., 1995).

Two animals also showed evidence of axon growth into grey matter beneath the lesion, into the lesion itself and in the white matter distal to the lesion. In non-transgenic animals, no labelled axons were seen distal to the lesion. However, both these transgenic animals received lesions at a slightly lower level (L3 as opposed to L2) and it was clear that collateral innervation of Clarke's nucleus extended a significant distance rostral to the lesion, which was not found in other animals. It is quite possible therefore that this growth into the distal white matter originated from these collaterals rather than from axons in the dorsal columns which had traversed the lesion, although the evidence for this interpretation is inconclusive. The axon growth into grey matter beneath the lesion clearly originated from collaterals in grey matter. It would be interesting if these axons show different properties with regard to growth-inhibitory molecules in CNS grey matter and white matter. It may be of course, that they are simply better placed to access distal white matter as they do not have to traverse the lesion.

iv. Further comments

Although the effects of GAP-43 overexpression were too inconsistent to conclude that it has more than a minor effect on regeneration, it seems likely that in the lumbar cord it promotes some axonal sprouting and regeneration. However, significant differences were observed between lesions performed at thoracic and at lumbar levels on transgenic animals. It is unclear what the reason for this may be. It is unlikely that lumbar dorsal column injury provokes a significant cell body response in the DRG neurons, as a more proximal injury to these axons in the dorsal roots fails to do so. The major difference between the two models is the presence of collaterals of dorsal column axons in the grey matter, where they

innervate Clarke's nucleus. Dorsal column transection at the lumbar level would also damage these collaterals, but it is not clear what effect, if any, this would have on the ascending dorsal column axons.

Neumann and Woolf (1999) reported significant growth into and around the lesion if rats were given a sciatic nerve injury 1 week prior to a dorsal column injury. In some animals extensive growth was seen into the lesion and beyond it into the distal grey matter. In others, axons avoided the lesion itself but grew within the grey matter, both caudally and rostrally under and past the lesion. Many axons were observed growing horizontally within the plane of the lesion. While the degree of growth found in these experiments exceeds that found in most of the animals studied here, neither pattern of axonal labelling observed by Neumann and Woolf (1999) is similar to that observed here. These authors did not report any tendency for axons to extend towards the dorsal surface of the cord, which was found consistently in the present study, and did not report any axonal growth out of the cord into the scar tissue. This last difference may be due to differences in gross morphology of the cord between the rat and the mouse after this type of injury.

v. Dorsal root injury

Following transection and re-anastomosis of the L4 and L5 dorsal roots, regeneration of the severed axons is slow without an accompanying lesion of the sciatic nerve. Little difference could be observed between transgenic and non-transgenic animals. In both groups, small numbers of axons regenerated as far as the dorsal root entry zone. A relatively high proportion of axons which reached the spinal cord were able to cross the DREZ and enter the grey matter, as compared to previous studies on the rat (Chong et al., 1999), when axons were rarely observed to enter the dorsal horn without an accompanying peripheral nerve lesion. Given the small number of labelled axons which regenerated this far, it was impossible to determine if this happened at a higher rate in transgenic than in non-transgenic animals.

Compared to the amount of regeneration which is seen when a peripheral nerve lesion accompanies the rhizotomy, GAP-43 overexpression did little to augment axon growth. Typically many hundreds of axons would be expected to arrive at the DREZ if the neuronal

cell bodies are properly stimulated by a peripheral axotomy (S. McNally and P. Anderson, personal communication). Therefore, GAP-43 expression alone is insufficient to promote regeneration of significant numbers of axons from the dorsal root into the spinal cord.

vi. A comparison with a related experiment

Recently, interesting results were reported by Bomze et al. (2001) in a related experiment. These authors studied the effects of overexpression of either GAP-43 alone or GAP-43 and CAP-23 together in DRG neurons on regeneration of their central axons. The model used was that of peripheral nerve implantation into the spinal cord, following which dorsal column axons may regenerate into the graft. The authors used retrograde labelling to quantify the numbers of neurons regenerating into the grafts, and found that GAP-43 expression alone had no effect, but combined expression of GAP-43 and CAP-23 resulted in a 60-fold increase. 7% of neurons were found to have regenerated axons into the graft. This compares favourably with 17% of neurons regenerating when the sciatic nerve was injured. Obviously it would be interesting to examine the effects of overexpression of both molecules together in the models used here. The results of Bomze et al. (2001) would suggest that such combined expression might have significant effects on these models and go much further towards mimicking the cell body response induced by a peripheral injury.

Although the differences in experimental models of Bomze et al. (2000) and the present study preclude a direct comparison, it is a little puzzling that combined expression of both molecules would have such a significantly greater growth-promoting effect than expression of GAP-43 alone, given the partial functional redundancy of GAP-43 and CAP-23. The effects of these two molecules were found to be additive in promoting sprouting at the neuromuscular junction (Caroni, 1997) but each molecule alone was sufficient to cause significant effects. Studies of cultured DRG neurons from double transgenic mice also indicated that combined production of CAP-23 and GAP-43 in these cells increased their propensity for neurite growth in the first 24 hours after dissection, and this was also seen to a lesser degree in DRG neurons from single transgenic mice overexpressing either GAP-43 or CAP-23. It remains to be seen whether the synergistic effects of GAP-43 and CAP-23 would be effective in the models used here.

A major difference between the the model used by Bomze et al. (2001) and those used in the present study is that in the former case the axons were presented with a highly favourable environment through which to grow, whereas after dorsal column injury alone axons are presented with a non-conducive environment. Nonetheless, following lumbar dorsal column injuries some axon growth was seen into the less inhibitory environment of the fibrous scar overlying the cord in the mice overexpressing GAP-43. In the models used here, it may very well be necessary to combine the expression of neuronal growth-associated molecules with treatments to neutralise or overcome inhibitory factors, in order to promote regeneration.

vii. Summary

Inducing expression of growth-associated molecules would appear to be a prerequisite for vigorous axon growth, so manipulation of gene expression in injured neurons may prove to be a useful tool in promoting regeneration in the CNS. While it is perhaps unsurprising that increasing production of a single molecule is not sufficient to promote extensive regeneration, expressing combinations of growth-associated molecules might be more successful. In addition, it is possible that vector-driven expression of a number of these molecules in injured neurons might be effective in promoting regeneration without the increased risk of cell death that accompanies the cell body response in some neurons following axotomy.

9.10 Gene expression in axotomised corticospinal and rubrospinal neurons

In the final experiments presented in this study, changes in gene expression were examined in axotomised corticospinal and rubrospinal neurons. Previous reports indicate that corticospinal neurons do not upregulate GAP-43 following axotomy at the level of the pyramids, but will do so if axotomised close to their cell bodies (Kalil and Skene, 1986; Reh et al., 1987; Tetzlaff and Giehl, 1991; Tetzlaff et al., 1994). Downregulation of cytoskeletal elements was also reported. However, the expression of other growth associated molecules has not been previously examined. Rubrospinal neurons have been reported to upregulate *GAP-43* and c-Jun following a cervical axotomy, as well as *Tα1* tubulin (Tetzlaff et al., 1991; Jenkins et al., 1993a; Fernandes et al., 1999) but not

following a thoracic injury. Therefore, proximally and distally axotomised corticospinal neurons and cervically axotomised rubrospinal neurons were examined by ISH to determine levels of mRNA for the following molecules: GAP-43, CAP-23, SCG10, L1, CHL1, c-Jun, ATF3 and Krox-24.

GAP-43, CAP-23 and SCG10 are, as has been discussed, proteins important for growth-cone function which are all upregulated by regenerating neurons. L1 and CHL1 are cell-adhesion molecules with growth-promoting properties, and may be of importance for regeneration into peripheral nerve grafts. C-Jun, ATF3 and Krox-24 are transciption factors which are associated with axotomy, although only c-Jun and ATF3 are associated with axonal regeneration. Expression of these would also be expected to have a bearing on subsequent regeneration.

i. Retrograde labelling

Both corticospinal and rubrospinal neurons were identified by retrogradely labelling them with CTB injected into the dorsal funiculus and the dorsal lateral funiculus respectively, 1-2mm above a laceration lesion. The large numbers of retrogradely labelled neurons identified by this technique indicate that almost all corticospinal and rubrospinal axons took up and retrogradely transported the tracer. CTB has been reported not to be taken up by axons of passage, so it is likely that the injection itself damaged large numbers of axons sufficiently for CTB-take-up. However, it is possible that some corticospinal or rubrospinal axons were not retrogradely labelled, but this has little bearing on the interpretation of the results. If axons of passage do take up the tracer, it is also possible that small numbers of axons terminated in grey matter between the injection and the lesion, and so were retrogradely labelled but not axotomised. The great majority of corticospinal and rubrospinal axons around the injection site would be expected to terminate below this level, and the distance between the lesion and the injection was minimal, so these are likely to be negligible.

ii. Corticospinal neurons

Evidence has been presented that corticospinal neurons upregulate *c-jun*, *ATF3*, *GAP-43*, *SCG10*, *L1* and *CHL1* following proximal axotomy, but not after axotomy in the cervical spinal cord. *CAP-23* and *krox-24* were not upregulated after either injury. The increased gene expression described was observed in layer V pyramidal neurons. Retrogradely labelled corticospinal neurons were identified on adjacent sections, sandwiching the sections reacted for ISH, and detailed additional analysis of adjacent sections enabled retrogradely labelled neurons present on both CTB and ISH-reacted sections to be identified.

Of the molecules being investigated, GAP-43, CAP-23, SCG10, L1 and CHL1 are expected to be directly involved in axon growth. Messenger RNAs for all of these are expressed at a moderately high level in layer V pyramidal neurons in the intact animal. Corticospinal neurons injured in the spinal cord show a limited sprouting response following injury, typically growing up to 1mm (e.g. Bregman et al., 1989; Schnell et al., 1994) which may be enabled by the constitutive expression of these molecules. The lack of change in gene expression following axotomy at the cervical level is consistent with previous findings that GAP-43 production and axonal transport does not change after pyramidotomy (Kalil and Skene, 1986; Reh et al., 1987). The findings presented here extend these observations, such that it is now clear that no upregulation of any of the growth-associated molecules takes place. However, it would be interesting to repeat the experiment with additional treatments which have been shown to promote corticospinal axon growth after injury, such as the application of NT-3 (Grill et al., 1997; Blits et al., 2000) or the IN-1 antibody (Schnell and Schwab, 1990). Such treatments might also result in upregulation of growth-associated molecules, as has been observed following delivery of IN-1 antibody in the cerebellum, which led to upregulation of c-Jun in Purkinje cells (Zagrebelsky et al., 1998).

It is of interest that corticospinal neurons will respond to axotomy if injured sufficiently proximally, and that in doing so they upregulate nearly all the mRNAs for growth-associated molecules examined, with the exception of *CAP-23*. Apart from ATF3, which has been little studied, each of these molecules has been associated with the cell body

response to axotomy, and with successful regeneration in a variety of neurons. This indicates that corticospinal neurons are capable of the same type of response to axotomy as other neurons which successfully regenerate, unlike, for example, Purkinje cells. The latter are equally poor at regenerating but also show a very limited subset of the typical changes in gene expression that were observed here, even after very proximal axotomy.

Evidently, as with other CNS neurons, axotomy of corticospinal neurons must be within a critical distance of the cell-body to provoke a response. Analysis of one animal suggested that the distance is between 350µm and 500µm, although the maximal distance at which a response was produced varied between molecules. The most sensitive molecule to axotomy in this experiment appeared to be *ATF3*, which was clearly upregulated 500µm from the lesion, and the least sensitive of those which responded at all was *GAP-43* which was not noticeably increased beyond 350µm from the lesion. These findings are in broad agreement with a finding reported by Tetzlaff (Tetzlaff and Giehl, 1991; Tetzlaff et al., 1994) that *GAP-43* was upregulated only within 200µm of a lesion, although they did not publish full data, preventing a comparison of lesion protocols. This implies the response of corticospinal neurons to axotomy begins, or continues, to decline sharply at this distance. It should also be noted that upregulation of *ATF3* was more easily detectable because of the low (almost zero) basal expression. Because retrogradely labelled corticospinal neurons were not found beyond 500µm from the lesion the it cannot be ruled out that these neurons upregulate *ATF3* if axotomised further than this from the cell body.

With these results, the possibility arises that a very proximal axotomy may promote regeneration of corticospinal axons into peripheral nerve grafts, but it is likely that only neurons very close to a graft or an injury tract would do so.

The proximal axotomy model used here resulted in a lesion within layer VI or the lower part of layer V of neocortex. While the factors that determine the strength of the cell body response have yet to be resolved, a key factor may be the proportion of axon terminals that are lost, so a neuron whose axon has many collateral branches may not respond to the loss of a small proportion of these. Corticospinal axons, as well as having terminals in the spinal cord, have collaterals which terminate in the mesencephalon and locally in neocortical layers V and VI (Catsman-Berrevoets and Kuypers, 1981; Tseng and Prince, 1993; Tseng

and Prince, 1996). This last type of collateral innervation may be of particular importance in preventing a cell body response to axotomy outside the cortex, or indeed to limited lesions within it: using a stab wound model, Elliott et al. (1997; 1999) found that proximal axotomy of transcallosal pyramidal neurons did not result in upregulation of GAP-43 or of Tα1 tubulin. Although transcallosal and corticospinal neurons are separate populations (Catsman-Berrevoets et al., 1980) this is somewhat surprising. However, a horizontal slice lesion as used here may also be more effective in severing collaterals in the lower layers of neocortex than a simple stab wound.

It is curious that *CAP-23* is not upregulated in proximally axotomised cortical neurons, when six other growth-associated molecules are. While its constitutive expression might render upregulation unnecessary, a common, though not universal, feature of the cell body response is the upregulation of growth-associated molecules even if they are already expressed (for example, this is the case for *SCG10* and *CAP-23* in all three models of regeneration presented in this study).

Lastly, no upregulation of *krox-24* was detected. The Krox-24 transcription factor is induced after axotomy in some CNS neurons of the rat, including retinal ganglion cells (Herdegen et al., 1993a; Herdegen et al., 1993b; Robinson, 1994) but is not expressed by retinal ganglion cells regenerating axons, in either the rat or goldfish (Herdegen et al., 1993b; Robinson, 1995). Injured corticospinal neurons do not express *krox-24* after either cervical or proximal injury, and therefore Krox-24 does not appear to be a factor in the response of these neurons to axotomy. It is not clear what the role of Krox-24 is in those neurons which do express it after axotomy.

iii. Rubrospinal neurons

The expression of putative growth-related genes was also examined in rubrospinal neurons following axotomy in cervical spinal cord. Only *c-jun*, *ATF3* and *L1* were upregulated in axotomised rubrospinal neurons. Notably, *GAP-43* was not upregulated. This contradicts several reports by Tetzlaff and colleagues, who by ISH with ^{35}S -radiolabelled cDNA probes found that the amount of *GAP-43* mRNA is increased by 3-5 fold (Tetzlaff et al. 1991; Fernandes et al. 1999). It is not clear what the reasons are for this difference. While

the non-radioactive ISH protocol used in the present study is less sensitive to small changes than the technique employed in these reports, a difference of the order of magnitude they reported should be easily detectable. In Tetzlaff et al. (1991), analysis was restricted to the caudal-most 400μm of the red nucleus, whereas in the present study neurons were examined in the middle of the rostro-caudal extent of the nucleus. It is possible that *GAP-43* upregulation is observed only in the caudal pole of the nucleus. None the less, the region examined contained cells which projected to the injured region of the spinal cord. Furthermore, Fernandes et al.(1999) et al. found upregulation in cells in the lateral part of the nucleus after cervical axotomy.

The upregulation of *c-jun* which was observed is in agreement with the previous findings of Jenkins et al. (1993a). In the present study it was also shown, for the first time, that *ATF3* is upregulated by axotomised rubrospinal neurons. It was notable that the number of neurons which expressed *ATF3* after injury was considerably less than the number which expressed *c-jun*, and less than the number of retrogradely labelled neurons visible on adjacent sections. This parallels the previous findings of Takeda et al. (2000) who examined ATF3 and c-Jun protein and mRNA expression in axotomised retinal ganglion cells. In their study, ATF3 was expressed in fewer injured neurons than c-Jun. In contrast, following peripheral nerve injury virtually all axotomised neurons expressed ATF3. This may reflect a difference between neurons injured within the CNS, and therefore not provided with a suitable environment for regeneration, and those injured within peripheral nerve. As suggested by Takeda et al. (2000), the ATF3 response may be more transient than that of c-Jun and therefore detectable only in a subset of neurons at any given time.

L1 was also upregulated by axotomised rubrospinal neurons, in similar numbers to ATF3. However its close relative *CHL1* was not. This is the reverse of the situation seen following peripheral nerve injury, where *L1* regulation is unaffected in the axotomised neurons but *CHL1* increases (Zhang et al., 2000), and in most types of CNS neuron which regenerate into peripheral nerve grafts both molecules are upregulated (Zhang et al., 1995; Chaisuksunt et al., 2000a; Chaisuksunt et al., 2000b). The basal levels of expression of these two mRNAs were similar in neurons of the red nucleus.

The previously existing data implied that these neurons exhibited most of the changes which commonly constitute the cell body response, for example after peripheral nerve injury, as all molecules previously examined (c-Jun, GAP-43, tubulins and neurofilament) had been reported to respond appropriately. On the evidence presented, it appears that rubrospinal neurons show a more limited response to axotomy, as assessed by changes in gene expression, than was thought. However, axotomy at the cervical level is still some distance from the red nucleus (approximately 20-30mm), and it is possible that a more proximal axotomy would induce upregulation of a wider range of molecules. Given the limited response observed, it is perhaps surprising that rubrospinal neurons are observed to regenerate into peripheral nerve grafts (Richardson et al., 1984; Fernandes et al., 1999). However, this is less surprising when one considers that the numbers observed to do so are consistently small, representing 1-2% of rubrospinal neurons at best. Some upregulation of *GAP-43*, *CAP-23*, etc. in a very small number of axotomised rubrospinal neurons cannot be ruled out. Additionally, trophic factors which are released by the grafts may also stimulate injured neurons to upregulate more growth-associated molecules.

iv. ATF3 as a marker for axotomy

It is of particular interest that *ATF3* is upregulated in both corticospinal and rubrospinal neurons after axotomy. ATF3 is upregulated in regenerating motor and sensory neurons following peripheral nerve injury, and in retinal ganglion cells after axotomy (Takeda et al., 2000; Tsujino et al., 2000). Taken together, these results suggest that ATF3 is consistently upregulated by populations of axotomised neurons. This may have interesting implications for the study of the transcriptional regulation of growth-associated molecules (see Section 9.11 *ii*). However, in the case of rubrospinal neurons and retinal ganglion cells, it is also clear that *ATF3* upregulation is not necessarily observed in all axotomised neurons, at least at a given time point. Whether this means that some neurons do not upregulate ATF3, or that upregulation is transient and occurs at varying time points, is a question which needs to be resolved. During peripheral nerve regeneration, *ATF3* showed a much more consistent correlation with axotomy and regeneration, being expressed in virtually all axotomised neurons identified by retrograde labelling (Tsujino et al., 2000). This might suggest that its expression correlates better with regeneration than axotomy alone; this

point too deserves investigation, and it will be interesting to see if ATF3 is also expressed by CNS neurons regenerating into peripheral nerve grafts.

