Investigating the expression of the topographic guidance molecules, EphA5 and ephrin-A2, as well as metallothionein function, in the injured and regenerating adult mammalian visual system

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Abstract

During development of the visual system, topographic connections between the retina and the *superior colliculus* are established using guidance molecules. The EphA family of tyrosine kinase receptors and their ephrin-A ligands are important for establishing topography between the temporo-nasal axis of the retina and the rostro-caudal axis of the *superior colliculus*. After injury to the visual system *via* unilateral optic nerve transection, adult mammalian retinal ganglion cells fail to regenerate axons spontaneously to their main visual centre, which in rodents, is the *superior colliculus*. The EphA5 gradient is down-regulated from a temporal^{high} to nasal^{low} gradient to a uniform low level in the few surviving retinal ganglion cells, but ephrin-A2 is up-regulated back to a significant rostral^{low} to caudal^{high} gradient in the *superior colliculus*, similar to that seen during development.

In this thesis, a number of experiments have been undertaken to investigate further how EphA5 and ephrin-A2 are regulated after injury and how they may play a role once regeneration has been encouraged through surgical intervention.

In the first study, targeted unilateral retinal laser lesions were used to ablate either dorso-nasal or ventro-temporal quadrants of the retina. These specific lesions either denervate the crossed projection only, or both the contralateral and ipsilateral projection. Retinae and brains were examined by immunohistochemistry and *in situ* hybridization for EphA5 in the retina and ephrin-A2 in the *superior colliculus*. The results imply that EphA5 and ephrin-A2 are regulated through trans-synaptic connections.

In the second study, the expression of guidance molecules was studied in the peripheral nerve graft model of adult mammalian central nervous system regeneration. It has been shown that the regenerated projection formed in the *superior colliculus* does not have a precise topographic map. However, there is a small, but significant tendency for pairs of retinal ganglion cells to project to a topographically correct location along the rostral-caudal axis of the *superior colliculus* in relation to each another. Peripheral nerve graft surgery was carried out, and *in situ* hybridization and immunohistochemistry used to investigate EphA5 expression in the retina and ephrin-A2 expression in the *superior colliculus* and peripheral nerve graft. These results suggest that EphA5 and ephrin-A2 expression have the potential to guide the regenerating axons which enter the *superior*

colliculus to a topographically appropriate location. The data also support an additional role of ephrin-A2 locally at the insertion site, inhibiting more regenerating axons from entering the *superior colliculus*.

The third study investigated the potential of metallothionein-I/II as a neuroprotective and neuroregenerative molecule after injury to the visual system. Metallothionein-I/II overexpression *in vitro* has been shown to increase neuronal survival and exogenous application increases neurite extension in culture, and overcomes inhibition by myelin and myelin-associated glycoprotein. After optic nerve transection, a bolus dose of metallothionein-I/II was administered. Surviving and regenerating retinal ganglion cells in the retina, and axons in the optic nerve, were analysed. The data suggest that metallothionein-I/II increases axonal regeneration through the optic nerve injury site but, at the dose administered, had no neuroprotective effects on retinal ganglion cells.

This thesis provides further insight into the response of guidance molecules to injury, and the potential of metallothionein-I/II as a neuroregenerative factor in the adult mammalian visual system. The regulation of both EphA5 and ephrin-A2 through trans-synaptic connections may be a response common to other guidance molecules. Such connectivity now needs to be studied further to understand how it may impact on various treatments designed to increase re-connectivity after other brain injuries, including stroke. The ectopic expression of ephrin-A2 at the insertion site of a peripheral nerve graft in the *superior colliculus*, implicate this guidance molecule in the glial scar for the first time. Therefore, to overcome inhibition by the glial scar, axons must also overcome ephrin-A2 mediated inhibition, potentially by the addition of EphA5 fusion proteins. Metallothionein-I/II's effect of increasing axonal regeneration through the optic nerve injury site suggests that it could be used to increase the number of regenerating axons reaching their target. Such strategies to increase the absolute number of regenerated axons should enable these axons to better use the EphA5 and ephrin-A2 topographic gradients to optimize regenerative success.

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List of Abbreviations Used in this Thesis

3’-UTR	3’-untranslated region
AP	Alkaline phosphatase
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
C	Caudal
cAMP	Cyclic adenosine triphosphate
CNS	Central nervous system
CNTF	Cillary neurotrophic factor
CSPG	Chondroitin sulphate proteoglycans
CTβ	Cholera toxin β
d	Day(s)
DEPC	Diethyl pyrocarbonate
DIG	Digoxygenin
DN	Dorso-nasal
dpi	Dots per inch
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fab	Fragment antigen binding
FG	FluroGold
g	Gram(s)
G	Gauge
GAP-43	Growth associated protein-43
GDP	Guanosine diphosphate
GFAP	Glial Fibrillary Acidic Protein
GPI	Glycosylphosphatidyl inositol
GTP	Guanosine triphosphate
h	Hour(s)
IGF-1	Insulin-like growth factor-1
IFN-γ	Interferon-γ
IL-1	Interleukin-1
kDa	KiloDaltons
kg	Kilogram(s)
LIF	Leukaemia inhibitory factor
MAG	Myelin-associated glycoprotein),

M	Molar
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar
mRNA	Messenger ribonucleic acid
mW	Milliwatt(s)
µl	Microlitre(s)
µm	Micrometre(s)
N	Nasal
NBT/BCIP	Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate
nm	Nanometre(s)
NT-4/5	Neurotrophin-4/5
OCT	Optimal cutting temperature
OMgp	Oligodendrocyte myelin glycoprotein
PBS	Phosphate buffered saline
PM	Plasma membrane
PN	Peripheral nerve
R	Rostral
RGCs	Retinal ganglion cells
RT	Room temperature
s	Second(s)
SSC	Saline sodium citrate
SC	*Superior colliculus*
SZ	*Stratum zonale*
SGS	*Stratum griseum superficiale*
SO	*Stratum opticum*
SGI	*Stratum griseum intermediale*
SAI	*Stratum album intermedium*
SGP	*Stratum griseum profundum*
SAP	*Stratum album profundum*
T	Temporal
TBS	Tris buffered saline
TNF-α	Tumour necrosis factor-α
VT	Ventro-temporal

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Statement of Candidate Contribution

Chapter 2 of this thesis has been published as an article with multiple authors:
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Eph/ephrin expression in the adult rat visual system following localized retinal lesions: localized and transneuronal up-regulation in the retina and *superior colliculus*

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I confirm that I, Andrew Symonds carried out 70% of experimental work and data collection, including tissue preparation and processing, immunohistochemistry, *in situ* hybridization and data analysis for all the immunohistochemistry and *in situ* hybridization. I also carried out 35% of the writing including input to the introduction, material and methods and discussion.

All other work in this thesis is the work of the candidate, Andrew Symonds.

Andrew Symonds
All the authors have given permission for the published work to be included in the thesis.

Andrew Symonds | Professor Lyn Beazley

Professor Sarah Dunlop | Dr. Jennifer Rodger

Chapter 1:

General Introduction

Background

The complexity of the nervous system is such that we are still very much in the infancy of our understanding. One of the great challenges facing neuroscience research is to understand the response of the nervous system to injury.

The adult mammalian central nervous system, composed of the retina, brain and spinal cord, cannot spontaneously regenerate axon tracts after injury (Ramon y Cajal, 1991). Central neurons do not regenerate their axons due to both extrinsic and intrinsic factors (Domeniconi & Filbin, 2005). Extrinsic factors include the numerous inhibitory molecules expressed at the lesion site and beyond (Sandvig *et al.*, 2004), lack of morphological substrate to support axon regeneration (Barron, 2004) and lack of neurotrophic factors secreted by neuroglia (Fawcett & Keynes, 1990). Intrinsic factors refer to the lack of survival and regeneration potential of central neurons themselves (Barron, 1984; Goldberg *et al.*, 2002). The consequence of this failure of axon regeneration is that injuries to the brain or spinal cord are permanent; there is currently no cure.

The impact of neurotrauma can be seen in a broad cross section of our community. While spinal cord injury can leave patients paralysed, after stroke, patients can lose various functions including movement and speech, and a major component of progressive neurodegenerative diseases, such as Alzheimer's disease, is the inability of damaged neurons to regenerate. The failure of adult mammalian central nervous system to regenerate axons means that neurotrauma affects both old and young and can have a broad and devastating impact.

Pre-requisites for successful functional recovery

To achieve successful functional recovery after injury to the central nervous system at least four steps are necessary. After the glial limiting membrane between the central nervous system and periphery has been re-established (Reier, 1985), cell death must be minimized, and secondly, surviving neurons must regenerate axons either through the lesion site (Goldshmit *et al.*, 2004) or through a reconstructed pathway to their original targets (Vidal-Sanz *et al.*, 1987). Once regenerating axons reach their targets they must form appropriate connections to re-establish pathways, with a similar organization to that seen before injury (Sperry, 1943). Finally, these connections must form functional

synapses that can relay information to other parts of the brain (Keirstead *et al.*, 1989; Sauvé *et al.*, 1995).