9.11 Regulation of growth-associated molecules

In the light of the results presented here, it is interesting to speculate on how the expression of growth-associated molecules is regulated in neurons following axotomy. Currently, these mechanisms are only beginning to be understood. However, a better understanding might lead to new approaches for the promotion of regeneration, perhaps by allowing interventions to increase expression of large numbers of growth-associated molecules.

i. Signals generated by axotomy

Little is known about the nature of the signals that lead from the injured axon to a response in the cell body. There is evidence that one component of these is a 'negative' signal, i.e. a signal which is lost due to disruption of retrograde transport of target-derived factors. Thus blocking retrograde axonal transport colchicine or vinblastine leads to GAP-43 and c-Jun upregulation in the affected neurons (Woolf et al., 1990; Leah et al., 1991; Yao and Kiyama, 1995). Similarly, blocking transport by cooling of the facial nerve leads to upregulation of *Tα1* tubulin and *p75* mRNAs in the facial nucleus (Wu et al., 1993). However, other types of signal are probably also involved, and indeed multiple signals may regulate separate aspects of the cell body response. Tetzlaff et al. (1994) argued for the existence of more than one negative signal in axons of the PNS, because the degree of *Tα1* tubulin upregulation after peripheral nerve injury is dependent on the distance of the axotomy from the cell bodies, while upregulation of GAP-43 is distance-independent (Liabotis and Schreyer, 1995; Fernandes et al., 1999). Bussmann and Sofroniew (1999) found that downregulation of choline acetyl transferase was inducible in hypoglossal motor neurons by colchicine blockade of axonal transport of the hypoglossal nerve. Expression of p75, a correlate of axotomy, was not induced by the blockade, however, and p75 was only upregulated if injured axons contacted damaged peripheral nervous tissue, implying a requirement for a positive signal retrogradely transported from the damaged nerve terminals.

Axotomised sympathetic, motor and sensory neurons upregulate the neuropeptide galanin following axotomy. The neurotrophic factor NGF has been implicated as putative negative signal while leukaemia-inhibitory factor (LIF) produced in the distal stump was found to be a positive signal for the regulation of galanin and other neuropeptides (reviewed by Zigmond and Sun, 1997). In superior cervical ganglion neurons, both the application of LIF and neutralisation of NGF with anti-NGF antibodies were required to induce galanin expression (Shadiack et al., 1998). However, while these factors appear to mimic axotomy-type responses it is not known whether they provoke changes in other growth-associated molecules. FGF2 has also been implicated as a possible constitutive target-derived signal as its application can prevent c-Jun upregulation in partially axotomised parasympathetic preganglionic neurons (Blottner and Herdegen, 1998).

ii. Transcriptional control of growth-associated molecules

However, while there may be several signalling molecules which transmit signals leading to a cell body response, and the pathways to changes in gene expression are not clearly defined, the common expression of c-Jun suggests a central role for this transcription factor. This is also supported by the finding that the increased c-Jun levels in axotomised neurons are accompanied by increased phosphorylation of this protein and increased JNK activity (Herdegen et al., 1998; Kenney and Kocsis, 1998). It is plausible, therefore, that JNK activation is part of a signalling pathway activated by axotomy. The simplest model would involve c-Jun activation by JNK, leading to transcription of genes for growth-associated molecules via AP-1 sites in their promoters. While c-Jun expression colocalises with a large number of molecules in axotomised neurons, little evidence has been found for direct activation of promoters for growth-associated molecules by AP-1 activity. The GAP-43 promoter was found to contain an AP-1 site (Eggen et al., 1994), and mutation of this reduced the activity of a reporter construct transfected into neurons (Weber and Skene, 1998), but its importance in the context of regeneration has not been demonstrated. So, while c-Jun is consistently expressed by regenerating neurons, it is recognised that the relationship between c-Jun and expression of other growth-associated molecules is unlikely to be as straightforward as simple AP-1 mediated transcriptional activation.

The findings in the present study, combined with those of Takeda et al. (2000) and Tsujino et al. (2000), show that ATF3 is upregulated by corticospinal neurons, rubrospinal neurons, motor and sensory neurons and retinal ganglion cells after axotomy. This suggests that this transcription factor is also involved in the response to axotomy, and therefore may participate in the transcriptional regulation of growth-associated genes. If ATF3 is consistently expressed by neurons regenerating their axons, this may have implications for the possible mechanisms by which growth-associated molecules are regulated. In particular, c-Jun/ATF3 dimers recognise the CRE motif as well as the AP-1 motif recognised by c-Jun homodimers (Hai and Curran, 1991; Hsu et al., 1992), so this may have hindered investigations into transcriptional regulation of growth-associated molecules by c-Jun, if it were assumed that c-Jun homodimers were the active transcriptional complex.

The sole example to date whereby a direct link was demonstrated between c-Jun and expression of an axotomy-induced molecule was a study of regulation of the neuropeptide VIP in cultured sensory neurons (Mulderry and Dobson, 1996). Microinjections of antisense oligonucleotides to *c-jun* reduced both c-Jun and VIP immunofluorescence. In addition, a CRE element was identified in the VIP promoter, which was shown to bind c-Jun/c-Fos heterodimers but did not bind c-Jun homodimers. Microinjection of competitive DNA containing the CRE sequence also reduced VIP expression, presumably by sequestering the active transcription factor complex. Although no attempt was made to investigate a role for ATF3, these findings would be consistent with regulation of the VIP promoter by c-Jun/ATF3 heterodimers, and do not support transcriptional activation by c-Jun homodimers via AP-1 sites. C-Jun/c-Fos heterodimer binding and activation is unlikely to occur in vivo after axotomy, as c-Fos expression is not induced except very transiently (Herdegen and Leah, 1998).

In order to fully understand how growth-associated molecules are regulated, further study needs to be made of the promoters of their genes and the transcription factors which bind them. The genes identified in the present study as associated with axon regeneration would be suitable candidates. Although no attempt was made in the present study to demonstrate colocalisation of c-Jun with *SCG10, CAP-23* or *FKBP12* in regenerating neurons, this would almost certainly occur. Clearly, however, the regulatory mechanisms are likely to be complex and may involve many pathways and molecules. For example, the exact set of

genes upregulated following axotomy varies between neurons, so a single transcription factor would most likely be insufficient to control such variations.

It is possible that the transcriptional activity of c-Jun and/or ATF3 is not involved in the regulation of growth-associated molecules. Both molecules are generally associated with cell stress and their upregulation may reflect this, although the prolonged upregulation of both molecules during axon regeneration argues against this. Alternatively they may simply regulate the general increase in cell metabolism required to grow an axon, while the growth-associated molecules are regulated via a separate pathway, albeit one triggered by the same mechanism. In studies of dissociated goldfish retinal ganglion cells in culture, Benowitz and co-workers have found evidence that a purine sensitive mechanism mediates signals which promote neurite outgrowth and also lead to upregulation of growth-associated molecules, including GAP-43 (Benowitz et al., 1998; Petrausch et al., 2000). The purine inosine stimulated these effects while the purine analogue 6-thioguanine (6-TG) blocked their induction by growth-promoting factors derived from goldfish optic nerve. Inosine also counteracted the effects of 6-TG. The authors argued that this signalling activity may involve a purine-sensitive kinase similar to one isolated from PC-12 cells (Volonte and Greene, 1992). While GAP-43 and other growth-associated molecules were apparently regulated by such a purine-sensitive mechanism, and were found largely in neurons where axon-growth had been induced, c-Jun expression was found in all neurons and was unaffected by purines. Whether this dissociation of c-Jun and GAP-43 regulation implies a c-Jun-independent pathway for regulation of growth-associated genes, or a purine-sensitive regulatory mechanism downstream of c-Jun or JNKs has not been determined.

iii. Post-transcriptional control of GAP-43 and SCG10 mRNAs

Considerations on the regulation of growth-associated molecules are further complicated by evidence that *GAP-43* and perhaps *SCG10* are subject to post-transcriptional regulation. The increase in transcription of *GAP-43* which occurs in neurons during development and nerve regeneration is insufficient to account for the increase in mRNA levels (Perrone-Bizzozero et al., 1991). Post-transcriptional regulation of *GAP-43* mRNA has been studied in PC12 cells, in which neuronal differentiation is induced by NGF, with a concomitant

increase in GAP-43 mRNA and protein levels. Perrone-Bizzozero et al. (1993) found that the increase in *GAP-43* mRNA could be induced by PKC activation, but by a 6-fold increase in the half-life of the mRNA rather than by an increase in transcription. Two separate regions in the 3' untranslated region were found to mediate the regulation of mRNA stability, and acted as protein-binding sites (Nishizawa, 1994; Kohn et al., 1996; Tsai et al., 1997). One of these RNA-binding proteins was identified as HuD (Chung et al., 1997), and in several models overexpression of HuD resulted in increases in *GAP-43* mRNA, while expression of *HuD* antisense RNA reduced *GAP-43* mRNA levels and blocked NGF-induction of neurite outgrowth (Anderson et al., 2000; Mobarak et al., 2000; Anderson et al., 2001).

It would appear then, that *GAP-43* mRNA is regulated partly by post-transcriptional stabilisation and this is likely to be the major control mechanism in PC12 cells. However, in vivo both transcriptional and post-transcriptional control contribute to the regulation of *GAP-43* mRNA (Cantallops and Routtenberg, 1999; Namgung and Routtenberg, 2000). By ISH for intron sequences in the *GAP-43* gene, Namgung and Routtenberg (2000) were able to compare primary transcript and mRNA expression, and in most (though not all) areas of the adult rat CNS these two measures were highly correlated, suggesting transcriptional control was the main mechanism used. Of greater interest was the finding that following facial nerve crush, both transcriptional and post-transcriptional mechanisms were found to contribute to the increase in mRNA levels in the axotomised facial neurons.

SCG10 mRNA may also be regulated by post-transcriptional mechanisms. Two forms of *SCG10* mRNA are found in both the rat and the chick nervous system, a 1kb and a 2kb transcript, formed by termination of transcription alternative polyadenylation sites (Stein et al., 1988b; Hannan et al., 1996). These two species of mRNA appear to be separately regulated during development and during differentiation of PC12 cells, with expression of only the 2kb mRNA being associated with neurite outgrowth in both cases (Hannan et al., 1996). It was suggested that this separate regulation may be achieved by selective stabilisation of the 2kb mRNA. Interestingly, during neuronal differentiation the 2kb mRNA was found to be localised to the base of the neurite and within the proximal neurite, suggesting a possible mechanism of targetting of SCG10 protein to the axon.

9.12 Conclusions

The experiments presented here provide new data on several aspects of the neuronal response to axotomy and, in particular, the changes in gene expression which occur. The mRNAs for the growth-cone molecules SCG10 and CAP-23 and the immunophilin FKBP12 have been shown to be upregulated by neurons regenerating their axons and this upregulation consistently forms a part of the cell body response. This strongly supports the idea that they are important for successful axonal regeneration. These molecules appear to be co-regulated during regeneration, and to a degree, *SCG10*, *CAP-23* and *GAP-43* also appear to be co-regulated in uninjured neurons. This suggests there may be common regulatory mechanisms for these molecules, although this is a subject about which much remains to be understood.

The cell body response to axotomy almost always includes upregulation of GAP-43, and this is associated with successful regeneration. In this study, it was also shown that constitutive overexpression of GAP-43 is insufficient to promote regeneration; on its own GAP-43 does not simulate the cell body response to axotomy. However the approach remains a valid one and subsequent experiments involving expression of more growth-associated molecules may be more successful in promoting regeneration. CAP-23, SCG10 and FKBP12 would be among a number of suitable molecules.

Lastly, the response of corticospinal and rubrospinal neurons to axotomy was examined. It was shown that corticospinal neurons do not upregulate any of these following axotomy in the cervical spinal cord. This may explain their failure to regenerate into peripheral nerve grafts, although other factors may also be important. In contrast, following a very proximal axotomy, almost all mRNAs studied were upregulated, except *CAP-23* and *krox-24*. Rubrospinal neurons showed a more limited response to axotomy in the cervical spinal cord than previously appeared to be the case, upregulating only *c-jun*, *ATF3* and *L1*. *GAP-43* was not found to be upregulated, in contrast to previous findings, but the reason for this discrepancy is unclear.

Future directions of this research

This work could be taken further in several possible directions. Having established that SCG10, CAP-23 and FKBP12 are likely to be of some importance for axon regeneration, more enquiries are needed into the importance of these molecules and of GAP-43 for axon regeneration. For example, in the case of FKBP12 it is not yet clear whether it functions in the cell body, the axon or in the growth-cone, or all three. FK506-binding activity was reported to be present in neonatal forebrain growth-cones (Lyons et al., 1995), but this may be due to the presence of other FK-binding proteins. The study of GAP-43 and CAP-23 knockout mice (Frey et al., 1998) has yielded limited information about axonal regeneration in these animals, presumably because their postnatal survival is poor. However, a mutant GAP-43 construct was reported to retard peripheral nerve regeneration. Nerve grafting or peripheral nerve injury experiments in with such animals might reveal what role GAP-43 and CAP-23 play in axonal regeneration. FKBP12-deficient mice rarely survive to adulthood (Shou et al., 1998) and no SCG10 knockout has been reported, but many axonal regeneration experiments could be envisaged on such animals, and indeed on animals which are deficient for other growth-associated molecules. However, such animals may not be viable. Another approach to overcome such problems is to use a viral vector driven recombinase to delete growth-related genes from a limited population of neurons in the adult animal, for example using the cre/lox system (Sauer, 1998). Useful information might also be obtained by experiments similar to those of (Meiri et al., 1998). Cultures of neurons deficient in one or more of GAP-43, CAP-23, SCG10 or FKBP12 on various substrates or in gradients of known attractive or repellant guidance molecules may reveal the importance of these neuronal molecules for the responses of growth-cones to guidance cues.

A curious finding which also deserves further investigation is that of the unusually high incidence of neuronal cell bodies in peripheral nerve grafts implanted in the cerebellum, and the possibility this raises that these cells may be derived from a local population of neural stem cells, probably the ependymal cells lining the fourth ventricle (Johansson et al., 1999). Pre-labelling of this population of cells by DiI injection into the CSF (Johansson et al. 1999) might provide evidence that factors present in injured peripheral nerve can promote migration and neurogenesis by these cells. Such a finding would be of interest as these cells

can proliferate after CNS injury but normally differentiate only into glia.

Corticospinal neurons have rarely been observed to regenerate axons into peripheral nerve grafts. It was shown in Chapter 8 that an intracortical axotomy provokes a cell body response in these neurons which was of a magnitude and character similar to that of neurons which will regenerate axons into grafts. The horizontal lesions used may be more effective in this than a simple stab wound, so it would be interesting to determine if the combination of such an intracortical axotomy and the implantation of a graft in the cortex would result in regeneration of corticospinal axons into the grafts. If not, this would support the idea that these neurons express receptors which guide them away from peripheral nervous tissue.

It might also be interesting to conduct more studies on the effects of overexpression of growth-associated molecules on axon regeneration. A possibility already mentioned would be to study regeneration of the central axon branches of DRG neurons in spinal cord and dorsal root injury models in the strain of transgenic mice overexpressing GAP-43 and CAP-23 together, to establish whether axon regeneration is augmented even in a non-conducive environment by the expression of both transgenes. So far, only the effects on axon regeneration into peripheral nerve grafts have been published (Bomze et al., 2001). It might be expected that the expression of combinations of molecules which are important for axon growth may yield greater effects in promoting regeneration than one or two alone. As overexpression of SCG10 also accelerated neurite outgrowth in PC12 cells (Riederer et al., 1997), it would be logical to study the effect of overexpression of SCG10 in these neurons in combination with GAP-43 and CAP-23.

In the long-term it might also be fruitful to develop more sophisticated approaches to stimulate the expression of growth-associated genes without causing an axotomy. However this will require a greater understanding than is currently available of the mechanisms of regulation of growth-associated molecules. For this reason it was of particular interest that *ATF3* was upregulated by axotomised corticospinal and rubrospinal neurons. This may have implications for studies into the transcriptional regulation of growth-associated molecules, although relatively little data is available in this area. Therefore it will be of particular interest to elucidate the role of ATF3 and c-Jun in axon regeneration, as these

transcription factors are well-placed to orchestrate at least some of the responses of neurons to axotomy. Mice lacking c-Jun die during development (Johnson et al., 1993) and currently there are no reports of ATF3 (-/-) mice, but the generation of these would allow experiments to establish how important this transcription factor is in the neuronal response to axotomy and in axonal regeneration. Another approach would be to study regeneration and gene expression in neurons expressing a dominant-negative version of c-Jun, such as that used by (Ham et al., 1995), either in transgenic mice or in neurons transfected by a vector construct. Dominant negative c-Jun is expected to block the transcriptional activity of all c-Jun-containing dimers and might therefore provide definitive evidence on the role of c-Jun and ATF3 in initiating or maintaining axon regeneration and the program of gene expression that accompanies it. If growth-related genes are downstream of these transcription factors, their upregulation after axotomy may be prevented or diminished by such manipulations.

In summary, it is hoped that the sum of the data presented provides useful information about regeneration and the neuronal responses to axotomy, and may lead to further studies to elucidate the exact roles of these molecules and the methods of their regulation, as well as assisting the development of strategies to promote regeneration in the injured CNS.

References

Aarts L.H., Schrama L.H., Hage W.J., Bos J.L., Gispen W.H. and Schotman P. (1998). B-50/GAP-43-induced formation of filopodia depends on Rho-GTPase. *Molecular Biology of the Cell* **9** 1279-1292.

Aarts L.H., van der Linden J.A., Hage W.J., van Rozen A.J., Oestreicher A.B., Gispen W.H. and Schotman P. (1995). N-terminal cysteines essential for Golgi sorting of B-50 (GAP-43) in PC12 cells. *NeuroReport* **6** 969-972.

Aarts L.H., Verkade P., van Dalen J.J., van Rozen A.J., Gispen W.H., Schrama L.H. and Schotman P. (1999). B-50/GAP-43 potentiates cytoskeletal reorganization in raft domains. *Molecular and Cellular Neuroscience* **14** 85-97.

Abraham W.C., Dragunow M. and Tate W.P. (1991). The role of immediate early genes in the stabilization of long-term potentiation. *Molecular Neurobiology* **5** 297-314.

Abraham W.C., Mason S.E., Demmer J., Williams J.M., Richardson C.L., Tate W.P., Lawlor P.A. and Dragunow M. (1993). Correlations between immediate early gene induction and the persistence of long-term potentiation. *Neuroscience* **56** 717-727.

Aguayo AJ (1985) Axonal regeneration from injured neurons in the adult mammalian central nervous system. In: Synaptic Plasticity (ed Cotman CW), pp. 457-484. The Guilford Press, New York.

Aigner L., Arber S., Kapfhammer J.P., Laux T., Schneider C., Botteri F., Brenner H.R. and Caroni P. (1995). Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell* **83** 269-278.

Aigner L. and Caroni P. (1993). Depletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. *Journal of Cell Biology* **123** 417-429.

Aigner L. and Caroni P. (1995). Absence of persistent spreading, branching, and adhesion in GAP-43- depleted growth cones. *Journal of Cell Biology* **128** 647-660.

Alexander K.A., Cimler B.M., Meier K.E. and Storm D.R. (1987). Regulation of

calmodulin binding to P-57. A neurospecific calmodulin binding protein. *Journal of Biological Chemistry* **262** 6108-6113.

Ambron R.T., Dulin M.F., Zhang X.P., Schmied R. and Walters E.T. (1995). Axoplasm enriched in a protein mobilized by nerve injury induces memory- like alterations in Aplysia neurons. *Journal of Neuroscience* **15** 3440-3446.

Andersen L.B. and Schreyer D.J. (1999). Constitutive expression of GAP-43 correlates with rapid, but not slow regrowth of injured dorsal root axons in the adult rat. *Experimental Neurology* **155** 157-164.

Anderson D.J. and Axel R. (1985). Molecular probes for the development and plasticity of neural crest derivatives. *Cell* **42** 649-662.

Anderson K.D., Morin M.A., Beckel-Mitchener A., Mobarak C.D., Neve R.L., Furneaux H.M., Burry R. and Perrone-Bizzozero N.I. (2000). Overexpression of HuD, but not of its truncated form HuD I+II, promotes GAP-43 gene expression and neurite outgrowth in PC12 cells in the absence of nerve growth factor. *Journal of Neurochemistry* **75** 1103-1114.

Anderson K.D., Sengupta J., Morin M., Neve R.L., Valenzuela C.F. and Perrone-Bizzozero N.I. (2001). Overexpression of HuD accelerates neurite outgrowth and increases GAP- 43 mRNA expression in cortical neurons and retinoic acid-induced embryonic stem cells in vitro. *Experimental Neurology* **168** 250-258.

Anderson P.N., Campbell G., Zhang Y. and Lieberman A.R. (1998). Cellular and molecular correlates of the regeneration of adult mammalian CNS axons into peripheral nerve grafts. *Progress in Brain Research* **117** 211-232.

Anderson P.N., Mitchell J., Mayor D. and Stauber V.V. (1983). An ultrastructural study of the early stages of axonal regeneration through rat nerve grafts. *Neuropathology and Applied Neurobiology* **9** 455-466.

Andreasen T.J., Luetje C.W., Heideman W. and Storm D.R. (1983). Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry* **22** 4615-4618.

Antonsson B., Kassel D.B., Di Paolo G., Lutjens R., Riederer B.M. and Grenningloh G. (1998). Identification of in vitro phosphorylation sites in the growth cone protein SCG10. Effect Of phosphorylation site mutants on microtubule- destabilizing activity. *Journal of Biological Chemistry* **273** 8439-8446.

Armistead D.M. and Harding M.W. (1993). Immunophilins and immunosuppressive drug action. *Annual Reports in Medicinal Chemistry, Vol 28* **28** 207-215.

Bachinger H.P., Morris N.P. and Davis J.M. (1993). Thermal stability and folding of the collagen triple helix and the effects of mutations in osteogenesis imperfecta on the triple helix of type I collagen. *American Journal of Medical Genetics* **45** 152-162.

Bamburg J.R., Bray D. and Chapman K. (1986). Assembly of microtubules at the tip of growing axons. *Nature* **321** 788-790.

Bandtlow C., Zachleder T. and Schwab M.E. (1990). Oligodendrocytes arrest neurite growth by contact inhibition. *Journal of Neuroscience* **10** 3837-3848.

Bartsch U., Bandtlow C.E., Schnell L., Bartsch S., Spillmann A.A., Rubin B.P., Hillenbrand R., Montag D., Schwab M.E. and Schachner M. (1995). Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. *Neuron* **15** 1375-1381.

Basbaum A.I. and Fields H.L. (1979). The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. *Journal of Comparative Neurology* **187** 513-531.

Bates C.A. and Stelzner D.J. (1993). Extension and regeneration of corticospinal axons after early spinal injury and the maintenance of corticospinal topography. *Experimental Neurology* **123** 106-117.

Bavetta S., Hamlyn P.J., Burnstock G., Lieberman A.R. and Anderson P.N. (1999). The effects of FK506 on dorsal column axons following spinal cord injury in adult rats: neuroprotection and local regeneration. *Experimental Neurology* **158** 382-393.

Beattie M.S., Bresnahan J.C., Komon J., Tovar C.A., Van Meter M., Anderson D.K., Faden A.I., Hsu C.Y., Noble L.J., Salzman S. and Young W. (1997). Endogenous repair

after spinal cord contusion injuries in the rat. *Experimental Neurology* **148** 453-463.

Beattie M.S., Farooqui A.A. and Bresnahan J.C. (2000). Review of current evidence for apoptosis after spinal cord injury. *Journal of Neurotrauma* **17** 915-925.

Becker D.B., Jensen J.N., Myckatyn T.M., Doolabh V.B., Hunter D.A. and Mackinnon S.E. (2000). Effects of FKBP-12 ligands following tibial nerve injury in rats. *Journal of Reconstructive Microsurgery* **16** 613-620.

Belmont L.D. and Mitchison T.J. (1996). Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell* **84** 623-631.

Bendotti C., Pende M. and Samanin R. (1994). Expression of GAP-43 in the granule cells of rat hippocampus after seizure-induced sprouting of mossy fibres: in situ hybridization and immunocytochemical studies. *European Journal of Neuroscience* **6** 509-515.

Benfey M. and Aguayo A.J. (1982). Extensive elongation of axons from rat brain into peripheral nerve grafts. *Nature* **296** 150-152.

Benfey M., Bunger U.R., Vidal Sanz M., Bray G.M. and Aguayo A.J. (1985). Axonal regeneration from GABAergic neurons in the adult rat thalamus. *Journal of Neurocytology* **14** 279-296.

Benowitz L.I., Jing Y., Tabibiazar R., Jo S.A., Petrausch B., Stuermer C.A., Rosenberg P.A. and Irwin N. (1998). Axon outgrowth is regulated by an intracellular purine-sensitive mechanism in retinal ganglion cells. *Journal of Biological Chemistry* **273** 29626-29634.

Benowitz L.I. and Routtenberg A. (1997). GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends in Neurosciences* **20** 84-91.

Benowitz L.I., Shashoua V.E. and Yoon M.G. (1981). Specific changes in rapidly transported proteins during regeneration of the goldfish optic nerve. *Journal of Neuroscience* **1** 300-307.

Bernstein J.J. and Bernstein M.E. (1971). Axonal regeneration and formation of synapses proximal to the site of lesion following hemisection of the rat spinal cord. *Experimental Neurology* **30** 336-351.

Berry M. (1982). Post-injury myelin-breakdown products inhibit axonal growth: an hypothesis to explain the failure of axonal regeneration in the mammalian central nervous system. *Bibliotheca Anatomica* **23** 1-11.

Berry M., Carlile J. and Hunter A. (1996). Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. *Journal of Neurocytology* **25** 147-170.

Berry M., Carlile J., Hunter A., Tsang W., Rosustrel P. and Sievers J. (1999). Optic nerve regeneration after intravitreal peripheral nerve implants: trajectories of axons regrowing through the optic chiasm into the optic tracts. *Journal of Neurocytology* **28** 721-741.

Berry M., Maxwell W.L., Logan A., Mathewson A., McConnell P., Ashhurst D.E. and Thomas G.H. (1983). Deposition of scar tissue in the central nervous system. *ACTA Neurochirurgica. Supplementum (Wien)* **32** 31-53.

Berry M., Rees L., Hall S., Yiu P. and Sievers J. (1988). Optic axons regenerate into sciatic nerve isografts only in the presence of Schwann cells. *Brain Research Bulletin* **20** 223-231.

Berry M, Rees L, Sievers J (1986) Unequivocal regeneration of rat optic nerve axons into sciatic nerve isografts. In: Neural transplantation and regeneration (eds Das GD, Wallace RB), pp. 63-79. Springer-Verlag, New York.

Bierer B.E., Somers P.K., Wandless T.J., Burakoff S.J. and Schreiber S.L. (1990). Probing immunosuppressant action with a nonnatural immunophilin ligand. *Science* **250** 556-559.

Biffo S., Verhaagen J., Schrama L.H., Schotman P., Danho W. and Margolis F.L. (1990). B-50/GAP43 expression correlates with process outgrowth in the embryonic mouse nervous system. *European Journal of Neuroscience* **2** 487-499.

Bisby M.A. and Tetzlaff W. (1992). Changes in cytoskeletal protein synthesis following axon injury and during axon regeneration. *Molecular Neurobiology* **6** 107-123.

Bixby J.L., Lilien J. and Reichardt L.F. (1988). Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro. *Journal of Cell Biology*

107 353-361.

Bjorklund A., Segal M. and Stenevi U. (1979). Functional reinnervation of rat hippocampus by locus coeruleus implants. *Brain Research* **170** 409-426.

Bjorklund A. and Stenevi U. (1979). Regeneration of monoaminergic and cholinergic neurons in the mammalian central nervous system. *Physiol Rev.* **59** 62-100.

Blanchard G.C., Taylor C.G., Busey B.R. and Williamson M.L. (1990). Regeneration of immunosorbent surfaces used in clinical, industrial and environmental biosensors. Role of covalent and non-covalent interactions. *Journal of Immunological Methods* **130** 263-275.

Blesch A., Uy H.S., Grill R.J., Cheng J.G., Patterson P.H. and Tuszynski M.H. (1999). Leukemia inhibitory factor augments neurotrophin expression and corticospinal axon growth after adult CNS injury. *Journal of Neuroscience* **19** 3556-3566.

Blits B., Dijkhuizen P.A., Boer G.J. and Verhaagen J. (2000). Intercostal nerve implants transduced with an adenoviral vector encoding neurotrophin-3 promote regrowth of injured rat corticospinal tract fibers and improve hindlimb function. *Experimental Neurology* **164** 25-37.

Blottner D. and Herdegen T. (1998). Neuroprotective fibroblast growth factor type-2 down-regulates the c-Jun transcription factor in axotomized sympathetic preganglionic neurons of adult rat. *Neuroscience* **82** 283-292.

Bomze H.M., Bulsara K.R., Iskandar B.J., Caroni P. and Skene J.H. (2001). Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nature Neuroscience* **4** 38-43.

Bovolenta P. and Fernaud-Espinosa I. (2000). Nervous system proteoglycans as modulators of neurite outgrowth. *Progress in Neurobiology* **61** 113-132.

Bovolenta P. and Mason C. (1987). Growth cone morphology varies with position in the developing mouse visual pathway from retina to first targets. *Journal of Neuroscience* **7** 1447-1460.

Bradbury E.J., Khemani S., King V.R., Priestley J.V. and McMahon S.B. (1999). NT-3

promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. *European Journal of Neuroscience* **11** 3873-3883.

Bravin M., Savio T., Strata P. and Rossi F. (1997). Olivocerebellar axon regeneration and target reinnervation following dissociated Schwann cell grafts in surgically injured cerebella of adult rats. *European Journal of Neuroscience* **9** 2634-2649.

Bregman B.S., Kunkel-Bagden E., McAtee M. and O'Neill A. (1989). Extension of the critical period for developmental plasticity of the corticospinal pathway. *Journal of Comparative Neurology* **282** 355-370.

Bregman B.S., Kunkel-Bagden E., Schnell L., Ning Dai H., Gao D. and Schwab M.E. (1995). Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. *Nature* **378** 498-501.

Bresnahan J.C., Beattie M.S., Stokes B.T. and Conway K.M. (1991). Three-dimensional computer-assisted analysis of graded contusion lesions in the spinal cord of the rat. *Journal of Neurotrauma* **8** 91-101.

Bridgman P.C., Kachar B. and Reese T.S. (1986). The structure of cytoplasm in directly frozen cultured cells. II. Cytoplasmic domains associated with organelle movements. *Journal of Cell Biology* **102** 1510-1521.

Brillantes A.B., Ondrias K., Scott A., Kobrinsky E., Ondriasova E., Moschella M.C., Jayaraman T., Landers M., Ehrlich B.E. and Marks A.R. (1994). Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77** 513-523.

Brown E.J., Albers M.W., Shin T.B., Ichikawa K., Keith C.T., Lane W.S. and Schreiber S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **369** 756-758.

Brown E.J. and Schreiber S.L. (1996). A signaling pathway to translational control. *Cell* **86** 517-520.

Brummendorf T., Hubert M., Treubert U., Leuschner R., Tarnok A. and Rathjen F.G. (1993). The axonal recognition molecule F11 is a multifunctional protein: specific domains mediate interactions with Ng-CAM and restrictin. *Neuron* **10** 711-727.

Buffo A., Holtmaat A.J., Savio T., Verbeek J.S., Oberdick J., Oestreicher A.B., Gispen W.H., Verhaagen J., Rossi F. and Strata P. (1997). Targeted overexpression of the neurite growth-associated protein B-50/GAP-43 in cerebellar Purkinje cells induces sprouting after axotomy but not axon regeneration into growth-permissive transplants. *Journal of Neuroscience* **17** 8778-8791.

Buffo A., Zagrebelsky M., Huber A.B., Skerra A., Schwab M.E., Strata P. and Rossi F. (2000). Application of neutralizing antibodies against NI-35/250 myelin- associated neurite growth inhibitory proteins to the adult rat cerebellum induces sprouting of uninjured purkinje cell axons. *Journal of Neuroscience* **20** 2275-2286.

Bunge M.B., Holets V.R., Bates M.L., Clarke T.S. and Watson B.D. (1994). Characterization of photochemically induced spinal cord injury in the rat by light and electron microscopy. *Experimental Neurology* **127** 76-93.

Bunge R.P., Puckett W.R., Becerra J.L., Marcillo A. and Quencer R.M. (1993). Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. *Advances in Neurology* **59** 75-89.

Burden-Gulley S.M., Pendergast M. and Lemmon V. (1997). The role of cell adhesion molecule L1 in axonal extension, growth cone motility, and signal transduction. *Cell and Tissue Research* **290** 415-422.

Bussmann K.A. and Sofroniew M.V. (1999). Re-expression of p75NTR by adult motor neurons after axotomy is triggered by retrograde transport of a positive signal from axons regrowing through damaged or denervated peripheral nerve tissue. *Neuroscience* **91** 273-281.

Cai D., Qiu J., Cao Z., McAtee M., Bregman B.S. and Filbin M.T. (2001). Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. *Journal of Neuroscience* **21** 4731-4739.

Cai D., Shen Y., De Bellard M., Tang S. and Filbin M.T. (1999). Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* **22** 89-101.

Cameron A.M., Nucifora F.C., Jr., Fung E.T., Livingston D.J., Aldape R.A., Ross C.A. and Snyder S.H. (1997). FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine- proline (1400-1401) and anchors calcineurin to this FK506-like domain. *Journal of Biological Chemistry* **272** 27582-27588.

Cameron A.M., Steiner J.P., Sabatini D.M., Kaplin A.I., Walensky L.D. and Snyder S.H. (1995). Immunophilin FK506 binding protein associated with inositol 1,4,5- trisphosphate receptor modulates calcium flux. *Proceedings of the National Academy of Sciences USA* **92** 1784-1788.

Campbell G., Anderson P.N., Lieberman A.R. and Turmaine M. (1991). Mammalian CNS neurons regenerating axons into peripheral nerve grafts express GAP-43. *Experimental Brain Research* **87** 67-74.

Campbell G., Holt J.K.L., Shotton H.R., Anderson P.N., Bavetta S. and Lieberman A.R. (1999). Spontaneous regeneration after optic nerve injury in adult rat. *NeuroReport* **10** 3955-3960.

Cantallops I. and Routtenberg A. (1996). Rapid induction by kainic acid of both axonal growth and F1/GAP-43 protein in the adult rat hippocampal granule cells. *Journal of Comparative Neurology* **366** 303-319.

Cantallops I. and Routtenberg A. (1999). Activity-dependent regulation of axonal growth: posttranscriptional control of the GAP-43 gene by the NMDA receptor in developing hippocampus. *Journal of Neurobiology* **41** 208-220.

Carlstedt T. (1985). Regenerating axons from nerve terminals at astrocytes. *Brain Research* **347** 188-191.

Caroni P. (1997). Intrinsic neuronal determinants that promote axonal sprouting and elongation. *Bioessays* **19** 767-775.

Caroni P., Aigner L. and Schneider C. (1997a). Intrinsic neuronal determinants locally regulate extrasynaptic and synaptic growth at the adult neuromuscular junction. *Journal of Cell Biology* **136** 679-692.