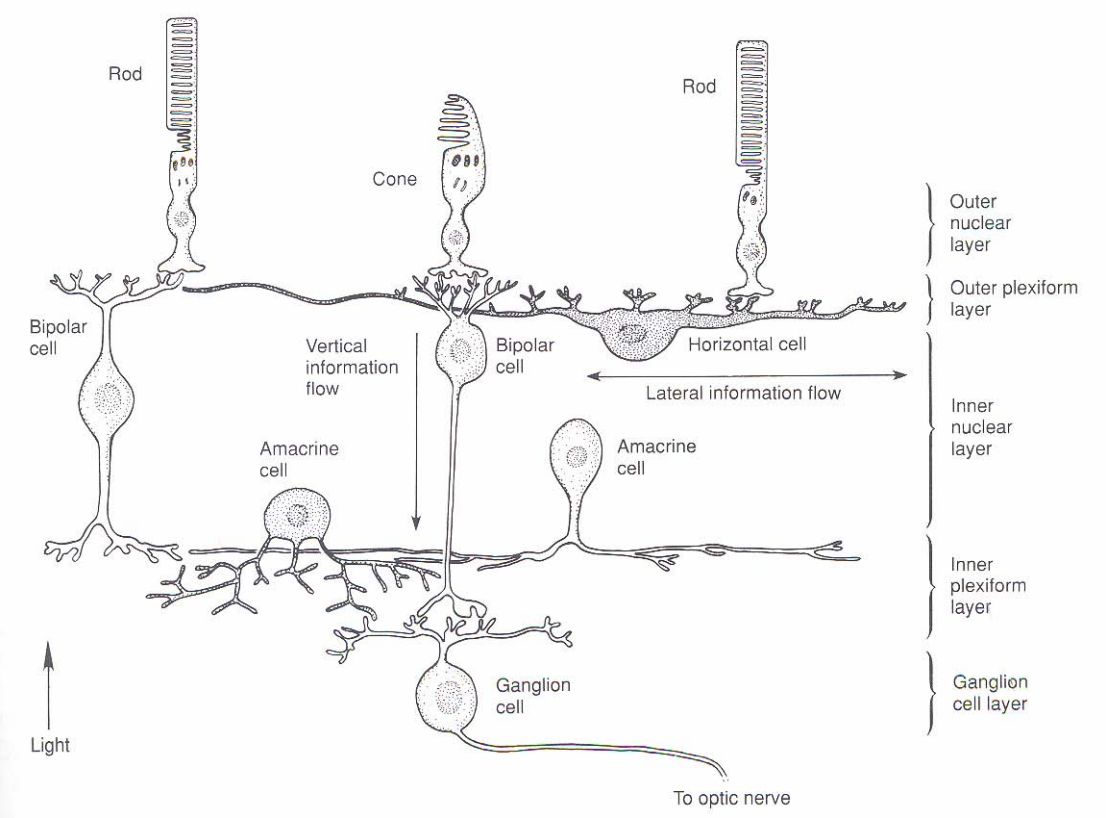

Figure 1. 1: The layered structure of the mammalian retina. Figure taken from Kandel *et al.*, 2000.

Most research into mammalian central nervous system repair over the last few decades has focused on minimizing cell death and encouraging axon regeneration (Cui *et al.*, 2003; Mansour-Robaey *et al.*, 1994; Meyer-Franke *et al.*, 1995; Thanos *et al.*, 1993; Weishaupt *et al.*, 2004). Fewer studies, however, have investigated the molecular mechanisms involved in directing regenerating axons to the appropriate locations within their target.

I have used the visual system to study the Eph and ephrin family of molecules to gain a better understanding of their response to both an injury and a regeneration model in the adult mammalian central nervous system. The Ephs and ephrins have been shown to be crucial in forming appropriate connections in the visual system during development, but their role after injury is unclear. As a preliminary to re-establishing connections in the brain, I have also assessed the neuroprotective and neuroregenerative effect of a potential therapeutic molecule, metallothionein-I/II (MT-I/II). MT-I/II is ubiquitously expressed in the body and has been shown to induce cortical healing after stab wound

injury (Chung *et al.*, 2003). I have investigated the potential of this molecule *in vivo* in the rodent visual system after injury.

The visual system

The retina (Fig. 1.1) is an outgrowth of the diencephalon that arises during development (Nolte, 2002), and therefore is part of the central nervous system. The optic nerve and optic tract connect the retina to the visual centres in the brain. The optic nerve/ tract is a pure white matter tract making it ideal to study the regenerative response of axons alone, without the complication of the gray matter. In addition, the optic nerve/ tract contains axons from only a single cell type termed retinal ganglion cells, compared to the numerous cell types that contribute to the white matter tracts of the brain and spinal cord. It is anticipated that any successful regenerative strategies discovered in the visual system could likely to be applicable to other parts of the central nervous system.

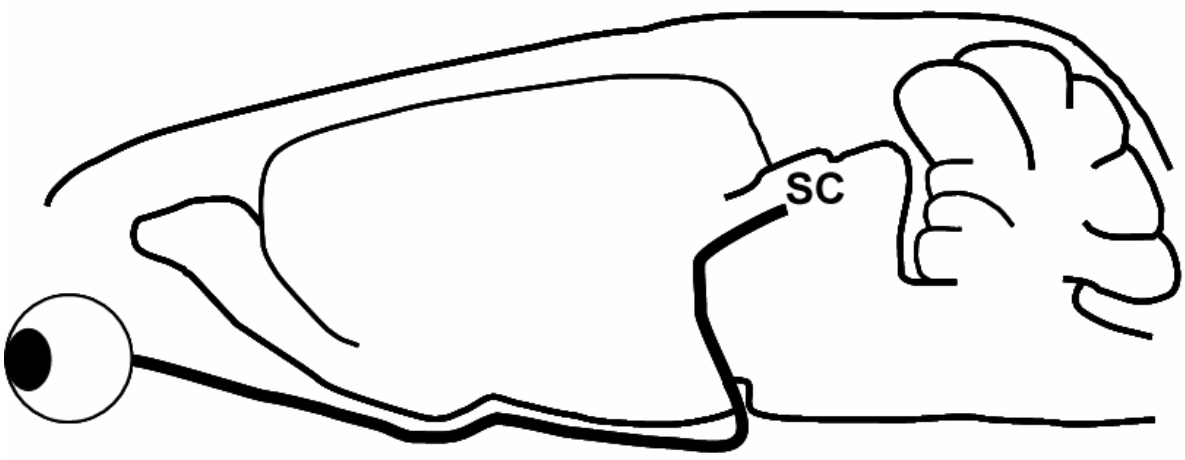

Figure 1. 2: Diagram of the rodent brain, indicating the location of the main visual centre in the rodent brain, the *superior colliculus* (SC).

The major visual centre in the brain of rodents is the *superior colliculus* (homologous to the optic tectum in non-mammalian vertebrates), a midbrain structure to which 95% of retinal ganglion cell axons project (Fig. 1.2) (Sefton *et al.*, 2004). The *superior colliculus* is a layered structure, reflecting its role as a prominent sensorimotor integrating centre of the midbrain (Fig. 1.3) (Huerta & Harting, 1984). The superficial layers of the *superior colliculus* receive most of their input from the retina. Retinal ganglion cell axons course through the rostrally positioned *superior brachium* and into the *superior colliculus* within the *stratum opticum* layer. Axons branch upwards,

towards the *pia mater*, out of the white matter rich *stratum opticum* to form topographically located terminal arbors in the cellular layer of the *superior colliculus* called the *stratum griseum superficiale* (Simon & O'Leary, 1992).

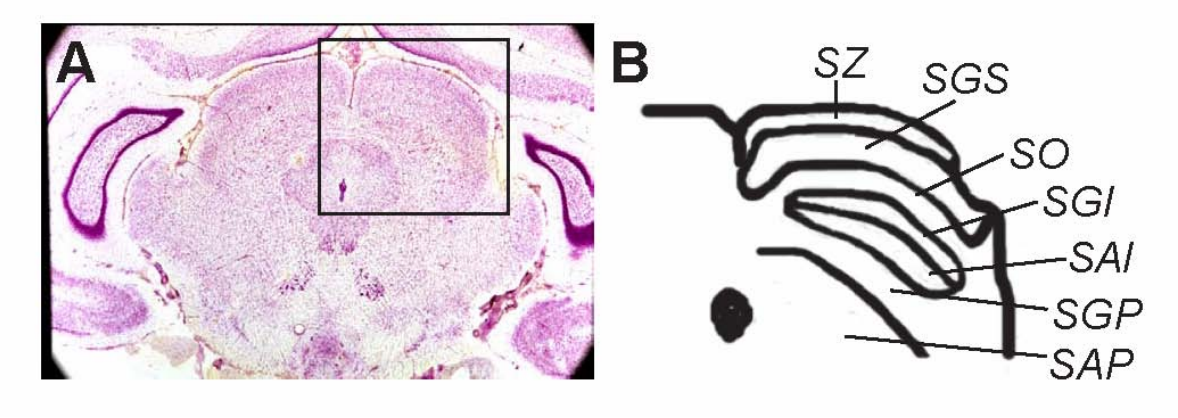

Figure 1. 3: The laminated structure of the *superior colliculus*. (A) is a cresyl stained coronal section of a rat brain taken from (Paxinos & Watson, 1998). The square denotes the location of the diagram in B. (B) The layers of the *superior colliculus, SZ, stratum zonale; SGS, stratum griseum superficiale; SO, stratum opticum, SGI, stratum griseum intermediale; SAI, stratum album intermedium; SGP, stratum griseum profundum, and SAP, stratum album profundum.*

Establishment of topographic maps

The visual system, as for most sensory inputs to the brain, is ordered topographically with adjacent retinal ganglion cells projecting to neighbouring cells in the *superior colliculus* (Siminoff *et al.*, 1966). The topographic organization allows recreation of maps of visual space in the brain and subsequent processing to be rapid and efficient (Nelson & Bower, 1990).

Topographic maps are established in the nervous system during development in a multiphase process. Guidance molecules direct axons to the approximately correct location and this initial path-finding is later refined by activity dependent mechanisms (Wong *et al.*, 1993). Initial path-finding in the visual system has been particularly well studied and fulfils the predictions of Sperry's chemoaffinity hypothesis (Sperry, 1963). In this model complementary gradients of receptor-ligand molecules are expressed across two orthogonal axes of the retina, temporo-nasal and dorso-ventral, and two orthogonal axes of the *superior colliculus*, rostro-caudal and medio-lateral. The resulting grid-like co-ordinates allow retinal ganglion cell axons to find their correct location.

Ephs and ephrins

The Eph tyrosine kinase receptors and their ephrin ligands have complementary and graded expression in the retina and *superior colliculus*, as required by Sperry's hypothesis, (Cheng *et al.*, 1995; Drescher *et al.*, 1995; Feldheim *et al.*, 1998; Frisén *et al.*, 1998). The Ephs and ephrins have subsequently been shown to have crucial roles in the topographic mapping of the *superior colliculus* (Brown *et al.*, 2000; Feldheim *et al.*, 2000; McLaughlin *et al.*, 2003).