Caroni P., Savio T. and Schwab M.E. (1988). Central nervous system regeneration:

oligodendrocytes and myelin as non-permissive substrates for neurite growth. *Progress in Brain Research* **78** 363-370.

Caroni P. and Schwab M.E. (1988b). Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* **1** 85-96.

Caroni P. and Schwab M.E. (1988a). Two membrane proteins fractions from rat central myelin with inhibitory properties for neurite outgrowth. *Journal of Cell Biology* **106** 1281-1288.

Caroni P. and Schwab M.E. (1989). Codistribution of neurite growth inhibitors and oligodendrocytes in rat CNS: appearance follows nerve fiber growth and precedes myelination. *Developmental Biology* **136** 287-295.

Caroni P. and Schwab M.E. (1993). Oligodendrocyte- and myelin-associated inhibitors of neurite growth in the adult nervous system. *Advances in Neurology* **61** 175-179.

Carreau A., Gueugnon J., Benavides J. and Vige X. (1997). Comparative effects of FK-506, rapamycin and cyclosporin A, on the in vitro differentiation of dorsal root ganglia explants and septal cholinergic neurons. *Neuropharmacology* **36** 1755-1762.

Catsman-Berrevoets C.E. and Kuypers H.G. (1981). A search for corticospinal collaterals to thalamus and mesencephalon by means of multiple retrograde fluorescent tracers in cat and rat. *Brain Research* **218** 15-33.

Catsman-Berrevoets C.E., Lemon R.N., Verburgh C.A., Bentivoglio M. and Kuypers H.G. (1980). Absence of callosal collaterals derived from rat corticospinal neurons. A study using fluorescent retrograde tracing and electrophysiological techniques. *Experimental Brain Research* **39** 433-440.

Chaisuksunt V. (1999) Differential Expression of Regeneration Relevant Molecules in Neurons of Adult Rat Brain After Injury and the Implantation of Peripheral Nerve Grafts. PhD Thesis, University College London.

Chaisuksunt V., Campbell G., Zhang Y., Schachner M., Lieberman A.R. and Anderson P.N. (2000b). The cell recognition molecule CHL1 is strongly upregulated by injured and

regenerating thalamic neurons. *Journal of Comparative Neurology* **425** 382-292.

Chaisuksunt V., Zhang Y., Anderson P.N., Campbell G., Vaudano E., Schachner M. and Lieberman A.R. (2000a). Axonal regeneration from CNS neurons in the cerebellum and brainstem of adult rats: correlation with the patterns of expression and distribution of messenger RNAs for L1, CHL1, c-jun and growth-associated protein-43. *Neuroscience* **100** 87-108.

Challacombe J.F., Snow D.M. and Letourneau P.C. (1997). Dynamic microtubule ends are required for growth cone turning to avoid an inhibitory guidance cue. *Journal of Neuroscience* **17** 3085-3095.

Chen M.S., Huber A.B., van der Haar M.E., Frank M., Schnell L., Spillmann A.A., Christ F. and Schwab M.E. (2000). Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1 [see comments]. *Nature* **403** 434-439.

Chen Y.G., Liu F. and Massague J. (1997). Mechanism of TGFbeta receptor inhibition by FKBP12. *European Molecular Biology Organization Journal* **16** 3866-3876.

Cheng H., Cao Y. and Olson L. (1996). Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function. *Science* **273** 510-513.

Choi J.H., Adames N.R., Chan T.F., Zeng C., Cooper J.A. and Zheng X.F. (2000). TOR signaling regulates microtubule structure and function. *Current Biology* **10** 861-864.

Chomczynski P. (1992). Solubilization in formamide protects RNA from degradation. *Nucleic Acids Research* **20** 3791-3792.

Chong M.-S., Fitzgerald M., Winter J., Hu-Tsai M., Emson P.C., Weise U. and Woolf C.J. (1992). GAP-43 mRNA in rat spinal cord and dorsal root ganglia neurons: developmental changes and re-expression following peripheral nerve injury. *European Journal of Neuroscience* **4** 883-895.

Chong M.-S., Reynolds M.L., Irwin N., Coggeshall R.E., Emson P.C., Benowitz L.I. and Woolf C.J. (1994a). GAP-43 expression in primary sensory neurons following central axotomy. *Journal of Neuroscience* **14(7)** 4375-4384.

Chong M.-S., Woolf C.J., Haque N.S.K. and Anderson P.N. (1999). Regeneration of axons from injured dorsal roots into the spinal cord in adult rats. *Journal of Comparative Neurology* **410** 42-54.

Chong M.-S., Woolf C.J., Turmaine M., Emson P.C. and Anderson P.N. (1996). Intrinsic vs extrinsic factors in determining the regeneration of the central processes of rat dorsal root ganglion neurons: the influence of a peripheral nerve graft. *Journal of Comparative Neurology* **370** 97-104.

Chong M.S., Woolf C.J., Andrews P., Turmaine M., Schreyer D.J. and Anderson P.N. (1994b). The downregulation of GAP-43 is not responsible for the failure of regeneration in freeze-killed nerve grafts in the rat. *Experimental Neurology* **129** 311-320.

Chung S., Eckrich M., Perrone-Bizzozero N., Kohn D.T. and Furneaux H. (1997). The Elav-like proteins bind to a conserved regulatory element in the 3'- untranslated region of GAP-43 mRNA. *Journal of Biological Chemistry* **272** 6593-6598.

Cimler B.M., Andreasen T.J., Andreasen K.I. and Storm D.R. (1985). P-57 is a neural specific calmodulin-binding protein. *Journal of Biological Chemistry* **260** 10784-10788.

Collins J.H. (1991). Sequence analysis of the ryanodine receptor: possible association with a 12K, FK506-binding immunophilin/protein kinase C inhibitor. *Biochemical and Biophysical Research Communications* **178** 1288-1290.

Compton L.A., Davis J.M., Macdonald J.R. and Bachinger H.P. (1992). Structural and functional characterization of Escherichia coli peptidyl-prolyl cis-trans isomerases. *European Journal of Biochemistry* **206** 927-934.

Crutcher K.A. (1989). Tissue sections from the mature rat brain and spinal cord as substrates for neurite outgrowth in vitro: extensive growth on gray matter but little growth on white matter. *Experimental Neurology* **104** 39-54.

Culotti J.G. and Merz D.C. (1998). DCC and netrins. *Current Opinion in Cell Biology* **10** 609-613.

Curmi P.A., Andersen S.S., Lachkar S., Gavet O., Karsenti E., Knossow M. and Sobel A. (1997). The stathmin/tubulin interaction in vitro. *Journal of Biological Chemistry* **272**

25029-25036.

Dale S.M., Kuang R.Z., Wei X. and Varon S. (1995). Corticospinal motor neurons in the adult rat: degeneration after intracortical axotomy and protection by ciliary neurotrophic factor (CNTF). *Experimental Neurology* **135** 67-73.

Dani J.W., Armstrong D.M. and Benowitz L.I. (1991). Mapping the development of the rat brain by GAP-43 immunocytochemistry. *Neuroscience* **40** 277-287.

Darian-Smith C. and Gilbert C.D. (1994). Axonal sprouting accompanies functional reorganization in adult cat striate cortex. *Nature* **368** 737-740.

Das A. and Gilbert C.D. (1995). Long-range horizontal connections and their role in cortical reorganization revealed by optical recording of cat primary visual cortex. *Nature* **375** 780-784.

David S. and Aguayo A.J. (1981). Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* **214** 931-933.

Davies S.J., Field P.M. and Raisman G. (1993). Long fibre growth by axons of embryonic mouse hippocampal neurons microtransplanted into the adult rat fimbria. *European Journal of Neuroscience* **5** 95-106.

Davies S.J., Field P.M. and Raisman G. (1994). Long interfascicular axon growth from embryonic neurons transplanted into adult myelinated tracts. *Journal of Neuroscience* **14** 1596-1612.

Davies S.J., Fitch M.T., Memberg S.P., Hall A.K., Raisman G. and Silver J. (1997). Regeneration of adult axons in white matter tracts of the central nervous system. *Nature* **390** 680-683.

Davies S.J.A., Goucher D.R., Doller C. and Silver J. (1999). Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. *Journal of Neuroscience* **19** 5810-5822.

Davis J.Q. and Bennett V. (1994). Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. *Journal of*

Biological Chemistry **269** 27163-27166.

Dawson T.M., Steiner J.P., Lyons W.E., Fotuhi M., Blue M. and Snyder S.H. (1994). The immunophilins, FK506 binding protein and cyclophilin, are discretely localized in the brain: relationship to calcineurin. *Neuroscience* **62** 569-580.

DeBellard M.E., Tang S., Mukhopadhyay G., Shen Y.J. and Filbin M.T. (1996). Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein. *Molecular and Cellular Neuroscience* **7** 89-101.

DeBernardo A.P. and Chang S. (1996). Heterophilic interactions of DM-GRASP: GRASP-NgCAM interactions involved in neurite extension. *Journal of Cell Biology* **133** 657-666.

Dent E.W. and Meiri K.F. (1998). Distribution of phosphorylated GAP-43 (neuromodulin) in growth cones directly reflects growth cone behavior. *Journal of Neurobiology* **35** 287-299.

Desai A. and Mitchison T.J. (1997). Microtubule polymerization dynamics. *Annual Review of Cell and Developmental Biology* **13:83-117.** 83-117.

Di Paolo G., Antonsson B., Kassel D., Riederer B.M. and Grenningloh G. (1997c). Phosphorylation regulates the microtubule-destabilizing activity of stathmin and its interaction with tubulin. *Federation European Biochemical Societies Letters* **416** 149-152.

Di Paolo G., Lutjens R., Osen-Sand A., Sobel A., Catsicas S. and Grenningloh G. (1997b). Differential distribution of stathmin and SCG10 in developing neurons in culture. *Journal of Neuroscience Research* **50** 1000-1009.

Di Paolo G., Lutjens R., Pellier V., Stimpson S.A., Beuchat M.H., Catsicas S. and Grenningloh G. (1997a). Targeting of SCG10 to the area of the Golgi complex is mediated by its NH2-terminal region. *Journal of Biological Chemistry* **272** 5175-5182.

Di Paolo G., Pellier V., Catsicas M., Antonsson B., Catsicas S. and Grenningloh G. (1996). The phosphoprotein stathmin is essential for nerve growth factor- stimulated differentiation. *Journal of Cell Biology* **133** 1383-1390.

DiTella M., Feiguin F., Morfini G. and Caceres A. (1994). Microfilament-associated growth cone component depends upon Tau for its intracellular localization. *Cell Motility and the Cytoskeleton* **29** 117-130.

Doherty P. and Walsh F.S. (1996). CAM-FGF receptor interactions: a model for axonal growth. *Molecular and Cellular Neuroscience* **8** 99-111.

Doolabh V.B. and Mackinnon S.E. (1999). FK506 accelerates functional recovery following nerve grafting in a rat model. *Plastic and Reconstructive Surgery* **103** 1928-1936.

Dooley, J. M. and Aguayo, A. J. Axonal elongation from cerebellum into peripheral nervous system grafts in the adult rat. Annals of Neurology 12, 221. 1982.

Doster S.K., Lozano A.M., Aguayo A.J. and Willard M.B. (1991). Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. *Neuron* **6** 635-647.

Dou C.L. and Levine J.M. (1994). Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. *Journal of Neuroscience* **14** 7616-7628.

Dragunow M. (1996). A role for immediate-early transcription factors in learning and memory. *Behavior Genetics* **26** 293-299.

Dragunow M., Young D., Hughes P., MacGibbon G., Lawlor P., Singleton K., Sirimanne E., Beilharz E. and Gluckman P. (1993). Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? *Brain Research. Molecular Brain Research* **18** 347-352.

Drazba J. and Lemmon V. (1990). The role of cell adhesion molecules in neurite outgrowth on Muller cells. *Developmental Biology* **138** 82-93.

Dumont F.J., Staruch M.J., Koprak S.L., Siekierka J.J., Lin C.S., Harrison R., Sewell T., Kindt V.M., Beattie T.R. and Wyvratt M. (1992). The immunosuppressive and toxic effects of FK-506 are mechanistically related: pharmacology of a novel antagonist of FK-506 and rapamycin. *Journal of Experimental Medicine* **176** 751-760.

Dyer J.K., Bourque J.A. and Steeves J.D. (1998). Regeneration of brainstem-spinal axons after lesion and immunological disruption of myelin in adult rat. *Experimental Neurology* **154** 12-22.

Eggen B.J., Nielander H.B., Rensen-de Leeuw M.G., Schotman P., Gispen W.H. and Schrama L.H. (1994). Identification of two promoter regions in the rat B-50/GAP-43 gene. *Brain Research. Molecular Brain Research* **23** 221-234.

Ehrlich Y.H. and Routtenberg A. (1974). Cyclic AMP regulates phosphorylation of three protein components of rat cerebral cortex membranes for thirty minutes. *Federation European Biochemical Societies Letters* **45** 237-243.

Elliott E.J., Parks D.A. and Fishman P.S. (1997). Effect of proximal axotomy on GAP-43 expression in cortical neurons in the mouse. *Brain Research* **755** 221-228.

Elliott E.J., Parks D.A. and Fishman P.S. (1999). Alpha 1-tubulin expression in proximally axotomized mouse cortical neurons. *Journal of Neurotrauma* **16** 333-339.

Eng L.F., Reier P.J. and Houle J.D. (1987). Astrocyte activation and fibrous gliosis: glial fibrillary acidic protein immunostaining of astrocytes following intraspinal cord grafting of fetal CNS tissue. *Progress in Brain Research* **71** 439-455.

Estep R.P., Alexander K.A. and Storm D.R. (1990). Regulation of free calmodulin levels in neurons by neuromodulin: relationship to neuronal growth and regeneration. *Current Topics in Cellular Regulation* **31** 161-180.

Fansa H., Keilhoff G., Horn T., Altmann S., Wolf G. and Schneider W. (1999). [Stimulation of Schwann cell growth and axon regeneration of peripheral nerves by the immunosuppressive drug FK 506]. *Handchirurgie, Mikrochirurgie, Plastische Chirurgie* **31** 323-329.

Farooque M., Badonic T., Olsson Y. and Holtz A. (1995). Astrocytic reaction after graded spinal cord compression in rats: immunohistochemical studies on glial fibrillary acidic protein and vimentin. *Journal of Neurotrauma* **12** 41-52.

Fawcett J.W. and Asher R.A. (1999). The glial scar and central nervous system repair. *Brain Research Bulletin* **49** 377-391.

Fawcett J.W. and Keynes R.J. (1990). Peripheral nerve regeneration. *Annual Review of Neuroscience* **13** 43-60.

Fawcett J.W., Rokos J. and Bakst I. (1989). Oligodendrocytes repel axons and cause axonal growth cone collapse. *Journal of Cell Science* **92 (Pt 1)** 93-100.

Felsenfeld D.P., Hynes M.A., Skoler K.M., Furley A.J. and Jessell T.M. (1994). TAG-1 can mediate homophilic binding, but neurite outgrowth on TAG-1 requires an L1-like molecule and beta 1 integrins. *Neuron* **12** 675-690.

Ferguson I.A., Koide T. and Rush R.A. (2001). Stimulation of corticospinal tract regeneration in the chronically injured spinal cord. *European Journal of Neuroscience* **13** 1059-1064.

Fernandes K.J., Fan D.P., Tsui B.J., Cassar S.L. and Tetzlaff W. (1999). Influence of the axotomy to cell body distance in rat rubrospinal and spinal motoneurons: differential regulation of GAP-43, tubulins, and neurofilament-M. *Journal of Comparative Neurology* **414** 495-510.

Fidler P.S., Schuette K., Asher R.A., Dobbertin A., Thornton S.R., Calle Patino Y., Muir E., Levine J.M., Geller H.M., Rogers J.H., Faissner A. and Fawcett J.W. (1999). Comparing astrocytic cell lines that are inhibitory or permissive for axon growth: the major axon-inhibitory proteoglycan is NG2. *Journal of Neuroscience* **19** 8778-8788.

Foster G.A., Schultzberg M., Gage F.H., Bjorklund A., Hokfelt T., Nornes H., Cuello A.C., Verhofstad A.A. and Visser T.J. (1985). Transmitter expression and morphological development of embryonic medullary and mesencephalic raphe neurones after transplantation to the adult rat central nervous system. I. Grafts to the spinal cord. *Experimental Brain Research* **60** 427-444.

Fournier A.E., GrandPre T. and Strittmatter S.M. (2001). Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* **409** 341-346.

Frey D., Laux T., Xu L., Schneider C. and Caroni P. (2000). Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. *Journal of Cell Biology* **149** 1443-1454.

Frey, D., Xu, L., Schneider, C., Meier, T., and Caroni, P. Role of CAP-23 in structural and functional plasticity at the adult neuromuscular junction. Neuroscience Abstracts 24, 1036-1036. 1998.

Friedlander D.R., Milev P., Karthikeyan L., Margolis R.K., Margolis R.U. and Grumet M. (1994). The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. *Journal of Cell Biology* **125** 669-680.

Frisen J., Johansson C.B., Torok C., Risling M. and Lendahl U. (1995). Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. *Journal of Cell Biology* **131** 453-464.

Frotscher M., Heimrich B. and Deller T. (1997). Sprouting in the hippocampus is layer-specific. *Trends in Neurosciences* **20** 218-223.

Fukura H., Komiya Y. and Igarashi M. (1996). Signaling pathway downstream of GABAA receptor in the growth cone. *Journal of Neurochemistry* **67** 1426-1434.

Funakoshi H., Frisen J., Barbany G., Timmusk T., Zachrisson O., Verge V.M. and Persson H. (1993). Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *Journal of Cell Biology* **123** 455-465.

Gavet O., Ozon S., Manceau V., Lawler S., Curmi P. and Sobel A. (1998). The stathmin phosphoprotein family: intracellular localization and effects on the microtubule network. *Journal of Cell Science* **111 (Pt 22)** 3333-3346.

Godement P., Salaun J. and Mason C.A. (1990). Retinal axon pathfinding in the optic chiasm: divergence of crossed and uncrossed fibers. *Neuron* **5** 173-186.

Gold B.G. (1997). FK506 and the role of immunophilins in nerve regeneration. *Molecular Neurobiology* **15** 285-306.

Gold B.G., Densmore V., Shou W., Matzuk M.M. and Gordon H.S. (1999). Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *J.Pharmacol.Exp.Ther.* **289** 1202-1210.