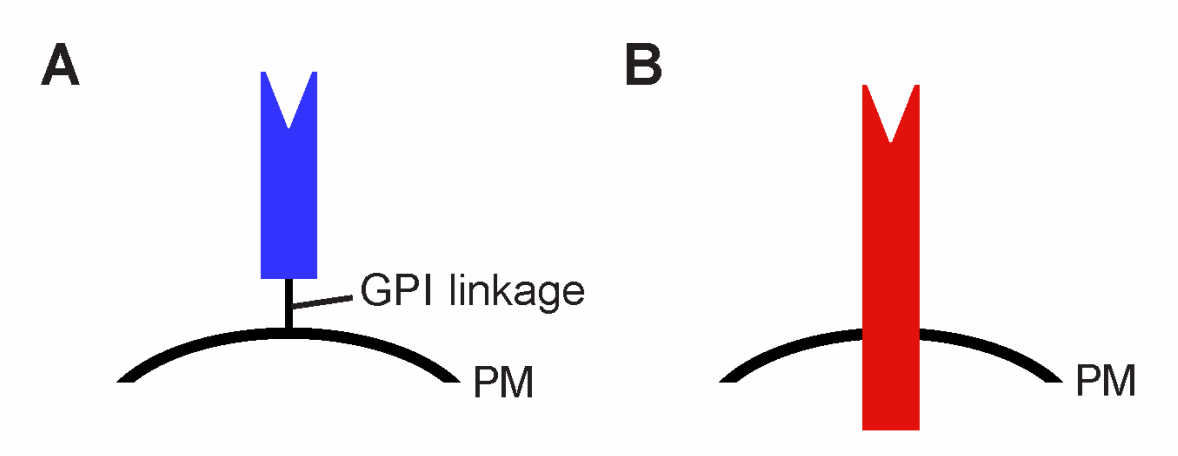

Figure 1. 4: The membrane linkage of ephrin-A and –B. (A) The diagram shows ephrin-A (blue) attached to the plasma membrane (PM) *via* a glycosylphosphatidyl inositol (GPI) linkage. (B) The diagram shows transmembrane ephrin-B (red) crossing the plasma membrane (PM).

The ephrin ligands are classified as A and B subfamilies depending on their mode of attachment to the membrane. Ephrin-As are attached to the membrane *via* a glycosylphosphatidyl inositol (GPI) sugar linkage, while ephrin-Bs are transmembrane proteins (Fig. 1.4) (Murai & Pasquale, 2003). The Eph tyrosine kinase receptors are also divided into A and B subfamilies, largely reflecting their binding to either A-ephrins or B-ephrins. The exceptions are EphA4, that binds to both ephrin-As and ephrin-Bs (Flanagan & Vanderhaeghen, 1998), and ephrin-A5 that can bind to both EphAs and EphB2 (Himanen *et al.*, 2004). Binding within subfamilies is promiscuous, although the affinity of each individual receptor-ligand interaction can vary greatly (Flanagan & Vanderhaeghen, 1998). The Eph receptor family is currently the largest family of known tyrosine kinase receptors. Ten EphA receptors, six of their ephrin-A ligands, along with six EphB receptors and three of their ephrin-B ligands have been characterized to date (Pasquale, 2004). Eph receptors and their ephrin ligands are involved in a host of cellular functions, including cell motility and attachment, angiogenesis, cell boundary formation and axon guidance (Surawska *et al.*, 2004).

In the visual system, the EphA/ ephrin-A subfamily is primarily involved with patterning the temporo-nasal axis of the retina to the rostro-caudal axis of the *superior colliculus* in birds and mammals (Hindges *et al.*, 2002), and has been studied most extensively. The EphB/ ephrin-B subfamily is primarily involved with patterning the dorso-ventral axis of the retina to the medio-lateral axis of the *superior colliculus* (Hindges *et al.*, 2002).

Ephrin-As are expressed in a rostral^{low} to caudal^{high} gradient in the *superior colliculus*, with a complementary temporal^{high} to nasal^{low} gradient of EphA expression in the retina (Cheng *et al.*, 1995; Frisén *et al.*, 1998). In zebrafish, chick and mouse, two ephrin-A molecules, -A2 and -A5 are expressed in the optic tectum/ *superior colliculus* (Brennan *et al.*, 1997; Cheng *et al.*, 1995; Drescher *et al.*, 1995; Frisén *et al.*, 1998). In mouse, the gradient of ephrin-A2 reaches its maximum three-quarters of the distance along the rostral-caudal axis of the *superior colliculus* and is reduced caudal to this point, while the ephrin-A5 gradient is expressed only in the caudal half colliculus (Frisén *et al.*, 1998). The function of this overlapping expression pattern is not fully understood, but it could be to create an exponential gradient of total ephrin-A (Brown *et al.*, 2000). The member of the EphA subfamily expressed as a gradient in the retina varies between species (Cheng *et al.*, 1995; Feldheim *et al.*, 1998). In the mouse and rat retina EphA5 is expressed in a complementary gradient, and binds to both ephrin-A2 and ephrin-A5 with high affinity (Fig. 1.5) (Feldheim *et al.*, 1998).

The EphB/ ephrin-Bs are expressed in a graded fashion similar to EphA/ ephrin-As, but are involved in the mediolateral patterning of the optic tectum/ *superior colliculus* in chick and mouse (Hindges *et al.*, 2002; McLaughlin *et al.*, 2003). In chick and mouse, ephrin-B1 is expressed in a lateral^{low} to medial^{high} gradient and three of its EphB receptors are expressed in dorsal^{low} to ventral^{high} gradients in the retina. EphB2, B3 and B4 are the receptors expressed in the appropriate complementary gradients in the retina (Connor *et al.*, 1998; Hindges *et al.*, 2002; Holash & Pasquale, 1995).

Unlike the Sperry model which proposed chemoaffinity, based on attraction, the mapping of the rostral-caudal axis of the *superior colliculus* is reliant primarily on repulsion (Yates *et al.*, 2001), while the medio-lateral axis requires both attraction and repulsion (McLaughlin *et al.*, 2003).

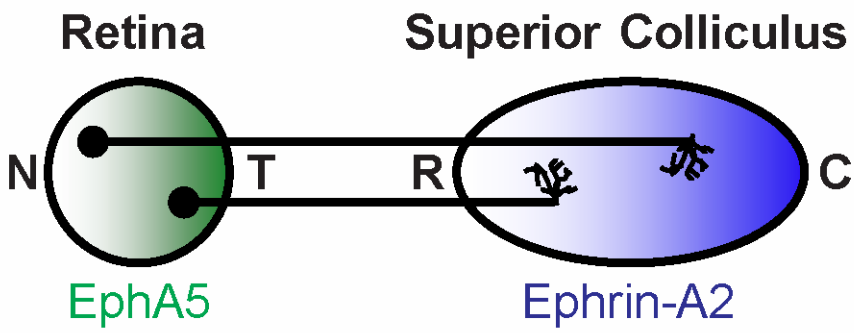

Figure 1. 5: A diagram showing the complementary gradients of EphA5 expression (green) in the retina, and ephrin-A2 (blue) in the *superior colliculus*. N, nasal; T, temporal; R, rostral; C, caudal.

Role of Ephs and ephrins in establishing topography during development

Although rodent retinal ganglion cells project axons in fascicles, directly to the optic disc, neighbour relationships are largely lost in the optic nerve where fascicles split and mix (Simon & O'Leary, 1991). While the tendency for retinotopic order increases in the optic tract, retinal ganglion cells essentially enter the *superior colliculus* in a poorly ordered arrangement. Cytochemical cues in the *superior colliculus* are largely responsible for guiding retinal ganglion cell axon to topographically correct locations (Simon & O'Leary, 1991).

During development, retinal ganglion cell axons grow through the rostrally located *superior brachium* to enter the *superior colliculus*, and their primary growth cones extend posteriorly towards the caudal pole (Nakamura & O'Leary, 1989; Paxinos & Watson, 1998; Simon & O'Leary, 1992). EphAs and ephrin-As have been shown to have a direct effect on primary retinal ganglion cell axons growing across the *superior colliculus* (Nakamoto *et al.*, 1996). The point along the rostro-caudal axis of the *superior colliculus* at which a retinal ganglion cell primary growth cone stops is thought to correspond to the `neutral-point' observed in a recently developed *in vitro* assay (Hansen *et al.*, 2004). Caudal to this neutral-point, retinal ganglion cell primary growth cones are repulsed by high levels of ephrin-As through EphA signalling mechanisms. Rostral to the neutral-point, retinal ganglion cell primary growth cones are promoted. The growth cone extension below the neutral-point was shown to be independent of intracellular signalling. It is likely to be a result of adhesion, due to EphA-ephrin-A2 binding, at concentrations low enough so as not to initiate down-stream signalling and subsequent growth cone collapse (Hansen *et al.*, 2004). The neutral-point also depends

on the retinal location from which the axon originates (Hansen *et al.*, 2004). The adhesion-mediated growth cone promotion observed suggests a mechanism by which primary retinal ganglion cell axons can extend across the *superior colliculus*, without the need for a matching gradient of a molecular attractant (Hansen *et al.*, 2004).

Chick and rodent retinal ganglion cell primary growth cones overshoot their target by considerable distances. Interstitial branches form along the axon shaft, at distances of up to a few millimetres behind the growth cone, and form the topographically correct termination zones (Fig. 1.6) (Nakamura & O'Leary, 1989; Simon & O'Leary, 1992; Yates *et al.*, 2001). It has been shown that ephrin-A2 and ephrin-A5 are also important for the topographically correct termination zones of these interstitial branches. These two molecules inhibit interstitial branching posterior to the correct termination zone (Sakurai *et al.*, 2002; Yates *et al.*, 2001). In addition, recent work has shown that EphA7, which is expressed in the *superior colliculus* in a rostral^{high} to caudal^{low} gradient, inhibits interstitial branching anterior to the correct termination zone (Rashid *et al.*, 2005).

Following interstitial branching at the topographically appropriate position along the rostral-caudal axis, branches must then form termination zones at the correct medio-lateral position (Nakamura & O'Leary, 1989). The guidance of interstitial branches in the correct direction along the medio-lateral axis has been shown to be due, in part, to branches which arise from axons lateral to their correct termination zone, being attracted medially up the increasing ephrin-B1 gradient (Hindges *et al.*, 2002). There is also evidence that the ephrin-B1 gradient can act bifunctionally, not only attracting branches, but also repelling medially located branches laterally down the ephrin-B1 gradient to their correct termination zone (McLaughlin *et al.*, 2003).

There is an added layer of complexity beyond the complementary gradients as predicted in Sperry's model: ephrin-As and ephrin-Bs are expressed in the retina in gradients opposing those of EphAs and EphBs (Connor *et al.*, 1998; Hindges *et al.*, 2002; Marcus *et al.*, 1996). In addition, EphAs are expressed in the *superior colliculus* in gradients opposing those of ephrin-As (Rashid *et al.*, 2005). Furthermore, Ephs and their corresponding ephrins are co-expressed on the same retinal ganglion cell growth cone, and it has been shown that over-expression of ephrin-A2 in the retina leads to topographic targeting errors (Hornberger *et al.*, 1999). It has been proposed that ephrin-

A expression can interact with EphA on the same cell (*cis*-interactions) to desensitize EphA to exogenous ephrin-A from other cells (*trans*-interactions) resulting in a `masking' affect (Flanagan, 2000; Hornberger *et al.*, 1999; Sobieszczuk & Wilkinson, 1999).

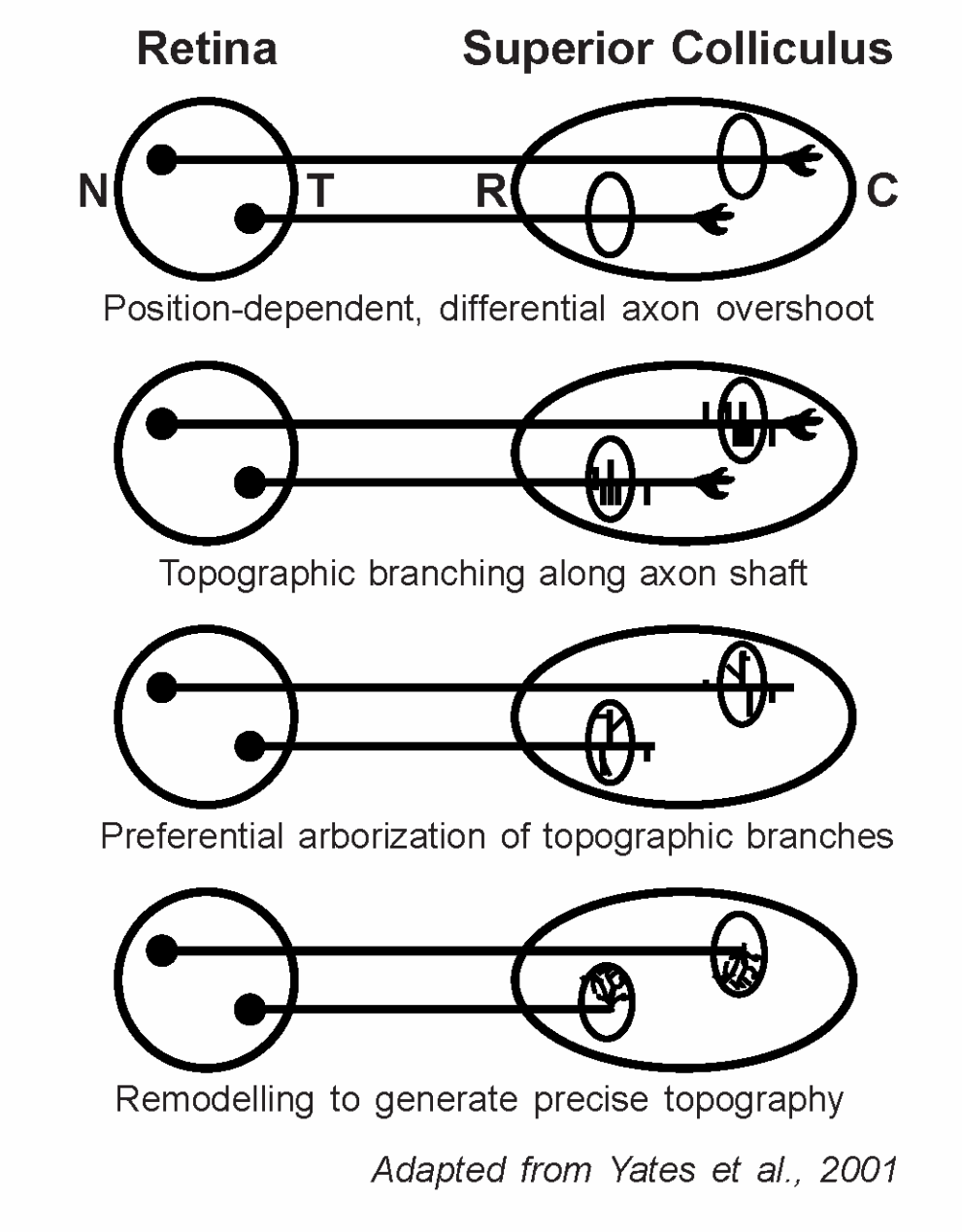

Figure 1. 6: Diagrams showing four important processes in the topographic formation of the retinocollicular projection. Adapted from (Yates *et al.*, 2001).

However, recent work on motor neurons has shown that masking does not occur physiologically in this system. Instead, the Ephs and ephrins that are co-expressed on the same growth cone are segregated into distinct membrane domains, preventing *cis*-interactions. It was found that growth cone-expressed EphA *trans*-interactions with ephrin-A on target tissue resulted in growth cone repulsion, while growth cone-

expressed ephrin-A *trans*-interactions with EphA receptors on target tissue resulted in adhesion (Marquardt *et al.*, 2005). In the same study, when Ephs and ephrins were forced to be expressed in the same membrane domains, they were found to interact in *cis*- and the resulting growth cone behaviour was consistent with a `masking' effect. Thus, masking is theoretically possible, but has not yet been shown to occur physiologically (Marquardt *et al.*, 2005). Whether or not co-expressed EphA and ephrins-A are segregated into distinct membrane domains in the retina still needs to be determined (Klein, 2005).

Regardless of whether distinct membrane domains or a masking mechanism is utilized in the visual system, intracellular down-stream signalling initiated by Eph-ephrin interactions are responsible for the subsequent growth cone behaviour (Murai & Pasquale, 2003). Ephs and ephrins have an intricate signalling network, that can activate numerous kinase-mediated signalling pathways and this is the most likely mechanism for the multiple functions of these molecules throughout the body (Egea *et al.*, 2005).

Signalling pathways

Eph receptors share many characteristics with other receptor tyrosine kinases, such as ligand binding induced autophosphorylation and subsequent kinase activation (Egea *et al.*, 2005). An important difference, however, is the requirement for their ephrin ligands to be membrane bound, enabling the receptor to form clusters and initiate signalling (Davis *et al.*, 1994; Stein *et al.*, 1998). The activation of Eph receptors seems to be a two stage process involving kinase activation and clustering of the Eph-ephrin complex (Egea *et al.*, 2005). Some of the signalling pathways and the cellular processes they control seem to explain the response of the growth cone to Ephs and ephrins. Indeed, activated EphAs and EphBs interact with different proteins, possibly explaining the differing responses of primary retinal ganglion cell axons to ephrin-As and ephrin-B in the topographic mapping of the *superior colliculus* (McLaughlin *et al.*, 2003; Murai & Pasquale, 2003; Yates *et al.*, 2001).

EphA receptor forward signalling

Important regulators of the actin cytoskeleton and therefore growth cone dynamics are the Rho family of small GTPases, which include members, RhoA, Rac1 and Cdc42 (Murai & Pasquale, 2003). While active Rac1 and Cdc42 are associated with growth

cone promotion and forward movement, RhoA activation is associated with growth cone collapse (Murai & Pasquale, 2005).

EphA receptor forward signalling activates RhoA and its effector Rho-kinase through the activation of ephexin. Ephexin is a GTPase exchange factor, catalysing the replacement of GDP by GTP on Rho, thereby activating it (Shamah *et al.*, 2001). Activation of EphA receptor leads not only to the activation of RhoA, but also to the initial reduction of Rac1 in the growth cone, although a subsequent recovery of Rac1 activity is required for growth cone collapse (Jurney *et al.*, 2002). To terminate signalling and allow growth cone collapse, the growth cone and the ephrin-expressing cell must be separated, through a combination of protein cleavage and endocytosis. Cleavage of the ephirn-A GPI linkage to the cell membrane is regulated by the metalloprotease Adam10/Kuzbanin (Hattori *et al.*, 2000), and the remaining Eph-ephrin complex is internalized by endocytosis.

It seems that changing the balance of GTPase activities by altering the ratio of activities of RhoA, Rac1 and Cdc42 is one of the key determinants of the growth cone response to ephrins or other ligands (Sahin *et al.*, 2005). Recent studies using ephexin1 knock out mice and ephexin1 knock down in chick have shown that ephexin1 is not only critical for appropriate growth cone collapse, but that is also required for proper axonal outgrowth (Sahin *et al.*, 2005). These contrasting results can be explained by the mechanism of ephexin1 regulation. Ephexin1 is tethered to Eph receptors, and in the absence of ephrin ligand can activate all three Rho family members equally. An equal ratio of GTPases could result in neurite outgrowth. When the EphA is phosphorylated at a specific residue, ephexin1 is altered such that it primarily activates RhoA, ultimately leading to growth cone collapse (Sahin *et al.*, 2005).

For the completion of growth cone collapse, not only must the cytoskeleton actin dynamics change, but excess plasma membrane must be removed (Jurney *et al.*, 2002). Excess plasma membrane is removed by endocytosis, which also serves the dual role of removing Eph-ephrin complexes from the cell surface, turning attraction of cells through adhesion into repulsion (Cowan *et al.*, 2005; Pasquale, 2005). It is believed that Rac1 activation, which occurs later in the growth cone collapse process, increases plasma membrane endocytosis (Marston *et al.*, 2003). A recent study has suggested that

the GTPase exchange factor, Vav2, is the molecular link between EphA forward signalling and Rac1 activity induced endocytosis (Cowan *et al.*, 2005).