Gold B.G., Katoh K. and Storm-Dickerson T. (1995). The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *Journal of Neuroscience* **15** 7509-7516.

Gold B.G., Storm-Dickerson T. and Austin D.R. (1994). The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury. *Restorative Neurology and Neurosciences* **6** 287-296.

Gold B.G., Yew J.Y. and Zeleny-Pooley M. (1998a). The immunosuppressant FK506 increases GAP-43 mRNA levels in axotomized sensory neurons. *Neuroscience Letters* **241** 25-28.

Gold B.G., Zeleny-Pooley M., Chaturvedi P. and Wang M.S. (1998b). Oral administration of a nonimmunosuppressant FKBP-12 ligand speeds nerve regeneration. *NeuroReport* **9** 553-558.

Gold B.G., Zeleny-Pooley M., Wang M.S., Chaturvedi P. and Armistead D.M. (1997). A nonimmunosuppressant FKBP-12 ligand increases nerve regeneration. *Experimental Neurology* **147** 269-278.

Gordon-Weeks P.R. (1991). Evidence for microtubule capture by filopodial actin filaments in growth cones. *NeuroReport* **2** 573-576.

Gradin H.M., Larsson N., Marklund U. and Gullberg M. (1998). Regulation of microtubule dynamics by extracellular signals: cAMP-dependent protein kinase switches off the activity of oncoprotein 18 in intact cells. *Journal of Cell Biology* **140** 131-141.

GrandPre T., Nakamura F., Vartanian T. and Strittmatter S.M. (2000). Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403** 439-444.

Greenberg S.G. and Lasek R.J. (1988). Neurofilament protein synthesis in DRG neurons decreases more after peripheral axotomy than after central axotomy. *Journal of Neuroscience* **8** 1739-1746.

Greensmith L. and Vrbova G. (1996). Motoneurone survival: a functional approach. *Trends in Neurosciences* **19** 450-455.

Grill R., Murai K., Blesch A., Gage F.H. and Tuszynski M.H. (1997). Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. *Journal of Neuroscience* **17** 5560-5572.

Grothe C., Meisinger C. and Claus P. (2001). In vivo expression and localization of the fibroblast growth factor system in the intact and lesioned rat peripheral nerve and spinal ganglia. *Journal of Comparative Neurology* **434** 342-357.

Grumet M., Friedlander D.R. and Edelman G.M. (1993). Evidence for the binding of Ng-CAM to laminin. *Cell Adhesion and Communication* **1** 177-190.

Gunstream J.D., Castro G.A. and Walters E.T. (1995). Retrograde transport of plasticity signals in Aplysia sensory neurons following axonal injury. *Journal of Neuroscience* **15** 439-448.

Hai T. and Curran T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proceedings of the National Academy of Sciences USA* **88** 3720-3724.

Hai T., Wolfgang C.D., Marsee D.K., Allen A.E. and Sivaprasad U. (1999). ATF3 and stress responses. *Gene Expression* **7** 321-335.

Hall A. and Nobes C.D. (2000). Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philosophical Transactions of the Royal Society of London.Series B: Biological Sciences* **355** 965-970.

Hall S.M. (1986). Regeneration in cellular and acellular autografts in the peripheral nervous system. *Neuropathology and Applied Neurobiology* **12** 27-46.

Ham J., Babij C., Whitfield J., Pfarr C.M., Lallemand D., Yaniv M. and Rubin L.L. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14** 927-939.

Hamilton G.S. and Steiner J.P. (1998). Immunophilins: beyond immunosuppression. *Journal of Medicinal Chemistry* **41** 5119-5143.

Hammarberg H., Piehl F., Cullheim S., Fjell J., Hokfelt T. and Fried K. (1996). GDNF

mRNA in Schwann cells and DRG satellite cells after chronic sciatic nerve injury. *NeuroReport* **7** 857-860.

Haney C.A., Sahenk Z., Li C., Lemmon V.P., Roder J. and Trapp B.D. (1999). Heterophilic binding of L1 on unmyelinated sensory axons mediates Schwann cell adhesion and is required for axonal survival. *Journal of Cell Biology* **146** 1173-1184.

Hannan A.J., Henke R.C., Weinberger R.P., Sentry J.W. and Jeffrey P.L. (1996). Differential induction and intracellular localization of SCG10 messenger RNA is associated with neuronal differentiation. *Neuroscience* **72** 889-900.

Hansel C., Linden D.J. and D'Angelo E. (2001). Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nature Neuroscience* **4** 467-475.

Harding M.W., Galat A., Uehling D.E. and Schreiber S.L. (1989). A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* **341** 758-760.

Harper S., Bilsland J., Young L., Bristow L., Boyce S., Mason G., Rigby M., Hewson L., Smith D., O'Donnell R., O'Connor D., Hill R.G., Evans D., Swain C., Williams B. and Hefti F. (1999). Analysis of the neurotrophic effects of GPI-1046 on neuron survival and regeneration in culture and in vivo. *Neuroscience* **88** 257-267.

He Q., Dent E.W. and Meiri K.F. (1997). Modulation of actin filament behavior by GAP-43 (neuromodulin) is dependent on the phosphorylation status of serine 41, the protein kinase C site. *Journal of Neuroscience* **17** 3515-3524.

Hens J.J., Benfenati F., Nielander H.B., Valtorta F., Gispen W.H. and De Graan P.N. (1993). B-50/GAP-43 binds to actin filaments without affecting actin polymerization and filament organization. *Journal of Neurochemistry* **61** 1530-1533.

Herdegen T., Bastmeyer M., Bahr M., Stuermer C., Bravo R. and Zimmermann M. (1993b). Expression of JUN, KROX, and CREB transcription factors in goldfish and rat retinal ganglion cells following optic nerve lesion is related to axonal sprouting. *Journal of Neurobiology* **24** 528-543.

Herdegen T., Brecht S., Mayer B., Leah J., Kummer W., Bravo R. and Zimmermann M. (1993a). Long-lasting expression of JUN and KROX transcription factors and nitric oxide

synthase in intrinsic neurons of the rat brain following axotomy. *Journal of Neuroscience* **13** 4130-4145.

Herdegen T., Claret F.X., Kallunki T., Martin Villalba A., Winter C., Hunter T. and Karin M. (1998). Lasting N-terminal phosphorylation of c-Jun and activation of c- Jun N-terminal kinases after neuronal injury. *Journal of Neuroscience* **18** 5124-5135.

Herdegen T., Fiallos-Estrada C.E., Schmid W., Bravo R. and Zimmermann M. (1992). The transcription factors c-JUN, JUN D and CREB, but not FOS and KROX- 24, are differentially regulated in axotomized neurons following transection of rat sciatic nerve. *Brain Research. Molecular Brain Research* **14** 155-165.

Herdegen T. and Leah J.D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Research. Brain Research Reviews* **28** 370-490.

Herdegen T., Skene P. and Bahr M. (1997). The c-Jun transcription factor--bipotential mediator of neuronal death, survival and regeneration. *Trends in Neurosciences* **20** 227-231.

Heumann R., Korsching S., Bandtlow C. and Thoenen H. (1987). Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. *Journal of Cell Biology* **104** 1623-1631.

Hill C.E., Beattie M.S. and Bresnahan J.C. (2001). Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. *Experimental Neurology* **171** 153-169.

Hillenbrand R., Molthagen M., Montag D. and Schachner M. (1999). The close homologue of the neural adhesion molecule L1 (CHL1): patterns of expression and promotion of neurite outgrowth by heterophilic interactions. *European Journal of Neuroscience* **11** 813-826.

Himi T., Okazaki T., Wang H., McNeill T.H. and Mori N. (1994). Differential localization of SCG10 and p19/stathmin messenger RNAs in adult rat brain indicates distinct roles for these growth-associated proteins. *Neuroscience* **60** 907-926.

Hoffman P.N. and Cleveland D.W. (1988). Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific beta-tubulin isotype. *Proceedings of the National Academy of Sciences USA* **85** 4530-4533.

Hoffman P.N., Cleveland D.W., Griffin J.W., Landes P.W., Cowan N.J. and Price D.L. (1987). Neurofilament gene expression: a major determinant of axonal caliber. *Proceedings of the National Academy of Sciences USA* **84** 3472-3476.

Hoke A. and Silver J. (1996). Proteoglycans and other repulsive molecules in glial boundaries during development and regeneration of the nervous system. *Progress in Brain Research* **108** 149-163.

Holm J., Hillenbrand R., Steuber V., Bartsch U., Moos M., Lubbert H., Montag D. and Schachner M. (1996). Structural features of a close homologue of L1 (CHL1) in the mouse: a new member of the L1 family of neural recognition molecules. *European Journal of Neuroscience* **8** 1613-1629.

Hortsch M. (2000). Structural and functional evolution of the L1 family: are four adhesion molecules better than one? *Molecular and Cellular Neuroscience* **15** 1-10.

Houle J.D. and Reier P.J. (1988). Transplantation of fetal spinal cord tissue into the chronically injured adult rat spinal cord. *Journal of Comparative Neurology* **269** 535-547.

Houle J.D. and Reier P.J. (1989). Regrowth of calcitonin gene-related peptide (CGRP) immunoreactive axons from the chronically injured rat spinal cord into fetal spinal cord tissue transplants. *Neuroscience Letters* **103** 253-258.

Howell B., Larsson N., Gullberg M. and Cassimeris L. (1999). Dissociation of the tubulin-sequestering and microtubule catastrophe- promoting activities of oncoprotein 18/stathmin. *Molecular Biology of the Cell* **10** 105-118.

Hsu J.C., Bravo R. and Taub R. (1992). Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G1 phase of liver regeneration. *Mol.Cell Biol.* **12** 4654-4665.

Hsu J.C., Laz T., Mohn K.L. and Taub R. (1991). Identification of LRF-1, a leucine-

zipper protein that is rapidly and highly induced in regenerating liver. *Proceedings of the National Academy of Sciences USA* **88** 3511-3515.

Huang D.W., McKerracher L., Braun P.E. and David S. (1999). A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. *Neuron* **24** 639-647.

Hull M. and Bahr M. (1994). Differential regulation of c-JUN expression in rat retinal ganglion cells after proximal and distal optic nerve transection. *Neuroscience Letters* **178** 39-42.

Hüll M. and Bähr M. (1994). Regulation of immediate-early gene expression in rat retinal ganglion cells after axotomy and during regeneration through a peripheral nerve graft. *Journal of Neurobiology* **25** 92-105.

Ide C. (1996). Peripheral nerve regeneration. *Neuroscience Research* **25** 101-121.

Igarashi M., Strittmatter S.M., Vartanian T. and Fishman M.C. (1993). Mediation by G proteins of signals that cause collapse of growth cones. *Science* **259** 77-79.

Iino S., Kobayashi S. and Maekawa S. (1999). Immunohistochemical localization of a novel acidic calmodulin-binding protein, NAP-22, in the rat brain. *Neuroscience* **91** 1435-1444.

Iino S. and Maekawa S. (1999). Immunohistochemical demonstration of a neuronal calmodulin-binding protein, NAP-22, in the rat spinal cord. *Brain Research* **834** 66-73.

Itoh Y., Sugawara T., Kowada M. and Tessler A. (1992). Time course of dorsal root axon regeneration into transplants of fetal spinal cord: I. A light microscopic study. *Journal of Comparative Neurology* **323** 198-208.

Iwashita Y., Kawaguchi S. and Murata M. (1994). Restoration of function by replacement of spinal cord segments in the rat. *Nature* **367** 167-170.

Jap Tjoen S.E., Schmidt-Michels M., Oestreicher A.B., Gispen W.H. and Schotman P. (1992). Inhibition of nerve growth factor-induced B-50/GAP-43 expression by antisense oligomers interferes with neurite outgrowth of PC12 cells. *Biochemical and Biophysical*

Research Communications **187** 839-846.

Jayaraman T., Brillantes A.M., Timerman A.P., Fleischer S., Erdjument-Bromage H., Tempst P. and Marks A.R. (1992). FK506 binding protein associated with the calcium release channel (ryanodine receptor). *Journal of Biological Chemistry* **267** 9474-9477.

Jeffery N.D. and Fitzgerald M. (2001). Effects of red nucleus ablation and exogenous neurotrophin-3 on corticospinal axon terminal distribution in the adult rat. *Neuroscience* **104** 513-521.

Jenkins R. and Hunt S.P. (1991). Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. *Neuroscience Letters* **129** 107-110.

Jenkins R., McMahon S.B., Bond A.B. and Hunt S.P. (1993b). Expression of c-Jun as a response to dorsal root and peripheral nerve section in damaged and adjacent intact primary sensory neurons in the rat. *European Journal of Neuroscience* **5** 751-759.

Jenkins R., Tetzlaff W. and Hunt S.P. (1993a). Differential expression of immediate early genes in rubrospinal neurons following axotomy in rat. *European Journal of Neuroscience* **5** 203-209.

Johansson C.B., Momma S., Clarke D.L., Risling M., Lendahl U. and Frisen J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* **96** 25-34.

Johnson R.S., van Lingen B., Papaioannou V.E. and Spiegelman B.M. (1993). A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes and Development* **7** 1309-1317.

Jones EG (1985) The Thalamus. Plenum Press, New York.

Jost S.C., Doolabh V.B., Mackinnon S.E., Lee M. and Hunter D. (2000). Acceleration of peripheral nerve regeneration following FK506 administration. *Restorative Neurology and Neurosciences* **17** 39-44.

Jourdain L., Curmi P., Sobel A., Pantaloni D. and Carlier M.F. (1997). Stathmin: a

tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules. *Biochemistry* **36** 10817-10821.

Jung M., Petrausch B. and Stuermer C.A. (1997). Axon-regenerating retinal ganglion cells in adult rats synthesize the cell adhesion molecule L1 but not TAG-1 or SC-1. *Molecular and Cellular Neuroscience* **9** 116-131.

Kalil K. and Skene J.H.P. (1986). Elevated synthesis of an axonally transported protein correlates with axonal outgrowth in normal and injured pyramidal tracts. *Journal of Neuroscience* **6** 2563-2570.

Kamiguchi H., Hlavin M.L. and Lemmon V. (1998). Role of L1 in neural development: what the knockouts tell us. *Molecular and Cellular Neuroscience* **12** 48-55.

Karin M., Liu Z. and Zandi E. (1997). AP-1 function and regulation. *Current Opinion in Cell Biology* **9** 240-246.

Kater S.B. and Rehder V. (1995). The sensory-motor role of growth cone filopodia. *Current Opinion in Neurobiology* **5** 68-74.

Keirstead H.S., Hasan S.J., Muir G.D. and Steeves J.D. (1992). Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. *Proceedings of the National Academy of Sciences USA* **89** 11664-11668.

Kennedy T.E. (2000). Cellular mechanisms of netrin function: long-range and short-range actions. *Biochemistry and Cell Biology* **78** 569-575.

Kenney A.M. and Kocsis J.D. (1997). Temporal variability of jun family transcription factor levels in peripherally or centrally transected adult rat dorsal root ganglia. *Brain Research. Molecular Brain Research* **52** 53-61.

Kenney A.M. and Kocsis J.D. (1998). Peripheral axotomy induces long-term c-Jun amino-terminal kinase-1 activation and activator protein-1 binding activity by c-Jun and junD in adult rat dorsal root ganglia In vivo. *Journal of Neuroscience* **18** 1318-1328.

Klintsova A.Y. and Greenough W.T. (1999). Synaptic plasticity in cortical systems. *Current Opinion in Neurobiology* **9** 203-208.

Kohn D.T., Tsai K.C., Cansino V.V., Neve R.L. and Perrone-Bizzozero N.I. (1996). Role of highly conserved pyrimidine-rich sequences in the 3' untranslated region of the GAP-43 mRNA in mRNA stability and RNA- protein interactions. *Brain Research. Molecular Brain Research* **36** 240-250.

Kruger S., Sievers J., Hansen C., Sadler M. and Berry M. (1986). Three morphologically distinct types of interface develop between adult host and fetal brain transplants: implications for scar formation in the adult central nervous system. *Journal of Comparative Neurology* **249** 103-116.

Kuhn T.B., Stoeckli E.T., Condrau M.A., Rathjen F.G. and Sonderegger P. (1991). Neurite outgrowth on immobilized axonin-1 is mediated by a heterophilic interaction with L1(G4). *Journal of Cell Biology* **115** 1113-1126.

Landreth G.E. and Agranoff B.W. (1976). Explant culture of adult goldfish retina: effect of prior optic nerve crush. *Brain Research* **118** 299-303.

Larsson N., Marklund U., Gradin H.M., Brattsand G. and Gullberg M. (1997). Control of microtubule dynamics by oncoprotein 18: dissection of the regulatory role of multisite phosphorylation during mitosis. *Mol.Cell Biol.* **17** 5530-5539.

Larsson N., Segerman B., Gradin H.M., Wandzioch E., Cassimeris L. and Gullberg M. (1999a). Mutations of oncoprotein 18/stathmin identify tubulin-directed regulatory activities distinct from tubulin association. *Mol.Cell Biol.* **19** 2242-2250.

Larsson N., Segerman B., Howell B., Fridell K., Cassimeris L. and Gullberg M. (1999b). Op18/stathmin mediates multiple region-specific tubulin and microtubule- regulating activities. *Journal of Cell Biology* **146** 1289-1302.

Laux T., Fukami K., Thelen M., Golub T., Frey D. and Caroni P. (2000). GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *Journal of Cell Biology* **149** 1455-1472.

Leah J.D., Herdegen T. and Bravo R. (1991). Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: evidence for

a role in the regeneration process. *Brain Research* **566** 198-207.

Leah J.D., Herdegen T., Murashov A., Dragunow M. and Bravo R. (1993). Expression of immediate early gene proteins following axotomy and inhibition of axonal transport in the rat central nervous system. *Neuroscience* **57** 53-66.

Lee M., Doolabh V.B., Mackinnon S.E. and Jost S. (2000). FK506 promotes functional recovery in crushed rat sciatic nerve. *Muscle Nerve* **23** 633-640.

Lee S.K. and Wolfe S.W. (2000). Peripheral nerve injury and repair. *Journal of the American Academy of Orthopaedic Surgery* **8** 243-252.

Lehmann M., Fournier A., Selles-Navarro I., Dergham P., Sebok A., Leclerc N., Tigyi G. and McKerracher L. (1999). Inactivation of Rho signaling pathway promotes CNS axon regeneration. *Journal of Neuroscience* **19** 7537-7547.

Lemmon V., Farr K.L. and Lagenaur C. (1989). L1-mediated axon outgrowth occurs via a homophilic binding mechanism. *Neuron* **2** 1597-1603.

Leon S., Yin Y., Nguyen J., Irwin N. and Benowitz L.I. (2000). Lens injury stimulates axon regeneration in the mature rat optic nerve. *Journal of Neuroscience* **20** 4615-4626.

Letourneau P.C. and Ressler A.H. (1984). Inhibition of neurite initiation and growth by taxol. *Journal of Cell Biology* **98** 1355-1362.

Lewin-Kowalik J., Gorka D., Larysz-Brysz M., Goka B., Swiech-Sabuda E., Malecka-Tendera E. and Krause M. (1997). Short-time predegenerated peripheral nerve grafts promote regrowth of injured hippocampal neurites. *ACTA Physiologica Hungarica (Budapest)* **85** 259-268.

Lewin-Kowalik J., Sieron A.L., Krause M., Barski J.J. and Gorka D. (1992). Time-dependent regenerative influence of predegenerated nerve grafts on hippocampus. *Brain Research Bulletin* **29** 831-835.

Lewis A.K. and Bridgman P.C. (1992). Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity. *Journal of Cell Biology* **119** 1219-1243.

Li M., Shibata A., Li C., Braun P.E., McKerracher L., Roder J., Kater S.B. and David S. (1996). Myelin-associated glycoprotein inhibits neurite/axon growth and causes growth cone collapse. *Journal of Neuroscience Research* **46** 404-414.

Li Y., Field P.M. and Raisman G. (1997). Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells [see comments]. *Science* **277** 2000-2002.

Li Y., Field P.M. and Raisman G. (1998). Regeneration of adult rat corticospinal axons induced by transplanted olfactory ensheathing cells. *Journal of Neuroscience* **18** 10514-10524.

Li Y. and Raisman G. (1993). Long axon growth from embryonic neurons transplanted into myelinated tracts of the adult rat spinal cord. *Brain Research* **629** 115-127.

Li Y. and Raisman G. (1994). Schwann cells induce sprouting in motor and sensory axons in the adult rat spinal cord. *Journal of Neuroscience* **14** 4050-4063.

Liabotis S. and Schreyer D.J. (1995). Magnitude of GAP-43 induction following peripheral axotomy of adult rat dorsal root ganglion neurons is independent of lesion distance. *Experimental Neurology* **135** 28-35.

Lieberman A.R. (1971). The axon reaction: a review of the principal features of perikaryal responses to axon injury. *International Review of Neurobiology* **14** 49-124.

Lieberman AR (2001) Some factors affecting retrograde neuronal responses to axonal lesions. In: Essays on the nervous system (eds Bellairs R, Grey EG), pp. 71-105. Clarendon Press, Oxford.

Lin C.H. and Forscher P. (1993). Cytoskeletal remodeling during growth cone-target interactions. *Journal of Cell Biology* **121** 1369-1383.

Lin C.H., Thompson C.A. and Forscher P. (1994). Cytoskeletal reorganization underlying growth cone motility. *Current Opinion in Neurobiology* **4** 640-647.

Liu Y., Fisher D.A. and Storm D.R. (1993). Analysis of the palmitoylation and membrane targeting domain of neuromodulin (GAP-43) by site-specific mutagenesis. *Biochemistry* **32** 10714-10719.

Liu Y., Fisher D.A. and Storm D.R. (1994). Intracellular sorting of neuromodulin (GAP-43) mutants modified in the membrane targeting domain. *Journal of Neuroscience* **14** 5807-5817.

Liu Y.C., Chapman E.R. and Storm D.R. (1991). Targeting of neuromodulin (GAP-43) fusion proteins to growth cones in cultured rat embryonic neurons. *Neuron* **6** 411-420.

Liu Y.C. and Storm D.R. (1989). Dephosphorylation of neuromodulin by calcineurin. *Journal of Biological Chemistry* **264** 12800-12804.

Liu Y.C. and Storm D.R. (1990). Regulation of free calmodulin levels by neuromodulin: neuron growth and regeneration. *Trends in Pharmacological Sciences* **11** 107-111.

Liuzzi F.J. and Lasek R.J. (1987). Astrocytes block axonal regeneration in mammals by activating the physiological stop mechanism. *Science* **237** 642-645.

Lodish H.F. and Kong N. (1991). Cyclosporin A inhibits an initial step in folding of transferrin within the endoplasmic reticulum. *Journal of Biological Chemistry* **266** 14835-14838.

Loewy A.D. and Schader R.E. (1977). A quantitative study of retrograde neuronal changes in Clarke's column. *Journal of Comparative Neurology* **171** 65-81.

Lombillo V.A., Stewart R.J. and McIntosh J.R. (1995). Minus-end-directed motion of kinesin-coated microspheres driven by microtubule depolymerization. *Nature* **373** 161-164.

Lu B. and Gottschalk W. (2000). Modulation of hippocampal synaptic transmission and plasticity by neurotrophins. *Progress in Brain Research* **128** 231-241.

Luckenbill-Edds L. (1997). Laminin and the mechanism of neuronal outgrowth. *Brain Research. Brain Research Reviews* **23** 1-27.

Luo L. (2000). Rho GTPases in neuronal morphogenesis. *Nature Reviews Neuroscience* **1** 173-180.

Lutjens R., Igarashi M., Pellier V., Blasey H., Di Paolo G., Ruchti E., Pfulg C., Staple J.K., Catsicas S. and Grenningloh G. (2000). Localization and targeting of SCG10 to the trans-

Golgi apparatus and growth cone vesicles. *European Journal of Neuroscience* **12** 2224-2234.

Lyons W.E., George E.B., Dawson T.M., Steiner J.P. and Snyder S.H. (1994). Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. *Proceedings of the National Academy of Sciences USA* **91** 3191-3195.

Lyons W.E., Steiner J.P., Snyder S.H. and Dawson T.M. (1995). Neuronal regeneration enhances the expression of the immunophilin FKBP- 12. *Journal of Neuroscience* **15** 2985-2994.

Ma M., Basso D.M., Walters P., Stokes B.T. and Jakeman L.B. (2001). Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. *Experimental Neurology* **169** 239-254.

Madsen J.R., MacDonald P., Irwin N., Goldberg D.E., Yao G.L., Meiri K.F., Rimm I.J., Stieg P.E. and Benowitz L.I. (1998). Tacrolimus (FK506) increases neuronal expression of GAP-43 and improves functional recovery after spinal cord injury in rats. *Experimental Neurology* **154** 673-683.

Maekawa S., Kumanogoh H., Funatsu N., Takei N., Inoue K., Endo Y., Hamada K. and Sokawa Y. (1997). Identification of NAP-22 and GAP-43 (neuromodulin) as major protein components in a Triton insoluble low density fraction of rat brain. *Biochimica et Biophysica ACTA* **1323** 1-5.

Maekawa S., Maekawa M., Hattori S. and Nakamura S. (1993). Purification and molecular cloning of a novel acidic calmodulin binding protein from rat brain. *Journal of Biological Chemistry* **268** 13703-13709.

Maekawa S., Matsuura Y. and Nakamura S. (1994a). Expression and myristoylation of NAP-22 using a baculovirus transfer vector system. *Biochimica et Biophysica ACTA* **1218** 119-122.

Maekawa S., Murofushi H. and Nakamura S. (1994b). Inhibitory effect of calmodulin on phosphorylation of NAP-22 with protein kinase C. *Journal of Biological Chemistry* **269** 19462-19465.

Maekawa S., Sato C., Kitajima K., Funatsu N., Kumanogoh H. and Sokawa Y. (1999). Cholesterol-dependent localization of NAP-22 on a neuronal membrane microdomain (raft). *Journal of Biological Chemistry* **274** 21369-21374.

Maier D.L., Mani S., Donovan S.L., Soppet D., Tessarollo L., McCasland J.S. and Meiri K.F. (1999). Disrupted cortical map and absence of cortical barrels in growth- associated protein (GAP)-43 knockout mice. *Proceedings of the National Academy of Sciences USA* **96** 9397-9402.

Marklund U., Larsson N., Gradin H.M., Brattsand G. and Gullberg M. (1996). Oncoprotein 18 is a phosphorylation-responsive regulator of microtubule dynamics. *European Molecular Biology Organization Journal* **15** 5290-5298.

Martini R. (1994). Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. *Journal of Neurocytology* **23** 1-28.

Martini R. and Schachner M. (1986). Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. *Journal of Cell Biology* **103** 2439-2448.

Martini R. and Schachner M. (1988). Immunoelectron microscopic localization of neural cell adhesion molecules (L1, NCAM and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerves. *Journal of Cell Biology* **106** 1735-1746.

Martini R., Schachner M. and Faissner A. (1990). Enhanced expression of the extracellular matrix molecule J1/tenascin in the regenerating adult mouse sciatic nerve. *Journal of Neurocytology* **19** 601-616.

Mason M.R.J., Campbell G., Caroni P., Anderson P.N. and Lieberman A.R. (2000). Overexpression of GAP-43 in thalamic projection neurons does not enable them to regenerate into peripheral nerve grafts. *Experimental Neurology* **165** 143-152.

McGuire C.B., Snipes G.J. and Norden J.J. (1988). Light-microscopic immunolocalization of the gr. *Brain Research* **469** 277-291.

McKeon R.J., Schreiber R.C., Rudge J.S. and Silver J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *Journal of Neuroscience* **11** 3398-3411.

McKerracher L., Chamoux M. and Arregui C.O. (1996). Role of laminin and integrin interactions in growth cone guidance. *Molecular Neurobiology* **12** 95-116.

McKerracher L., David S., Jackson D.L., Kottis V., Dunn R.J. and Braun P.E. (1994). Identification of myelin-associated glycoprotein as a major myelin- derived inhibitor of neurite growth. *Neuron* **13** 805-811.

McNally F.J. (1999). Microtubule dynamics: Controlling split ends. *Current Biology* **9** R274-R276.

McNeill T.H., Mori N. and Cheng H.W. (1999). Differential regulation of the growth-associated proteins, GAP-43 and SCG-10, in response to unilateral cortical ablation in adult rats. *Neuroscience* **90** 1349-1360.

Meberg P.J. and Routtenberg A. (1991). Selective expression of protein F1/(GAP-43) mRNA in pyramidal but not granule cells of the hippocampus. *Neuroscience* **45** 721-733.

Meiri K.F. and Gordon-Weeks P.R. (1990). GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. *Journal of Neuroscience* **10** 256-266.

Meiri K.F., Hammang J.P., Dent E.W. and Baetge E.E. (1996). Mutagenesis of ser41 to ala inhibits the association of GAP-43 with the membrane skeleton of GAP-43-deficient PC12B cells: effects on cell adhesion and the composition of neurite cytoskeleton and membrane. *Journal of Neurobiology* **29** 213-232.

Meiri K.F., Saffell J.L., Walsh F.S. and Doherty P. (1998). Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. *Journal of Neuroscience* **18** 10429-10437.

Melander G.H., Marklund U., Larsson N., Chatila T.A. and Gullberg M. (1997).

Regulation of microtubule dynamics by Ca2+/calmodulin-dependent kinase IV/Gr-dependent phosphorylation of oncoprotein 18. *Mol.Cell Biol.* **17** 3459-3467.

Mellitzer G., Xu Q. and Wilkinson D.G. (2000). Control of cell behaviour by signalling through Eph receptors and ephrins. *Current Opinion in Neurobiology* **10** 400-408.

Mesulam M-M (1982) Tracing Neural Connections with Horseradish Peroxidase. Wiley Interscience, Chichester.

Meyer M., Matsuoka I., Wetmore C., Olson L. and Thoenen H. (1992). Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *Journal of Cell Biology* **119** 45-54.

Mikucki S.A. and Oblinger M.M. (1991). Corticospinal neurons exhibit a novel pattern of cytoskeletal gene expression after injury. *Journal of Neuroscience Research* **30** 213-225.

Milbrandt J. (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* **238** 797-799.

Milev P., Friedlander D.R., Sakurai T., Karthikeyan L., Flad M., Margolis R.K., Grumet M. and Margolis R.U. (1994). Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *Journal of Cell Biology* **127** 1703-1715.

Miller F.D., Tetzlaff W., Bisby M.A., Fawcett J.W. and Milner R.J. (1989). Rapid induction of the major embryonic alpha-tubulin mRNA, T alpha 1, during nerve regeneration in adult rats. *Journal of Neuroscience* **9** 1452-1463.

Miller M.W. (1987). The origin of corticospinal projection neurons in rat. *Experimental Brain Research* **67** 339-351.

Ming G.L., Song H.J., Berninger B., Holt C.E., Tessier-Lavigne M. and Poo M.M. (1997). cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19** 1225-1235.

Mirsky R., Jessen K.R., Schachner M. and Goridis C. (1986). Distribution of the adhesion molecules N-CAM and L1 on peripheral neurons and glia in adult rats. *Journal of Neurocytology* **15** 799-815.

Misantone L.J., Gershenbaum M. and Murray M. (1984). Viability of retinal ganglion cells after optic nerve crush in adult rats. *Journal of Neurocytology* **13** 449-465.

Mitchison T. and Kirschner M. (1984). Dynamic instability of microtubule growth. *Nature* **312** 237-242.

Mobarak C.D., Anderson K.D., Morin M., Beckel-Mitchener A., Rogers S.L., Furneaux H., King P. and Perrone-Bizzozero N.I. (2000). The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP- 43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. *Molecular Biology of the Cell* **11** 3191-3203.

Moffett S.B. (1995). Neural regeneration in gastropod molluscs. *Progress in Neurobiology* **46** 289-330.

Montgomery A.M., Becker J.C., Siu C.H., Lemmon V.P., Cheresh D.A., Pancook J.D., Zhao X. and Reisfeld R.A. (1996). Human neural cell adhesion molecule L1 and rat homologue NILE are ligands for integrin alpha v beta 3. *Journal of Cell Biology* **132** 475-485.

Moon L.D., Asher R.A., Rhodes K.E. and Fawcett J.W. (2001). Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nature Neuroscience* **4** 465-466.

Morrow D.R., Campbell G., Lieberman A.R. and Anderson P.N. (1993). Differential regenerative growth of CNS axons into tibial and peroneal nerve grafts in the thalamus of adult rats. *Experimental Neurology* **120** 60-69.

Moscoso L.M. and Sanes J.R. (1995). Expression of four immunoglobulin superfamily adhesion molecules (L1, Nr-CAM/Bravo, neurofascin/ABGP, and N-CAM) in the developing mouse spinal cord. *Journal of Comparative Neurology* **352** 321-334.

Mosevitsky M.I., Capony J.P., Skladchikova G.Y., Novitskaya V.A., Plekhanov A.Y. and Zakharov V.V. (1997). The BASP1 family of myristoylated proteins abundant in axonal

termini. Primary structure analysis and physico-chemical properties. *Biochimie* **79** 373-384.

Mosevitsky M.I., Novitskaya V.A., Plekhanov A.Y. and Skladchikova G.Y. (1994). Neuronal protein GAP-43 is a member of novel group of brain acid- soluble proteins (BASPs). *Neuroscience Research* **19** 223-228.

Moss D.J., Fernyhough P., Chapman K., Baizer L., Bray D. and Allsopp T. (1990). Chicken growth-associated protein GAP-43 is tightly bound to the actin- rich neuronal membrane skeleton. *Journal of Neurochemistry* **54** 729-736.

Mukhopadhyay G., Doherty P., Walsh F.S., Crocker P.R. and Filbin M.T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13** 757-767.

Mulderry P.K. and Dobson S.P. (1996). Regulation of VIP and other neuropeptides by c-Jun in sensory neurons: implications for the neuropeptide response to axotomy. *European Journal of Neuroscience* **8** 2479-2491.

Muma N.A., Hoffman P.N., Slunt H.H., Applegate M.D., Lieberburg I. and Price D.L. (1990). Alterations in levels of mRNAs coding for neurofilament protein subunits during regeneration. *Experimental Neurology* **107** 230-235.

Murray M., Wang S.D., Goldberger M.E. and Levitt P. (1990). Modification of astrocytes in the spinal cord following dorsal root or peripheral nerve lesions. *Experimental Neurology* **110** 248-257.

Nadim W., Anderson P.N. and Turmaine M. (1990). The role of Schwann cells and basal lamina tubes in the regeneration of axons through long lengths of freeze-killed nerve grafts. *Neuropathology and Applied Neurobiology* **16** 411-421.

Namgung U. and Routtenberg A. (2000). Transcriptional and post-transcriptional regulation of a brain growth protein: regional differentiation and regeneration induction of GAP-43. *European Journal of Neuroscience* **12** 3124-3136.

Neumann S. and Woolf C.J. (1999). Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron* **23** 83-91.

Neve R.L., Finch E.A., Bird E.D. and Benowitz L.I. (1988). Growth-associated protein GAP-43 is expressed selectively in associative regions of the adult human brain. *Proceedings of the National Academy of Sciences USA* **85** 3638-3642.

Neve R.L., Perrone Bizzozero N.I., Finklestein S., Zwiers H., Bird E., Kurnit D.M. and Benowitz L.I. (1987). The neuronal growth-associated protein GAP-43 (B-50, F1): neuronal specificity, developmental regulation and regional distribution of the human and rat mRNAs. *Brain Research* **388** 177-183.

Ng K.K. and Weis W.I. (1998). Coupling of prolyl peptide bond isomerization and Ca2+ binding in a C-type mannose-binding protein. *Biochemistry* **37** 17977-17989.

Nicholls J. and Saunders N. (1996). Regeneration of immature mammalian spinal cord after injury. *Trends in Neurosciences* **19** 229-234.

Nielander H.B., French P., Oestreicher A.B., Gispen W.H. and Schotman P. (1993). Spontaneous morphological changes by overexpression of the growth- associated protein B-50/GAP-43 in a PC12 cell line. *Neuroscience Letters* **162** 46-50.

Nishizawa K. (1994). NGF-induced stabilization of GAP-43 mRNA is mediated by both 3' untranslated region and a segment encoding the carboxy-terminus peptide. *Biochemical and Biophysical Research Communications* **200** 789-796.

Noble L.J. and Wrathall J.R. (1985). Spinal cord contusion in the rat: morphometric analyses of alterations in the spinal cord. *Experimental Neurology* **88** 135-149.