EphB receptor forward signalling

In contrast to the growth cone collapse response to EphA signalling through Rho, EphB receptor forward signalling activates Rac1 and Cdc42 through the association with two different exchange factors, intersectin-1 and kalirin-7 (Murai & Pasquale, 2005). The down stream effector of these pathways, Pak-kinase does not play a large role in growth cone collapse and is more associated with dendrite spine formation and morphogenesis (Murai & Pasquale, 2003). However, Rac1-dependent endocytosis is also important in turning certain EphB-ephrin-B interactions from attraction to repulsion. EphBs can also interact with Vav2 (Pasquale, 2005), suggesting a similar role in endocytosis to that seen in EphA forward signalling.

The roles of EphBs and ephrin-Bs as bifunctional molecules in the formation of the retinocollicular projection are supported by these complex signalling networks that can actively signal both attraction and repulsion.

Ephrin ligand reverse signalling

Eph-ephrin interaction also results in reverse signalling through the ephrin ligand which is attached to the membrane. The difference in membrane attachment of the GPI-linked ephrin-As and the transmembrane ephrin-Bs requires them to signal using difference mechanisms. As yet, neither ephrin-A or ephrin-B signalling pathways have been shown to directly regulate growth cone dynamics (Murai & Pasquale, 2003).

Involvement of other signalling pathways

Another layer of complexity is that Eph receptors can interact with a number of other cell receptors. Although a role for this cross-talk has not yet been described in the initial formation of a retinocollicular projection, the direct association of EphB receptors with NMDA receptors at synapses suggests a possible role during the later refinement stage of the topographic map (Dalva *et al.*, 2000; Murai & Pasquale, 2003).

Changing attraction to repulsion

The response of a growth cone to guidance molecules such as the ephrins, is also dependent on the endogenous activity of Rho and its down-stream effector, Rho-kinase

(Ellezam *et al.*, 2002). Rho activity leads to growth cone collapse (Murai & Pasquale, 2003), but if Rho activity is inhibited, growth cone collapse is restricted and attraction can occur (Ellezam *et al.*, 2002).

It has been shown that endogenous levels of cyclic adenosine triphosphate (cAMP) levels are important for determining the activity of Rho, in that increasing intracellular cAMP inactivates Rho (Lang *et al.*, 1996). The effect of increased cAMP levels on cytoskeletal changes can be overcome, however, by using a constitutively active Rho mutant protein (Dong *et al.*, 1998). It therefore seems that cAMP and Rho have antagonistic roles in determining the growth cone response to guidance molecules, although it appears that Rho is down-stream of cAMP (Dong *et al.*, 1998; Ellezam *et al.*, 2002).

The varied response of neurite growth cones to another guidance molecule, netrin-1, has previously been shown to be dependent on intracellular cAMP levels. Netrin-1 is an attractant for growth cones when cAMP levels are high, but repulsive when cAMP levels are low (Ming *et al.*, 1997). Moreover, because intracellular levels of cAMP can be affected by various extracellular cues, an additional layer of control is imposed on the response of growth cones to guidance molecules (Ellezam *et al.*, 2002).

Visual system as a model for regeneration

Optic nerve transection is a useful model to study the response of retinal ganglion cells to injury. Moreover, the varied response to this injury in different species provides an excellent comparative model. The poor regenerative response after optic nerve axotomy in mammals contrasts sharply with that of Anamniotes, such as fish and frogs. Optic nerve transection in mammals results in massive retinal ganglion cell death and a failure of the few surviving cells to regenerate their axons. The lack of axon regeneration is due to the intrinsic and extrinsic factors that are responsible for the regenerative failure of axons in other areas of the adult mammalian central nervous system (Qiu *et al.*, 2002).

Optic nerve axotomy in rat results in delayed cell death of nearly 90% of retinal ganglion cells by 14 days after injury (Berkelaar *et al.*, 1994; Villegas-Perez *et al.*, 1993) and most of the death has been shown to be apoptotic (Berkelaar *et al.*, 1994; Garcia-Valenzuela *et al.*, 1994). This is in contrast to the response of goldfish retinal

ganglion cells, in which there is almost no retinal ganglion cell death (Humphrey, 1988; Matsukawa *et al.*, 2004).

A major difference between the mammalian central nervous system that fails to undergo axonal regeneration and systems that do have some regenerative capacity is the amount of survival and growth factors secreted by glial cells after injury (Fawcett & Keynes, 1990). Such factors are not secreted by adult mammalian optic nerve glia in response to axotomy, however, it has been reported that such factors are secreted by neonatal mammalian optic nerve. Furthermore, the addition of these neonatal derived molecules can induce a regeneration-associated response after injury in the adult mammalian optic nerve (Hadani *et al.*, 1984).

Axonal regeneration does not occur even when mammalian retinal ganglion cells are encouraged to survive for up to one month after optic nerve section, by the addition of various trophic factors (Cohen *et al.*, 1994; Mansour-Robaey *et al.*, 1994; van Adel *et al.*, 2003; Weishaupt *et al.*, 2004). Survival of the nerve cell body and regeneration of its axon have been shown to act *via* two separate pathways (Goldberg & Barres, 2000). In addition, it has been suggested that adult mammalian retinal ganglion cells lack intrinsic growth potential (Barron, 2004; Goldberg *et al.*, 2002). Even when the regenerative response of retinal ganglion cells can be increased, as evidenced by increases in growth associated proteins (Soto *et al.*, 2003), extrinsic factors can nevertheless preclude successful regeneration in the mammalian optic nerve.

The reformation of the glial limiting membrane at the lesion site results in a glial-fibrotic scar (Bundesen *et al.*, 2003). The glial scar formed after optic nerve injury is similar to that formed in other areas of the mammalian central nervous system (Bundesen *et al.*, 2003; Fitch *et al.*, 1999), and as a result, contains many of the same inhibitory molecules such as chondroitin sulphate proteoglycans (CSPG) and tenascin-C (Sandvig *et al.*, 2004). As a result of such inhibitory molecules, the rat optic nerve glial scar is inhibitory even to goldfish retinal ganglion cells axons, which normally can regenerate *in vivo* (Bahr *et al.*, 1995; Bastmeyer *et al.*, 1991).

In most studies in which rodent retinal ganglion cells have been encouraged to grow through the lesion site, by either blocking inhibitory molecules (Ellezam *et al.*, 2002) or by priming retinal ganglion cells with neuroregenerative molecules (Leon *et al.*, 2000;

Yin *et al.*, 2003), regeneration does not extend far beyond the injury site (Ellezam *et al.*, 2002; Sapieha *et al.*, 2003). The failure of axonal regeneration is thought to reflect both the lack of morphological substratum for retinal ganglion cell axons, and myelin-derived inhibitory molecules that remain in the distal nerve and tract (Koeppen, 2004). One of the main morphological substrates for the regeneration of goldfish retinal ganglion cell axons, laminin, is up-regulated in goldfish, but not in rat after optic nerve after transection (Ford-Holevinski *et al.*, 1986).

Myelin-derived factors include myelin-associated glycoprotein (MAG), NOGO and oligodendrocyte myelin glycoprotein (OMgp). These proteins are not exposed in healthy myelin, but after damage, and during degeneration, they are presented to the extracellular environment. Each of these myelin-derived factors has been shown to be inhibitory to neurites in culture and most likely carry out a similar role *in vivo*. There has recently been a reinterpretation of older data, however, suggesting that this type of myelin-derived inhibition may be required to guide regenerating axons and to prevent aberrant sprouting in white matter tracts (Raisman, 2004).

Unlike adult mammals, goldfish have the ability to regenerate their retinal ganglion cell axons after injury, allowing reconstruction of connections between the retina and the brain. In addition, the key to functional recovery of vision is that these regenerating axons form an appropriate topographic map in the tectum, similar to that seen during development in normal animals. The topographic order enables Anamniotes to respond appropriately to visual stimuli in terms of spatial vision (Sperry, 1943). For this reason Eph and ephrin expression in the tectum/ *superior colliculus* has recently been studied after optic nerve axotomy in a number of species (Bach *et al.*, 2003; Becker *et al.*, 2000; Knoll *et al.*, 2001; Rodger *et al.*, 2001).

Response of Ephs and ephrins to injury

Optic Tectum/ Superior colliculus

To date, work on expression of the EphBs and ephrin-Bs involved in topographic mapping has been restricted to development rather than after optic nerve injury, although ephrin-B2 mRNA has been reported to be up-regulated in the retina after injury by subtractive hybridization (Wang *et al.*, 2002). The expression patterns of EphAs and ephrin-As after injury, however, have been the focus of a number of studies.

After optic nerve transection in all species examined to date (zebrafish: Becker *et al.*, 2000; goldfish: Rodger et al., 2000; frog: Bach et al., 2003; mouse: Knoll et al., 2001; and rat: Rodger et al., 2001), there is an appropriate topographic gradient of ephrin-A2 (Rodger *et al.*, 2000; Rodger *et al.*, 2001), ephrin-A2 and -A5 (Becker *et al.*, 2000), or ephrin-As (Bach *et al.*, 2003; Knoll *et al.*, 2001). It is interesting that rodents, which are incapable of spontaneous regeneration of retinal ganglion cell axons after injury, nevertheless express an appropriate ephrin-A gradient in the *superior colliculus* (Knoll *et al.*, 2001; Rodger *et al.*, 2001).

However, there is still some controversy as to the exact response of ephrin-As and EphA to injury. Some studies suggest that optic nerve section elicits up-regulation of ephrin-A2 to mirror those seen in development (Rodger *et al.*, 2000; Rodger *et al.*, 2001), while others have suggested that ephrin-As are already in a topographic gradient in adult normal animals, and are unaffected by optic nerve injury (Bach *et al.*, 2003; Becker *et al.*, 2000; Knoll *et al.*, 2001).

Studies in chick and mouse during development, unlike those in frog (Bach *et al.*, 2003), have shown that topographic gradients of EphA and ephrin-A tend to disappear postnatally (Cheng *et al.*, 1995; Feldheim *et al.*, 1998). Therefore it seems contradictory to suggest that in rodents ephrin-A is already expressed in a topographic gradient in the adult (Knoll *et al.*, 2001), when it has been previously shown to be down-regulated at birth (Feldheim *et al.*, 1998). These conflicting views can, at least in part, be reconciled by consideration of methodological differences.