Nornes H., Bjorklund A. and Stenevi U. (1983). Reinnervation of the denervated adult spinal cord of rats by intraspinal transplants of embryonic brain stem neurons. *Cell and Tissue Research* **230** 15-35.

Nothias F. and Peschanski M. (1990). Homotypic fetal transplants into an experimental model of spinal cord neurodegeneration. *Journal of Comparative Neurology* **301** 520-534.

O'Connor T.P., Duerr J.S. and Bentley D. (1990). Pioneer growth cone steering decisions mediated by single filopodial contacts in situ. *Journal of Neuroscience* **10** 3935-3946.

Obata S., Obata J., Das A. and Gilbert C.D. (1999). Molecular correlates of topographic reorganization in primary visual cortex following retinal lesions. *Cerebral Cortex* **9** 238-248.

Oblinger M.M. and Lasek R.J. (1988). Axotomy-induced alterations in the synthesis and transport of neurofilaments and microtubules in dorsal root ganglion cells. *Journal of Neuroscience* **8** 1747-1758.

Ocain T.D., Longhi D., Steffan R.J., Caccese R.G. and Sehgal S.N. (1993). A nonimmunosuppressive triene-modified rapamycin analog is a potent inhibitor of peptidyl prolyl cis-trans isomerase. *Biochemical and Biophysical Research Communications* **192** 1340-1346.

Oestreicher A.B., De Graan P.N., Gispen W.H., Verhaagen J. and Schrama L.H. (1997). B-50, the growth associated protein-43: modulation of cell morphology and communication in the nervous system. *Progress in Neurobiology* **53** 627-686.

Ohara P.T. and Lieberman A.R. (1985). The thalamic reticular nucleus of the adult rat: experimental anatomical studies. *Journal of Neurocytology* **14** 365-411.

Ohbayashi K., Fukura H., Inoue H.K., Komiya Y. and Igarashi M. (1998). Stimulation of L-type Ca2+ channel in growth cones activates two independent signaling pathways. *Journal of Neuroscience Research* **51** 682-696.

Okazaki T., Yoshida B.N., Avraham K.B., Wang H., Wuenschell C.W., Jenkins N.A., Copeland N.G., Anderson D.J. and Mori N. (1993). Molecular diversity of the SCG10/stathmin gene family in the mouse. *Genomics* **18** 360-373.

Okuno H. and Miyashita Y. (1996). Expression of the transcription factor Zif268 in the temporal cortex of monkeys during visual paired associate learning. *European Journal of Neuroscience* **8** 2118-2128.

Oudega M., Varon S. and Hagg T. (1994). Regeneration of adult rat sensory axons into intraspinal nerve grafts: promoting effects of conditioning lesion and graft predegeneration. *Experimental Neurology* **129** 194-206.

Ozon S., Byk T. and Sobel A. (1998). SCLIP: a novel SCG10-like protein of the stathmin

family expressed in the nervous system. *Journal of Neurochemistry* **70** 2386-2396.

Ozon S., El Mestikawy S. and Sobel A. (1999). Differential, regional, and cellular expression of the stathmin family transcripts in the adult rat brain. *Journal of Neuroscience Research* **56** 553-564.

Ozon S., Maucuer A. and Sobel A. (1997). The stathmin family -- molecular and biological characterization of novel mammalian proteins expressed in the nervous system. *European Journal of Biochemistry* **248** 794-806.

Paino C.L., Fernandez-Valle C., Bates M.L. and Bunge M.B. (1994). Regrowth of axons in lesioned adult rat spinal cord: promotion by implants of cultured Schwann cells. *Journal of Neurocytology* **23** 433-452.

Parker E.M., Monopoli A., Ongini E., Lozza G. and Babij C.M. (2000). Rapamycin, but not FK506 and GPI-1046, increases neurite outgrowth in PC12 cells by inhibiting cell cycle progression. *Neuropharmacology* **39** 1913-1919.

Paulsen O. and Sejnowski T.J. (2000). Natural patterns of activity and long-term synaptic plasticity. *Current Opinion in Neurobiology* **10** 172-179.

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. Academic Press, Sydney.

Perrone-Bizzozero N., Neve R.L., Irwin N., Lewis S., Fischer I. and Benowitz L.I. (1991). Post-transcriptional regulation of GAP-43 levels during neuronal differentiation and nerve regeneration. *Molecular and Cellular Neuroscience* **2** 402-409.

Perrone-Bizzozero N.I., Cansino V.V. and Kohn D.T. (1993). Posttranscriptional regulation of GAP-43 gene expression in PC12 cells through protein kinase C-dependent stabilization of the mRNA. *Journal of Cell Biology* **120** 1263-1270.

Pesheva P. and Probstmeier R. (2000). The yin and yang of tenascin-R in CNS development and pathology. *Progress in Neurobiology* **61** 465-493.

Petrausch B., Tabibiazar R., Roser T., Jing Y., Goldman D., Stuermer C.A., Irwin N. and Benowitz L.I. (2000). A purine-sensitive pathway regulates multiple genes involved in

axon regeneration in goldfish retinal ganglion cells. *Journal of Neuroscience* **20** 8031-8041.

Prinjha R., Moore S.E., Vinson M., Blake S., Morrow R., Christie G., Michalovich D., Simmons D.L. and Walsh F.S. (2000). Inhibitor of neurite outgrowth in humans. *Nature* **403** 383-384.

Pu S.F., Zhuang H.X. and Ishii D.N. (1995). Differential spatio-temporal expression of the insulin-like growth factor genes in regenerating sciatic nerve. *Brain Research. Molecular Brain Research* **34** 18-28.

Quesada M.H., Millar D.B. and Smejkal R. (1986). Tubulin synthesis in the regenerating rat superior cervical ganglion: a biphasic response. *Journal of Neurobiology* **17** 77-82.

Ramon-Cueto A. (2000). Olfactory ensheathing glia transplantation into the injured spinal cord. *Progress in Brain Research* **128** 265-272.

Ramon-Cueto A., Cordero M.I., Santos-Benito F.F. and Avila J. (2000). Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. *Neuron* **25** 425-435.

Ramon-Cueto A., Plant G.W., Avila J. and Bunge M.B. (1998). Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. *Journal of Neuroscience* **18** 3803-3815.

Raper J.A. (2000). Semaphorins and their receptors in vertebrates and invertebrates. *Current Opinion in Neurobiology* **10** 88-94.

Rathjen F.G. (1988). A neurite outgrowth-promoting molecule in developing fiber tracts. *Trends in Neurosciences* **11** 183-184.

Reh T.A., Redshaw J.D. and Bisby M.A. (1987). Axons of the pyramidal tract do not increase their transport of growth-associated proteins after axotomy. *Brain Research* **388** 1-6.

Reier P.J. and Houle J.D. (1988). The glial scar: its bearing on axonal elongation and transplantation approaches to CNS repair. *Advances in Neurology* **47** 87-138.

Reier PJ, Stensaas LJ, Guth L (1983) The Astrocytic Scar As an Impediment to Regeneration in the Central Nervous System. In: Spinal Cord Reconstruction (eds Kao CC, Bunge RP, Reier PJ), pp. 163-194. Raven Press, New York.

Renaudie F., Yachou A.K., Grandchamp B., Jones R. and Beaumont C. (1992). A second ferritin L subunit is encoded by an intronless gene in the mouse. *Mammalian Genome* **2** 143-149.

Richardson P.M., Issa V.M. and Aguayo A.J. (1984). Regeneration of long spinal axons in the rat. *Journal of Neurocytology* **13** 165-182.

Richardson P.M., Issa V.M. and Shemie S. (1982b). Regeneration and retrograde degeneration of axons in the rat optic nerve. *Journal of Neurocytology* **11** 949-966.

Richardson P.M. and Issa V.M.K. (1984). Peripheral injury enhances central regeneration of primary sensory neurones. *Nature* **309** 791-793.

Richardson P.M., McGuinness U.M. and Aguayo A.J. (1980). Axons from CNS neurons regenerate into PNS grafts. *Nature* **284** 264-265.

Richardson P.M., McGuinness U.M. and Aguayo A.J. (1982a). Peripheral nerve autografts to the rat spinal cord: studies with axonal tracing methods. *Brain Research* **237(1)** 147-162.

Richardson P.M. and Verge V.M.K. (1986). The induction of a regenerative propensity in sensory neurons following peripheral axonal injury. *Journal of Neurocytology* **15** 585-594.

Richardson P.M. and Verge V.M.K. (1987). Axonal regeneration in dorsal spinal roots is accelerated by peripheral axonal transection. *Brain Research* **411** 406-408.

Riederer B.M., Pellier V., Antonsson B., Di Paolo G., Stimpson S.A., Lutjens R., Catsicas S. and Grenningloh G. (1997). Regulation of microtubule dynamics by the neuronal growth-associated protein SCG10. *Proceedings of the National Academy of Sciences USA* **94** 741-745.

Robinson G.A. (1994). Immediate early gene expression in axotomized and regenerating

retinal ganglion cells of the adult rat. *Brain Research. Molecular Brain Research* **24** 43-54.

Robinson G.A. (1995). Axotomy-induced regulation of c-Jun expression in regenerating rat retinal ganglion cells. *Brain Research. Molecular Brain Research* **30** 61-69.

Ruppert M., Aigner S., Hubbe M., Yagita H. and Altevogt P. (1995). The L1 adhesion molecule is a cellular ligand for VLA-5. *Journal of Cell Biology* **131** 1881-1891.

Rutishauser U., Acheson A., Hall A.K., Mann D.M. and Sunshine J. (1988). The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science* **240** 53-57.

Sabatini D.M., Erdjument-Bromage H., Lui M., Tempst P. and Snyder S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78** 35-43.

Saffell J.L., Williams E.J., Mason I.J., Walsh F.S. and Doherty P. (1997). Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. *Neuron* **18** 231-242.

Salzer J.L. and Bunge R.P. (1980). Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. *Journal of Cell Biology* **84** 739-752.

Sauer B. (1998). Inducible gene targeting in mice using the Cre/lox system. *Methods* **14** 381-392.

Saunders N.R., Balkwill P., Knott G., Habgood M.D., Mollgard K., Treherne J.M. and Nicholls J.G. (1992). Growth of axons through a lesion in the intact CNS of fetal rat maintained in long-term culture. *Proceedings of the Royal Society of London.Series B: Biological Sciences* **250** 171-180.

Savio T. and Schwab M.E. (1989). Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. *Journal of Neuroscience* **9** 1126-1133.

Savio T. and Schwab M.E. (1990). Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. *Proceedings of the National Academy of Sciences USA* **87** 4130-4133.

Schiene C. and Fischer G. (2000). Enzymes that catalyse the restructuring of proteins. *Current Opinion in Structural Biology* **10** 40-45.

Schinder A.F. and Poo M. (2000). The neurotrophin hypothesis for synaptic plasticity. *Trends in Neurosciences* **23** 639-645.

Schlingensiepen K.H., Luno K. and Brysch W. (1991). High basal expression of the zif/268 immediate early gene in cortical layers IV and VI, in CA1 and in the corpus striatum--an in situ hybridization study. *Neuroscience Letters* **122** 67-70.

Schneider S., Bosse F., D'Urso D., Muller H., Sereda M.W., Nave K., Niehaus A., Kempf T., Schnolzer M. and Trotter J. (2001). The AN2 protein is a novel marker for the Schwann cell lineage expressed by immature and nonmyelinating Schwann cells. *Journal of Neuroscience* **21** 920-933.

Schnell L., Schneider R., Kolbeck R., Barde Y.A. and Schwab M.E. (1994). Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* **367** 170-173.

Schnell L. and Schwab M.E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* **343** 269-272.

Schnell L. and Schwab M.E. (1993). Sprouting and regeneration of lesioned corticospinal tract fibres in the adult rat spinal cord. *European Journal of Neuroscience* **5** 1156-1171.

Schreiber S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* **251** 283-287.

Schreyer D.J. and Skene J.H. (1993). Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *Journal of Neurobiology* **24** 959-970.

Schubart U.K., Banerjee M.D. and Eng J. (1989). Homology between the cDNAs

encoding phosphoprotein p19 and SCG10 reveals a novel mammalian gene family preferentially expressed in developing brain. *DNA* **8** 389-398.

Schwab M.E. and Caroni P. (1988). Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *Journal of Neuroscience* **8** 2381-2393.

Seilheimer B. and Schachner M. (1988). Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neuron growth on Schwann cells in culture. *Journal of Cell Biology* **107** 341-351.

Sendtner M., Stockli K.A. and Thoenen H. (1992). Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *Journal of Cell Biology* **118** 139-148.

Shadiack A.M., Vaccariello S.A., Sun Y. and Zigmond R.E. (1998). Nerve growth factor inhibits sympathetic neurons' response to an injury cytokine. *Proceedings of the National Academy of Sciences USA* **95** 7727-7730.

Shea T.B., Perrone-Bizzozero N.I., Beermann M.L. and Benowitz L.I. (1991). Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neuritogenesis. *Journal of Neuroscience* **11** 1685-1690.

Shen, Y. P., Schwob, J. E., and Meiri, K. F. GAP-43 is required for forebrain commissure formation in mice. Society for Neuroscience Abstracts 108.6. 2000.

Shibuya Y., Mizoguchi A., Takeichi M., Shimada K. and Ide C. (1995). Localization of N-cadherin in the normal and regenerating nerve fibers of the chicken peripheral nervous system. *Neuroscience* **67** 253-261.

Shimizu I., Oppenheim R.W., O'Brien M. and Shneiderman A. (1990). Anatomical and functional recovery following spinal cord transection in the chick embryo. *Journal of Neurobiology* **21** 918-937.

Shou W., Aghdasi B., Armstrong D.L., Guo Q., Bao S., Charng M.J., Mathews L.M., Schneider M.D., Hamilton S.L. and Matzuk M.M. (1998). Cardiac defects and altered

ryanodine receptor function in mice lacking FKBP12. *Nature* **391** 489-492.

Siekierka J.J., Hung S.H., Poe M., Lin C.S. and Sigal N.H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341** 755-757.

Sievers J., Hausmann B. and Berry M. (1989). Fetal brain grafts rescue adult retinal ganglion cells from axotomy- induced cell death. *Journal of Comparative Neurology* **281** 467-478.

Sievers J., Hausmann B., Unsicker K. and Berry M. (1987). Fibroblast growth factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. *Neuroscience Letters* **76** 157-162.

Silver J. (1994). Inhibitory molecules in development and regeneration. *Journal of Neurology* **242** S22-4.

Simons K. and Toomre D. (2000). Lipid rafts and signal transduction. *Nature Reviews Molecular Cell Biology* **1** 31-39.

Skene J.H. (1989). Axonal growth-associated proteins. *Annual Review of Neuroscience* **12** 127-156.

Skene J.H. and Virag I. (1989). Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *Journal of Cell Biology* **108** 613-624.

Skene J.H. and Willard M. (1981). Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. *Journal of Cell Biology* **89** 86-95.

Slemmon J.R. and Martzen M.R. (1994). Neuromodulin (GAP-43) can regulate a calmodulin-dependent target in vitro. *Biochemistry* **33** 5653-5660.

Smith-Thomas L.C., Fok-Seang J., Stevens J., Du J.S., Muir E., Faissner A., Geller H.M., Rogers J.H. and Fawcett J.W. (1994). An inhibitor of neurite outgrowth produced by astrocytes. *Journal of Cell Science* **107 (Pt 6)** 1687-1695.

Smith G.V. and Stevenson J.A. (1988). Peripheral nerve grafts lacking viable Schwann

cells fail to support central nervous system axonal regeneration. *Experimental Brain Research* **69** 299-306.

Snow D.M., Lemmon V., Carrino D.A., Caplan A.I. and Silver J. (1990). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Experimental Neurology* **109** 111-130.

So K.F. and Aguayo A.J. (1985). Lengthy regrowth of cut axons from ganglion cells after peripheral nerve transplantation into the retina of adult rats. *Brain Research* **328** 349-354.

Sofroniew M.V. and Isacson O. (1988). Distribution of degeneration of cholinergic neurons in the septum following axotomy in different portions of the fimbria-fornix: a correlation between degree of cell loss and proximity of neuronal somata to the lesion. *Journal of Chemical Neuroanatomy* **1** 327-337.

Song H., Ming G., He Z., Lehmann M., McKerracher L., Tessier-Lavigne M. and Poo M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* **281** 1515-1518.

Song H.J., Ming G.L. and Poo M.M. (1997). cAMP-induced switching in turning direction of nerve growth cones. *Nature* **388** 275-279.

Song H.J. and Poo M.M. (1999). Signal transduction underlying growth cone guidance by diffusible factors. *Current Opinion in Neurobiology* **9** 355-363.

Sretavan D.W. and Kruger K. (1998). Randomized retinal ganglion cell axon routing at the optic chiasm of GAP-43-deficient mice: association with midline recrossing and lack of normal ipsilateral axon turning. *Journal of Neuroscience* **18** 10502-10513.

Stallcup W.B. and Beasley L. (1985). Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. *Proceedings of the National Academy of Sciences USA* **82** 1276-1280.

Stein R., Mori N., Matthews K., Lo L.C. and Anderson D.J. (1988b). The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. *Neuron* **1** 463-476.

Stein R., Orit S. and Anderson D.J. (1988a). The induction of a neural-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, and glucocorticoid inhibitable. *Developmental Biology* **127** 316-325.

Steiner J.P., Connolly M.A., Valentine H.L., Hamilton G.S., Dawson T.M., Hester L. and Snyder S.H. (1997b). Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin and cyclosporin A. *Nature Medicine* **3** 421-428.

Steiner J.P., Hamilton G.S., Ross D.T., Valentine H.L., Guo H., Connolly M.A., Liang S., Ramsey C., Li J.H., Huang W., Howorth P., Soni R., Fuller M., Sauer H., Nowotnik A.C. and Suzdak P.D. (1997a). Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proceedings of the National Academy of Sciences USA* **94** 2019-2024.

Steinmann B., Bruckner P. and Superti-Furga A. (1991). Cyclosporin A slows collagen triple-helix formation in vivo: indirect evidence for a physiologic role of peptidyl-prolyl cis-trans-isomerase. *Journal of Biological Chemistry* **266** 1299-1303.