The study by Knoll *et al.* (2001) used EphA fusion proteins that bind all ephrin-A ligands present in the *superior colliculus*, the main two being ephrin-A2 and ephrin-A5 (Feldheim *et al.*, 1998; Frisén *et al.*, 1998). In contrast, Rodger *et al.* (2001) used quantitative immunohistochemical techniques for a specific family member, ephrin-A2. It is possible that the shallow gradient observed in normal *superior colliculus* by ephrin-A2 immunohistochemistry constitutes only part of the gradient seen by fusion proteins, and if ephrin-A5 immunolabelling was also included, the gradients observed in the two studies would be the similar. Another possibility is that the quantification method used by Rodger *et al.* (2001) was able to detect subtle changes between normal and experimental animals in the staining intensity and ephrin-A2 gradient, which were unable to be measured after fusion protein staining. Finally, Knoll *et al.* (2001)

investigated ephrin-A expression two weeks after surgery, whereas Rodger *et al.* (2001) used a four week time point. It is possible that the later time point allowed further change in ephrin-A2 expression across the rostro-caudal axis of the *superior colliculus*.

The unilateral optic nerve lesion carried out by Rodger *et al.* (2001), denervated virtually the entire contralateral colliculus to which 97% of retinal ganglion cell axons project (Sefton, 1968). The response of ephrin-A2 in a topographically appropriate gradient, regardless of whether it was up-regulated or already present, suggests that the brain is ready to receive a regenerated input that will not spontaneously arrive. Interestingly, the up-regulation of ephrin-A2 is not confined to the contralateral colliculus, but is also expressed in the ipsilateral colliculus (Rodger *et al.*, 2001). The reasons for such bilateral up-regulation are unknown, but it seems unlikely to be induced by denervation of the small (~3%) ipsilateral input (Thanos, 1991) because this input is confined to the rostral *superior colliculus*, while most of the ephrin-A2 up-regulation is in caudal *superior colliculus*. These observations led to the first part of my thesis in which I investigated the pathways responsible for such a bilateral response following denervation (Chapter 2).

Retina

Although the Eph and ephrin response in the brain across species seems to be topographically appropriate, only one study has addressed EphA expression in the retina of rat after unilateral optic nerve section. After unilateral optic nerve section EphA5 expression is down-regulated in both eyes. In the experimental eye, down-regulation is presumably due to most of the retinal ganglion cells dying, rather than an inappropriate guidance molecule response; however, it is interesting that the non-experimental eye is also affected (Rodger *et al.*, 2001). This aspect has been further investigated in my thesis (Chapter 2).

In summary, it seems that in both the brain and the retina of rodents, injuries affecting the expression of guidance molecules on one side of the body are relayed across the midline thus producing a bilateral response. If we can better understand the mechanisms involved in changing the expression of guidance molecules, it may become possible to manipulate these levels to aid successful regeneration.

Regenerative strategies in the visual system

In goldfish, it has been possible to correlate the expression of guidance molecules after injury to the topographic re-innervation of visual brain centres leading to functional recovery. Such correlations have not been possible so far in the mammalian visual system, even though it seems that the *superior colliculus* is ready to organize input appropriately, with topographic collicular gradients of ephrin-A2 expressed after injury (Knoll *et al.*, 2001; Rodger *et al.*, 2001). With the development of various surgical interventions, however, it is now possible to stimulate both increased survival (Hu *et al.*, 2005; Vidal-Sanz *et al.*, 1987), and at least limited regeneration in adult mammalian retinal ganglion cells (Cui *et al.*, 2003).

Currently, one of the most successful approaches for such survival and regeneration is the peripheral nerve graft model (Fig. 1.7) (So & Aguayo, 1985). This model not only achieves survival and regeneration of retinal ganglion cells, but can direct regenerating axons to the *superior colliculus* (Vidal-Sanz *et al.*, 1987), where they form functional synaptic connections (Keirstead *et al.*, 1989; Sauvé *et al.*, 1995).

The peripheral nerve graft model

The peripheral nerve graft model encourages retinal ganglion cells to regenerate back to their major visual brain centre. Rather than attempting to encourage axons to regenerate through the lesion site of the optic nerve/ tract, the inhibitory environment is replaced by a length of autologous peripheral nerve. Because the peripheral (sciatic) nerve segment has been injured by removal from the leg, it undergoes Wallerian degeneration (Barron, 2004). This well characterized process provides both morphological support and secreted trophic factors to retinal ganglion cells, features they would not receive from the environment of the injured mammalian central nervous system (David & Aguayo, 1981; Fawcett & Keynes, 1990). The outcome is retinal ganglion cell survival and axon regeneration (So & Aguayo, 1985).

Despite provision of a superior environment, at best 10% of the retinal ganglion cell population can be rescued by attaching a piece of peripheral nerve to the back of the eye after optic nerve section (Cui *et al.*, 2003; Villegas-Perez *et al.*, 1988). However, only a subset of surviving retinal ganglion cells, are able to extend axons into and along the peripheral nerve graft (Aviles-Trigueros *et al.*, 2000; Vidal-Sanz *et al.*, 1987). This subset of regenerated retinal ganglion cell axons corresponds to one percent of the

original retinal ganglion cell population (Danias *et al.*, 2002; Cui *et al.*, 2003). The distal end of the peripheral nerve can be inserted into the *superior colliculus* to "bridge" the retina and the *superior colliculus* in rodents after optic nerve section (Fig. 1.7) (Vidal-Sanz *et al.*, 1987).

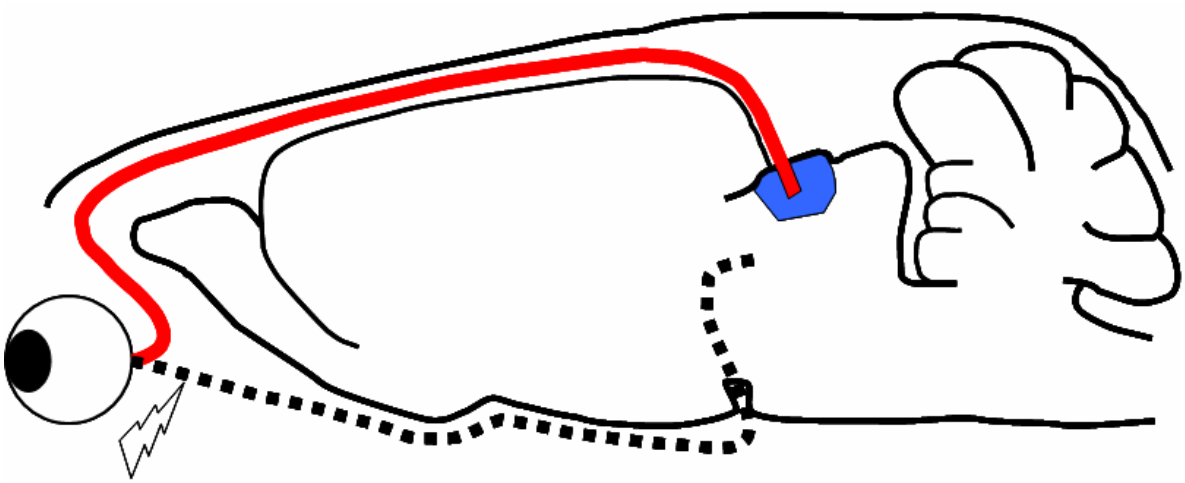

Figure 1. 7: A diagram showing the peripheral nerve graft model. The optic nerve (dashed line) is transected and a length of sciatic nerve (red) is attached to the optic nerve stump. The distal end of the nerve is inserted into the main visual centre of the rodent brain, the *superior colliculus* (blue). Using this model, retinal ganglion cells in the eye can be encouraged to regenerate back to their original targets in the brain.

Topography of the regenerated projection

In approximately a third of animals undergoing peripheral nerve graft procedure followed by insertion of the graft into the brain, a small regenerated projection is established (Aviles-Trigueros *et al.*, 2000) in the appropriate visual layers of the *superior colliculus* (Carter *et al.*, 1994, 1989, 1998; Vidal-Sanz *et al.*, 1987) and functional synaptic connections formed (Keirstead *et al.*, 1989; Sauvé *et al.*, 1995). Electrophysiological recordings from the *superior colliculus* of peripheral nerve grafted animals distinguished a maximum of 60 retinal ganglion cell axons contributing to the regenerated projection (Sauvé *et al.*, 1995). The most extensive re-innervation of the regenerated projection has been shown to cover approximately one-third of the surface area of the *superior colliculus* (Carter *et al.*, 1994; Sauvé *et al.*, 1995). The limited coverage of the collicular area suggests that re-establishing appropriate connectivity within the regenerated projection will be a major challenge.

It has been shown that there is no topographic order of retinal ganglion cell axons in the peripheral nerve graft itself as they regenerate to the *superior colliculus* (Sauvé *et al.*, 2001). These results were obtained by separating the distal end of the peripheral nerve graft in two and applying different fluorescent tracer to each side. The resulting back labelling was observed in retinal flat mount preparations and no order was seen. The result contrasts with an earlier study suggesting that some ordering of retinal ganglion cell axons occurs in the graft. However, in the earlier study, the peripheral nerve graft was not physically separated before dye application and may have allowed leakage of dye (Thanos *et al.*, 1997). The lack of order of retinal ganglion cell axons in the graft implies that any topographic order seen in the regenerating projection is due to guidance molecules expressed in the *superior colliculus*, and is reminiscent of the situation during development (Simon & O'Leary, 1991).

Electrophysiological recordings from the *superior colliculus* have failed to find evidence of topographic order in the regenerated projection (Sauvé *et al.*, 1995, 2001). It was observed that, 1) retinal ganglion cells in similar locations along the temporo-nasal axis in the retina can project to quite different and inappropriate locations in the *superior colliculus*; and 2) retinal ganglion cells from distant locations along the temporo-nasal axis in the retina can project to the same location in the *superior colliculus*.

Further investigation of the regenerated projection, however, showed some small, but significant tendency for topography. Pairs of retinal ganglion cells were scored on the appropriateness of their projections along the rostral-caudal axis of the *superior colliculus*, in relation to their soma position along the temporal nasal axis of the retina. The data showed a 3/2 significant tendency for the more nasal of the retinal ganglion cell pair to project more caudally in the *superior colliculus*. Thus, it seems that axons re-innervating the *superior colliculus* may be able to respond to guidance cues to a limited degree (Sauvé *et al.*, 2001). No similar tendency towards topographic order was seen along the medio-lateral axis of the *superior colliculus* (Sauvé *et al.*, 2001).

These results suggest that the topographically appropriate gradients of ephrin-As previously described in the mammalian *superior colliculus* after optic nerve transection (Knoll *et al.*, 2001; Rodger *et al.*, 2001), may provide some guidance information to the regenerating axons regenerating through a peripheral nerve graft (Sauvé *et al.*, 2001).

Two-thirds of peripheral nerve grafted animals fail to re-innervate the superior colliculus robustly

In the remaining two-thirds of animals undergoing peripheral nerve graft surgery, no regenerating axons were found to exit the distal end of the graft to form connections in the *superior colliculus*. The outcome is due to either formation of a neuroma at the distal end of the graft ($1/3^{rd}$ animals) or scarce innervation of the *superior colliculus* ($1/3^{rd}$) animals (Aviles-Trigueros *et al.*, 2000).

Inserting a peripheral nerve graft into the *superior colliculus* is akin to a penetrating injury of the central nervous system. It is often assumed that the typical response to this sort of injury is activated in the *superior colliculus*, leading to a glial-fibrotic scar (Bundesen *et al.*, 2003) composed of numerous inhibitory molecules (Sandvig *et al.*, 2004). The formation of a glial scar at the site of insertion of the peripheral nerve graft into the *superior colliculus*, however, has only been postulated, but not been directly addressed (Sauvé *et al.*, 2001).

A recent study of the spinal cord addressed the issue of the inhibitory glial-fibrotic scar preventing axons from exiting Schwann cell grafts. Some of the main inhibitory molecules associated with the scar were digested by using chondroitinase ABC at the insertion site (Fouad *et al.*, 2005; Yick *et al.*, 2000). An alternative process to reduce or remove inhibition is to disrupt the down-stream regulators of the inhibitory molecules within the growth cone (Ellezam *et al.*, 2002).

All the inhibitory molecules known to be associated with the glial-fibrotic scar are ligands that bind to their receptors on the membrane of the growth cone. Each inhibitory molecule has one or more receptors which signal down-stream changes within the growth cone leading to cytoskeleton collapse and repulsion. Many of the inhibitory molecules, including CSPG, MAG and NOGO bind receptors that signal the actin cytoskeleton to collapse through the Rho family of GTPases (Ellezam *et al.*, 2002). Activation of Rho by GTPase exchange factors, results in the activation of Rho-associated kinase which is the down-stream effector changing actin cytoskeleton dynamics (Ellezam *et al.*, 2002; Murai & Pasquale, 2003). By inactivating Rho or its down-stream effector, Rho-associated kinase, inhibition of regenerating axons is reduced, resulting in increased regeneration (Ellezam *et al.*, 2002; Lehmann *et al.*, 1999; Matsui & Oohira, 2004; Qi *et al.*, 2004).

An additional inhibitory molecule associated with the glial scar is the secreted factor semaphorin-3A, which is up-regulated at the site of penetrating injuries in the central nervous system (Pasterkamp *et al.*, 1999). Semaphorin-3A is a repulsive guidance molecule during development, which binds to its neuropillin-1 receptor on the growth cone surface. Binding to semaphorin-3A causes neuropilin-1 to associate with plexin-A, which transduces a down-stream signal (Negishi *et al.*, 2005). Similar to the inhibitory molecules mentioned above, plexin-1 signals through the Rho family of GTPase leading to growth cone collapse (Deo *et al.*, 2004). Although the signalling interactions leading to growth cone collapse after plexin-1 activation are still unclear, they appear to be different from those of Eph receptor-induced growth cone collapse: they primarily activate different members of the Rho family of GTPases which in turn activate different down-stream effector kinases (Deo *et al.*, 2004; Murai & Pasquale, 2004; Negishi *et al.*, 2005).

Given that during development EphAs also signal through Rho GTPases, resulting in growth cone repulsion (Ellezam *et al.*, 2002), recent work by Goldshmit and colleagues used EphA4 knock-out mice to investigate a possible inhibitory role for EphA4 after injury. The EphA4 knock-out mice received spinal cord hemisections which fully axotomizes both motor and sensory connections on one side of the animal (Goldshmit *et al.*, 2004). At various time points after injury, EphA4 knock-out mice had considerably better anatomical and behavioural outcomes compared to wild-type mice receiving comparable injuries. Anatomically, at six weeks after injury, 70% of axons crossed the lesion site and were seen at 100 µm past the injury site in EphA4 knock-out mice compared to four percent in wild-type; 55% and 15% of axons were maintained at 1 mm and 5 mm respectively, beyond the lesion site in EphA4 knock-out mice, while in wild-type mice, virtually no axons were seen at the corresponding distances (Goldshmit *et al.*, 2004). These results suggest that EphA4 is part of the glial-fibrotic scar, directly inhibiting regenerating axons.

However, an unexpected and exciting finding of this study was that EphA4 regulated gliosis after spinal cord injury. In EphA4 knockout mice, there is almost no gliosis compared to wild-type mice, as measured by the number of glial fibrillary acidic protein-(GFAP)-positive hypertrophic astrocytes at the lesion site. The reduction results in a much smaller glial scar at six weeks post injury, as measured by CSPG immunohistochemistry. The small glial scar in EphA4 knock-out mice, however,

seemed to be sufficient to re-establish the glial limiting membrane, without having the inhibitory affect of the larger glial scar of wild-type mice (Goldshmit *et al.*, 2004).

It has been shown previously that gliosis is mediated by inflammatory cytokines (Sugiura *et al.*, 2000; Yong *et al.*, 1991). Wild-type cultured astrocytes showed that EphA4 is up-regulated from basal levels, by greater than 50%, and its phosphorylation increased by twofold, in the presence of the cytokines interferon-γ (IFN-γ) and leukaemia inhibitory factor (LIF); interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) had no affect (Goldshmit *et al.*, 2004). Phosphorylation of EphA4 in both wild-type tissue removed from the lesion site, and in cultured astrocytes, presumably results in the increased Rho activation observed in this tissue (Goldshmit *et al.*, 2004). Increased Rho activation was not seen, however, in tissue removed from the lesion site of EphA4 knock-out mice (Goldshmit *et al.*, 2004). These results strongly suggest that EphA4 is required to mediate cytokine-induced activation of astrocytes, leading to their proliferation and formation of the glial scar.

These data support a possible role for ephrins and their Eph receptors as inhibitory molecules at the insertion site in the *superior colliculus* after peripheral nerve graft insertion. In addition, Eph receptors in the *superior colliculus* may play a role in activating the local inflammatory response and glial scaring at the site of insertion.

Regeneration and guidance molecules in adult mammalian central nervous system

It is becoming increasingly clear that functions requiring topographic organization of the retinocollicular projection will not be restored if expression of guidance molecules in the target is inappropriate, regardless of the size of the regenerated projection (Sauvé *et al.*, 2001; Sperry, 1943; Thanos *et al.*, 1997).

I have used the peripheral nerve graft model to investigate the response of Eph/ephrin guidance molecules to peripheral nerve graft surgery. Specifically, I have examined whether the expression of these molecules can explain both the electrophysiological observations of a small topographic tendency in the regenerated projection along the rostro-caudal axis of the *superior colliculus,* as well as a role for Eph/ephrins in the glial-fibrotic scar contributing to the lack of penetration of most regenerating axons into the *superior colliculus* (Chapter 3).

Size of projection and topography

It is still unclear whether the absolute number of axons entering the *superior colliculus* is important for the correct interpretation of guidance cues in mammals, but experiments in fish suggest that this is the case. In the goldfish retino-tectal projection, a minimum of 10-15% of the original population of retinal ganglion cell axons were required to establish a retinotopic map; lower numbers lead to the breakdown of the ordered map (Udin & Gaze, 1983). One reason for the breakdown of the retinotopic map is the lack of axon-axon interaction when there is only low axon numbers (Honda, 2004; Olson & Meyer, 1994; Udin & Gaze, 1983).

The number of axons constituting the regenerated projection in rodents after peripheral nerve graft surgery it is thought to be approximately 0.1% of the total population (Sauvé *et al.*, 1995). Based on the above-mentioned studies in fish, the number would seem too low to establish a topographic map, even if the appropriate guidance molecules were available in the *superior colliculus*.

If more retinal ganglion cell axons can be encouraged to regenerate along the graft, axon-axon interaction mechanisms may enable greater re-innervation of the *superior colliculus* than is currently seen (Honda, 2004; Olson & Meyer, 1994). With a greater number of retinal ganglion cell axons forming the regenerated projection, better topographic order may be established. As a result, numerous molecules have been tested *in vitro* and *in vivo* for neuroregenerative properties to encourage more retinal ganglion cell axons to the distal end of the graft (Mansour-Robaey *et al.*, 1994; Meyer-Franke *et al.*, 1995; Shen *et al.*, 1999; Thanos *et al.*, 1993; Yin *et al.*, 2003).

Metallothionein

The peripheral nerve graft model was originally developed to show that the adult mammalian central nervous system could regenerate if presented with a supportive environment (So & Aguayo, 1985). However, the model has the additional benefit that it can be used to test the efficacy of potential trophic factors to further increase cell survival and regeneration (Cui *et al.*, 2003; Thanos *et al.*, 1993). One such factor, which has shown potential as a neuroregenerative molecule in culture and *in vivo* is the low-molecular-weight, heavy-metal-binding protein MT (Chung *et al.*, 2003).

Mammalian MT constitute a superfamily are polypeptides of 61–68 amino acids and are characterized by a low molecular weight (6–7 kDa). MT has a distinctive amino acid composition consisting of a high cysteine content and no or low histidine content. During physiological conditions MT mostly bind zinc, however, the can also bind copper, cadmium and mercury *in vivo* (Penkowa, 2006).