Stichel C.C., Hermanns S., Luhmann H.J., Lausberg F., Niermann H., D'Urso D., Servos G., Hartwig H.G. and Muller H.W. (1999). Inhibition of collagen IV deposition promotes regeneration of injured CNS axons. *European Journal of Neuroscience* **11** 632-646.

Strata P. and Rossi F. (1998). Plasticity of the olivocerebellar pathway. *Trends in Neurosciences* **21** 407-413.

Strittmatter S.M., Fankhauser C., Huang P.L., Mashimo H. and Fishman M.C. (1995). Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell* **80** 445-452.

Strittmatter S.M., Vartanian T. and Fishman M.C. (1992). GAP-43 as a plasticity protein in neuronal form and repair. *Journal of Neurobiology* **23** 507-520.

Sudo Y., Valenzuela D., Beck-Sickinger A.G., Fishman M.C. and Strittmatter S.M. (1992). Palmitoylation alters protein activity: blockade of G(o) stimulation by GAP-43. *European Molecular Biology Organization Journal* **11** 2095-2102.

Sugiura Y. and Mori N. (1995). SCG10 expresses growth-associated manner in developing rat brain, but shows a different pattern to p19/stathmin or GAP-43. *Develomental Brain Research* **90** 73-91.

Suter D.M. and Forscher P. (2000). Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance. *Journal of Neurobiology* **44** 97-113.

Takasaki A., Hayashi N., Matsubara M., Yamauchi E. and Taniguchi H. (1999). Identification of the calmodulin-binding domain of neuron-specific protein kinase C substrate protein CAP-22/NAP-22. Direct involvement of protein myristoylation in calmodulin-target protein interaction. *Journal of Biological Chemistry* **274** 11848-11853.

Takeda M., Kato H., Takamiya A., Yoshida A. and Kiyama H. (2000). Injury-specific expression of activating transcription factor-3 in retinal ganglion cells and its colocalized expression with phosphorylated c-Jun. *Investigative Ophthalmology and Visual Science* **41** 2412-2421.

Tanaka E., Ho T. and Kirschner M.W. (1995). The role of microtubule dynamics in growth cone motility and axonal growth. *Journal of Cell Biology* **128** 139-155.

Tanaka E. and Kirschner M.W. (1995). The role of microtubules in growth cone turning at substrate boundaries. *Journal of Cell Biology* **128** 127-137.

Tang S., Woodhall R.W., Shen Y.J., DeBellard M.E., Saffell J.L., Doherty P., Walsh F.S. and Filbin M.T. (1997). Soluble myelin-associated glycoprotein (MAG) found in vivo inhibits axonal regeneration. *Molecular and Cellular Neuroscience* **9** 333-346.

Tatagiba M., Brosamle C. and Schwab M.E. (1997). Regeneration of injured axons in the adult mammalian central nervous system. *Neurosurgery* **40** 541-546.

Tello F. (1911). La influencia del neurotropismo en la regeneracion de los centros nerviosos. *Trab.Lab.Invest.Biol.* **9** 123-159.

Terenghi G. (1999). Peripheral nerve regeneration and neurotrophic factors. *Journal of Anatomy* **194 (Pt 1)** 1-14.

Tessler A., Himes B.T., Houle J. and Reier P.J. (1988). Regeneration of adult dorsal root

axons into transplants of embryonic spinal cord. *Journal of Comparative Neurology* **270** 537-548.

Tetzlaff W., Alexander S.W., Miller F.D. and Bisby M.A. (1991). Response of facial and rubrospinal neurons to axotomy: changes in cytoskeletal proteins and GAP-43. *Journal of Neuroscience* **11** 2528-2544.

Tetzlaff W., Bisby M.A. and Kreutzberg G.W. (1988). Changes in cytoskeletal proteins in the rat facial nucleus following axotomy. *Journal of Neuroscience* **8** 3181-3189.

Tetzlaff, W. and Giehl, K. M. G. Axotomized corticospinal neurons increase GAP-43 mRNA after subcortical lesions but not after pyramidial lesions. Third IBRO World Congress of Neuroscience, p220. 1991.

Tetzlaff W., Kobayashi N.R., Giehl K.M., Tsui B.J., Cassar S.L. and Bedard A.M. (1994). Response of rubrospinal and corticospinal neurons to injury and neurotrophins. *Progress in Brain Research* **103** 271-286.

Thoenen H. (2000). Neurotrophins and activity-dependent plasticity. *Progress in Brain Research* **128** 183-191.

Timerman A.P., Wiederrecht G., Marcy A. and Fleischer S. (1995). Characterization of an exchange reaction between soluble FKBP-12 and the FKBP.ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl-prolyl isomerase activity. *Journal of Biological Chemistry* **270** 2451-2459.

Timpl R. (1996). Macromolecular organization of basement membranes. *Current Opinion in Cell Biology* **8** 618-624.

Tobin G.R., Chvapil M. and Gildenberg P.L. (1979). Collagen biosynthesis in healing spinal cord wounds. *Surg.Forum* **30** 454-456.

Tran P.T., Walker R.A. and Salmon E.D. (1997). A metastable intermediate state of microtubule dynamic instability that differs significantly between plus and minus ends. *Journal of Cell Biology* **138** 105-117.

Tsai K.C., Cansino V.V., Kohn D.T., Neve R.L. and Perrone-Bizzozero N.I. (1997). Post-

transcriptional regulation of the GAP-43 gene by specific sequences in the 3' untranslated region of the mRNA. *Journal of Neuroscience* **17** 1950-1958.

Tseng G.F. and Prince D.A. (1993). Heterogeneity of rat corticospinal neurons. *Journal of Comparative Neurology* **335** 92-108.

Tseng G.F. and Prince D.A. (1996). Structural and functional alterations in rat corticospinal neurons after axotomy. *Journal of Neurophysiology* **75** 248-267.

Tsujino H., Kondo E., Fukuoka T., Dai Y., Tokunaga A., Miki K., Yonenobu K., Ochi T. and Noguchi K. (2000). Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. *Molecular and Cellular Neuroscience* **15** 170-182.

Tucker R.P. (1990). The roles of microtubule-associated proteins in brain morphogenesis: a review. *Brain Research. Brain Research Reviews* **15** 101-120.

Tuszynski M.H., Gabriel K., Gage F.H., Suhr S., Meyer S. and Rosetti A. (1996). Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. *Experimental Neurology* **137** 157-173.

Tuszynski M.H., Peterson D.A., Ray J., Baird A., Nakahara Y. and Gage F.H. (1994). Fibroblasts genetically modified to produce nerve growth factor induce robust neuritic ingrowth after grafting to the spinal cord. *Experimental Neurology* **126** 1-14.

van der Zee C.E., Nielander H.B., Vos J.P., Lopes d.S., Verhaagen J., Oestreicher A.B., Schrama L.H., Schotman P. and Gispen W.H. (1989). Expression of growth-associated protein B-50 (GAP43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. *Journal of Neuroscience* **9** 3505-3512.

van der Zee C.E.E.M., Nielander H.B., Vos J.P., da Silva S.L., Verhaagen J., Oestreicher A.B., Schrama L.H., Schotman P. and Gispen W.H. (1989). Expression of growth-associated protein B-50 (GAP-43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. *Journal of Neuroscience* **9** 3505-3512.

Vaudano E., Campbell G., Anderson P.N., Davies A.P., Woolhead C., Schreyer D.J. and

Lieberman A.R. (1995). The effects of a lesion or a peripheral nerve graft on GAP-43 upregulation in the adult rat brain: an in situ hybridization and immunocytochemical study. *Journal of Neuroscience* **15** 3594-3611.

Vaudano E., Campbell G., Hunt S.P. and Lieberman A.R. (1998). Axonal injury and peripheral nerve grafting in the thalamus and cerebellum of the adult rat: upregulation of c-jun and correlation with regenerative potential. *European Journal of Neuroscience* **10** 2644-2656.

Verge V.M.K., Tetzlaff W., Richardson P.M. and Bisby M.A. (1990). Correlation between GAP-43 and nerve growth factor receptors in rat sensory neurons. *Journal of Neuroscience* **10** 926-934.

Verhaagen J., Hermens W.T., Oestreicher A.B., Gispen W.H., Rabkin S.D., Pfaff D.W. and Kaplitt M.G. (1994). Expression of the growth-associated protein B-50/GAP43 via a defective herpes-simplex virus vector results in profound morphological changes in non-neuronal cells. *Brain Research. Molecular Brain Research* **26** 26-36.

Volonte C. and Greene L.A. (1992). Nerve growth factor-activated protein kinase N. Characterization and rapid near homogeneity purification by nucleotide affinity-exchange chromatography. *Journal of Biological Chemistry* **267** 21663-21670.

von Euler M., Seiger A. and Sundstrom E. (1997). Clip compression injury in the spinal cord: a correlative study of neurological and morphological alterations. *Experimental Neurology* **145** 502-510.

von Meyenburg J., Brosamle C., Metz G.A. and Schwab M.E. (1998). Regeneration and sprouting of chronically injured corticospinal tract fibers in adult rats promoted by NT-3 and the mAb IN-1, which neutralizes myelin-associated neurite growth inhibitors. *Experimental Neurology* **154** 583-594.

Walczak C.E. (2000). Microtubule dynamics and tubulin interacting proteins. *Current Opinion in Cell Biology* **12** 52-56.

Waller A. (1850). Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of

their primitive fibres. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **140** 423-429.

Walsh F.S. and Doherty P. (1997). Neural cell adhesion molecules of the immunoglobulin superfamily: role in axon growth and guidance. *Annual Review of Cell and Developmental Biology* **13** 425-456.

Wang M.S. and Gold B.G. (1999). FK506 increases the regeneration of spinal cord axons in a predegenerated peripheral nerve autograft. *Journal of Spinal Cord Medicine* **22** 287-296.

Wang T., Li B.Y., Danielson P.D., Shah P.C., Rockwell S., Lechleider R.J., Martin J., Manganaro T. and Donahoe P.K. (1996). The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* **86** 435-444.

Watanabe E. and Murakami F. (1989). Preferential adhesion of chick central neurons to the gray matter of the central nervous system. *Neuroscience Letters* **97** 69-74.

Watanabe E. and Murakami F. (1990). Cell attachment to and neurite outgrowth on tissue sections of developing, mature and lesioned brain: the role of inhibitory factor(s) in the CNS white matter. *Neuroscience Research* **8** 83-99.

Weber J.R. and Skene J.H. (1998). The activity of a highly promiscuous AP-1 element can be confined to neurons by a tissue-selective repressive element. *Journal of Neuroscience* **18** 5264-5274.

Weidner N., Grill R.J. and Tuszynski M.H. (1999). Elimination of basal lamina and the collagen "scar" after spinal cord injury fails to augment corticospinal tract regeneration. *Experimental Neurology* **160** 40-50.

Wictorin K. and Bjorklund A. (1992). Axon outgrowth from grafts of human embryonic spinal cord in the lesioned adult rat spinal cord. *NeuroReport* **3** 1045-1048.

Wictorin K., Brundin P., Gustavii B., Lindvall O. and Bjorklund A. (1990). Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. *Nature* **347** 556-558.

Widmer F. and Caroni P. (1990). Identification, localization, and primary structure of CAP-23, a particle-bound cytosolic protein of early development. *Journal of Cell Biology* **111** 3035-3047.

Widmer F. and Caroni P. (1993). Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *Journal of Cell Biology* **120** 503-512.

Wiederkehr A., Staple J. and Caroni P. (1997). The motility-associated proteins GAP-43, MARCKS, and CAP-23 share unique targeting and surface activity-inducing properties. *Experimental Cell Research* **236** 103-116.

Williams E.J., Doherty P., Turner G., Reid R.A., Hemperly J.J. and Walsh F.S. (1992). Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. *Journal of Cell Biology* **119** 883-892.

Williams E.J., Furness J., Walsh F.S. and Doherty P. (1994b). Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron* **13** 583-594.

Williams E.J., Walsh F.S. and Doherty P. (1994a). The production of arachidonic acid can account for calcium channel activation in the second messenger pathway underlying neurite outgrowth stimulated by NCAM, N-cadherin, and L1. *Journal of Neurochemistry* **62** 1231-1234.

Williamson T., Gordon-Weeks P.R., Schachner M. and Taylor J. (1996). Microtubule reorganization is obligatory for growth cone turning. *Proceedings of the National Academy of Sciences USA* **93** 15221-15226.

Winder D.G. and Schramm N.L. (2001). Plasticity and behavior. New genetic techniques to address multiple forms and functions. *Physiology and Behavior* **73** 763-780.

Wong E.V., Kenwrick S., Willems P. and Lemmon V. (1995). Mutations in the cell adhesion molecule L1 cause mental retardation. *Trends in Neurosciences* **18** 168-172.

Wong E.V., Schaefer A.W., Landreth G. and Lemmon V. (1996). Involvement of p90rsk in neurite outgrowth mediated by the cell adhesion molecule L1. *Journal of Biological*

Chemistry **271** 18217-18223.

Wong J. and Oblinger M.M. (1987). Changes in neurofilament gene expression occur after axotomy of dorsal root ganglion neurons: an in situ hybridization study. *Metabolic Brain Disease* **2** 291-303.

Woolf C.J., Reynolds M.L., Chong M.S., Emson P., Irwin N. and Benowitz L.I. (1992b). Denervation of the motor endplate results in the rapid expression by terminal Schwann cells of the growth-associated protein GAP-43. *Journal of Neuroscience* **12** 3999-4010.

Woolf C.J., Reynolds M.L., Molander C., O'Brien C., Lindsay R.M. and Benowitz L.I. (1990). GAP-43 a growth associated protein, appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. *Neuroscience* **34** 465-478.

Woolf C.J., Shortland P. and Coggeshall R.E. (1992a). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* **355** 75-78.

Woolf C.J., Shortland P., Reynolds M.L., Ridings J., Doubell T.P. and Coggeshall R.E. (1995). Central regenerative sprouting: the reorganization of the central terminals of myelinated primary afferents in the rat dorsal horn following peripheral nerve section or crush. *Journal of Comparative Neurology* **360** 121-134.

Woolhead C.L., Zhang Y., Lieberman A.R., Schachner M., Emson P.C. and Anderson P.N. (1998). Differential effects of autologous peripheral nerve grafts to the corpus striatum of adult rats on the regeneration of axons of striatal and nigral neurons and on the expression of GAP-43 and the cell adhesion molecules N-CAM and L1. *Journal of Comparative Neurology* **391** 259-273.

Wu W., Mathew T.C. and Miller F.D. (1993). Evidence that the loss of homeostatic signals induces regeneration- associated alterations in neuronal gene expression. *Developmental Biology* **158** 456-466.

Xu X.M., Guenard V., Kleitman N., Aebischer P. and Bunge M.B. (1995). A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. *Experimental Neurology* **134** 261-272.

Xu X.M. and Martin G.F. (1991). Evidence for new growth and regeneration of cut axons in developmental plasticity of the rubrospinal tract in the North American opossum. *Journal of Comparative Neurology* **313** 103-112.

Yankner B.A., Benowitz L.I., Villa Komaroff L. and Neve R.L. (1990). Transfection of PC12 cells with the human GAP-43 gene: effects on neurite outgrowth and regeneration. *Brain Research. Molecular Brain Research* **7** 39-44.

Yao G.L. and Kiyama H. (1995). Colchicine induces the GAP-43 gene expression in rat hypothalamus. *Brain Research. Molecular Brain Research* **30** 373-377.

Ye J.H. and Houle J.D. (1997). Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. *Experimental Neurology* **143** 70-81.

Zagrebelsky M., Buffo A., Skerra A., Schwab M.E., Strata P. and Rossi F. (1998). Retrograde regulation of growth-associated gene expression in adult rat Purkinje cells by myelin-associated neurite growth inhibitory proteins. *Journal of Neuroscience* **18** 7912-7929.

Zarow C. and Finch C.E. (1995). Limited responses of neuronal mRNAs to unilateral lesions of the rat entorhinal cortex. *Neuroscience Letters* **185** 87-90.

Zhang Y., Campbell G., Anderson P.N., Martini R., Schachner M. and Lieberman A.R. (1995). Molecular basis of interactions between regenerating adult rat thalamic axons and Schwann cells in peripheral nerve grafts I. Neural cell adhesion molecules. *Journal of Comparative Neurology* **361** 193-209.

Zhang Y., Dijkhuizen P.A., Anderson P.N., Lieberman A.R. and Verhaagen J. (1998). NT-3 delivered by an adenoviral vector induces injured dorsal root axons to regenerate back into the spinal cord. *Journal of Neuroscience Research* **54** 554-562.

Zhang Y., Roslan R., Lang D., Schachner M., Lieberman A.R. and Anderson P.N. (2000). Expression of CHL1 and L1 by neurons and glia following sciatic nerve and dorsal root injury. *Molecular and Cellular Neuroscience* **16** 71-86.

Zhang Y., Tohyama K., Winterbottom J.K., Haque N.S., Schachner M., Lieberman A.R.

and Anderson P.N. (2001). Correlation between Putative Inhibitory Molecules at the Dorsal Root Entry Zone and Failure of Dorsal Root Axonal Regeneration. *Molecular and Cellular Neuroscience* **17** 444-459.

Zhang Y., Winterbottom J.K., Schachner M., Lieberman A.R. and Anderson P.N. (1997). Tenascin-C expression and axonal sprouting following injury to the spinal dorsal columns in the adult rat. *Journal of Neuroscience Research* **49** 433-450.

Zhu Q. and Julien J.P. (1999). A key role for GAP-43 in the retinotectal topographic organization. *Experimental Neurology* **155** 228-242.

Zigmond R.E. and Sun Y. (1997). Regulation of neuropeptide expression in sympathetic neurons. Paracrine and retrograde influences. *Annals of the New York Academy of Science* **814** 181-197.

Zuber M.X., Goodman D.W., Karns L.R. and Fishman M.C. (1989). The neuronal growth-associated protein GAP-43 induces filopodia in non-neuronal cells. *Science* **244** 1193-1195.

Zuo J., Hernandez Y.J. and Muir D. (1998). Chondroitin sulfate proteoglycan with neurite-inhibiting activity is up- regulated following peripheral nerve injury. *Journal of Neurobiology* **34** 41-54.

Zwiers H., Veldhuis H.D., Schotman P. and Gispen W.H. (1976). ACTH, cyclic nucleotides, and brain protein phosphorylation *in vitro*. *Neurochemical Research* **1** 669-677.