MT has four isoforms, two of which, MT-I and MT-II, are so similar, both in structure and in their chronological and spatial expression patterns, that they are often considered together as MT-I/II (Chung *et al.*, 2003). MT-I/II is expressed ubiquitously in the body, but in the central nervous system, it is primarily expressed in astrocytes (Nakajima *et al.*, 1991; Nishimura *et al.*, 1992). MT-III expression is confined to the central nervous system, and MT-IV is expressed in squamous epithelium (Blaauwgeers *et al.*, 1996; Palmiter *et al.*, 1992; Quaife *et al.*, 1994).

Although the precise physiological role of MT is not known (Chung & West, 2004; Palmiter, 1998) they are involved in a number of cellular processes. MT plays a role in protecting cells from heavy metal and free radical toxicity (Palmiter, 1998; Sato & Bremner, 1993), and are potentially involved in neurodegenerative diseases, such as Alzheimer's Disease (Adlard *et al.*, 1998; Uchida *et al.*, 1991) and multiple sclerosis (Penkowa *et al.*, 2003; Penkowa & Hidalgo, 2003).

The importance of MT-I/II expression after brain damage has been demonstrated using various injury models (Molinero *et al.*, 2003; Penkowa *et al.*, 2005; Penkowa *et al.*, 2004), and is closely linked with the inflammatory response of the central nervous system. In mice over-expressing MT-I, there is a reduction of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, and an increase in growth factors, such as basic fibroblast growth factor, transforming growth factor-β and vascular endothelial growth factor, compared to wild-type mice (Giralt *et al.*, 2002). In contrast, MT-I/II knock-out mice have more cells expressing pro-inflammatory cytokines, and fewer cells expressing growth factors compared to wild-type mice (Penkowa *et al.*, 2000).

Metallothionein-I/II has neuroregenerative properties

The neuroregenerative potential of MT in the central nervous system has recently been demonstrated, since ubiquitously expressed MT-I/II can increase neurite elongation by exogenous application *in vitro* (Chung *et al.*, 2003). In addition, *in vivo* application of exogenous MT-I/II at the site of cortical needle stab injury, not only increased wound healing, but encouraged reactive axonal processes to enter the injury site 4 and 7 days after lesion (Chung *et al.*, 2003). It has been proposed that MT-I/II enhances wound healing by two mechanisms. The first is by decreasing inflammation at the injury site, while increasing astrocytic activation and migration (Penkowa *et al.*, 2000; Penkowa *et al.*, 1999a; Penkowa *et al.*, 1999b). Secondly, it seems that MT-I/II can act directly on neurons to promote axonal regeneration into injury tracts (Chung *et al.*, 2003). Furthermore, peripheral nerve crush injury in MT-I/II knock-out mice results in inferior regeneration compared to wild-type mice (Ceballos *et al.*, 2003).

Extracellular metallothionein

Although MT is often considered solely intracellular proteins, mainly because they do not contain a signalling peptide sequence or have a known extracellular trafficking signal (Palmiter *et al.*, 1992). Nevertheless, evidence is accumulating that they can be released by astrocytes into the extracellular environment (Chung *et al.*, 2004). Following focal injury to the rat neocortex, gelfoam which had absorbed the extracellular fluid around the injury site, detected high levels of MT by Western blotting (Chung & West, *unpublished observations*).

In neuron-astrocyte co-cultures, *in vitro* injury results in increased MT-I/II expression, however, injury to pure astrocyte cultures results in no change of MT-I/II expression (Chung *et al.*, 2004). The demonstration that MT-I/II is probably released by astrocytes after injury to local neurons, albeit in culture, provides a potential mechanism by which MT could act on injured neurons after injury. Signals from injured neurons could stimulate up-regulation of MT-I/II expression in astrocytes, which is then released into the extracellular environment (Chung & West, 2004). These *in vitro* and *in vivo* data suggest that exogenous application of MT-I/II into the nervous system after axonal injury may increase regeneration *in vivo*. To test the neuroregeneration potential in the visual system, the final part of my thesis investigated the effect of exogenous MT-I/II administration on the regeneration of retinal ganglion cells after peripheral nerve grafting (Chapter 4).

Summary of aims

The aims of my thesis investigate three inter-related areas important for the understanding of an animal's response to injury and regeneration and are as follows:

1. Investigate which anatomical pathways are involved in the bilateral regulation of EphA5 in the retina and ephin-A2 in the *superior colliculus* after injury (Chapter 2).
2. Investigate expression of ephrin-A2 and its EphA5 receptor in the peripheral nerve graft model (Chapter 3).
3. Investigate if axon regeneration into a peripheral nerve graft is improved by the addition of a putative neuroregeneration molecule, MT-I/II (Chapter 4).

Chapter 2:

Eph/ephrin expression in the adult rat visual system following localized retinal lesions: localized and transneuronal up-regulation in the retina and *superior colliculus*

Introduction

Ephs/Ephrins are membrane-bound proteins with highly regulated developmental expression patterns within the central nervous system that control diverse processes including topographic map formation (Conover *et al.*, 2000; Cooke *et al.*, 2001; Cooke & Moens, 2002; Durbin *et al.*, 1998; McLaughlin *et al.*, 2003; Wang *et al.*, 1998). In the visual system, temporal^{high} to nasal^{low} gradients of EphA receptors in retinal ganglion cells and rostral^{low} to caudal^{high} gradients of ephrin-A2 and ephrin-A5 in the *superior colliculus* are involved in establishing topography across these axes (Brown *et al.*, 2000; Feldheim *et al.*, 2000). Retinal ganglion cell axonal guidance across the medio-lateral *superior colliculus* axis occurs *via* interactions between EphB and ephrin-B gradients (Braisted *et al.*, 1997; Connor *et al.*, 1998; McLaughlin *et al.*, 2003).

In adults, EphA and ephrin-A gradients are maintained although at lower levels, suggesting that developmental positional identity in the retina and *superior colliculus* is maintained throughout life (Knoll *et al.*, 2001; Rodger *et al.*, 2001; Sajjadi & Pasquale, 1993). Gradients may also contribute to topographic stability, since changed EphA and ephrin-A protein expression occurs when connectivity is disrupted by unilateral optic nerve or optic tract lesions to sever all retinal ganglion cell axons (Rodger *et al.*, 2000; Rodger *et al.*, 2001; Symonds *et al.*, 2001). Unilateral optic nerve crush in rat results in up-regulation of ephrin-A2 in caudal, but not rostral, *superior colliculus* to form a steep (rostral^{low} to caudal^{high}) gradient that persists for six months despite the absence of retinal ganglion cell axon regeneration (Rodger *et al.*, 2001). The differential up-regulation suggests intrinsic differences between rostral and caudal *superior collicular* cells. By contrast, in the retina, EphA5 expression in surviving retinal ganglion cells decreases to yield a uniform distribution.

Comparable changes were also observed in the ipsilateral *superior colliculus* and in retinal ganglion cells of the opposite eye. Bilateral changes were unexpected since the retinocollicular projection in rat is mostly crossed with only 3% of retinal ganglion cells innervating far rostral ipsilateral *superior colliculus* (Sefton *et al.*, 2004). However, ephrin-A2 up-regulation in caudal ipsilateral *superior colliculus* could be due to propagation of a signal from the small denervated rostral region. Alternatively, bilateral changes could be transferred *via* intercollicular pathways (Tu *et al.*, 2000; Withington & McCrossan, 1996). Changes in the opposite retina require information to be transferred

by the minimal retino-retinal projection (Muller & Hollander, 1988) and/or *via* multiple synaptic relays involving both superior colliculi (Smith *et al.*, 2000).

To investigate mechanisms governing denervation-induced changes in Eph/ephrin expression, we took advantage of the decussation pattern in rat (Sefton *et al.*, 2004). Localized retinal ablations in dorso-nasal (DN) retina removed retinal ganglion cell axons projecting exclusively to caudal contralateral *superior colliculus*; ablations in ventro-temporal (VT) retina removed retinal ganglion cell axons projecting to rostral *superior colliculus* contralaterally and ipsilaterally. We also lesioned the entire retina (Total group) to confirm previous findings (Rodger *et al.*, 2001). Intercollicular transfer of ephrin-A2 expression was investigated using unilateral collicular ablation. *In situ* hybridization and semi-quantitative immunohistochemistry were used to examine ephrin-A2 expression in both *superior colliculi*, and in the remaining *superior colliculus* after ablation, and EphA5 in both retinae. Animals were examined at one month, when changes after optic nerve lesion are maximal (Rodger *et al.*, 2001). Results have been presented in abstract form (Beazley *et al.*, 2002).

Methods

Animals and Anaesthesia

Adult female pigmented rats (PVG/c hooded, 150 grams) were used. Unilateral retinal ablations were undertaken for either DN (n = 6) or VT (n = 6) retina, or the total retinal extent (n = 3). Unilateral collicular ablations were performed in separate animals (n=6). Normal animals were also used (n = 5). For retinal and collicular ablations, rats were anaesthetised by intraperitoneal injection of 6 mg/kg ilium xylazil-20 (Troy Laboratories Pty. Ltd) combined with 50 mg/kg ketamine (Parnell Laboratories, Australia Pty. Ltd). For cholera toxin β (CTβ) injections, we used intraperitoneal injections of 35 mg/kg ketamine (Parnell Laboratories, Australia Pty Ltd) supplemented by 5 mg/kg ilium xylazil-20 (Troy Laboratories Pty. Ltd). All animals were terminally anaesthetized by intraperitoneal injection of 120 mg/kg. pentobarbitone (Valabarb, Jurox, Australia). Procedures were approved by the Animal Ethics and Experimentation Committee of the University of Western Australia and conform to the statement of ethical standards for manuscripts submitted to the *European Journal of Neuroscience*.

Retinal lesions

Phenylephrine (2.5%, Chauvin Pharmaceuticals Ltd., England) plus 1% tropicamide (Alcon, Belgium) was applied to the experimental (left) eye to dilate the pupil and visualise the fundus. Krypton laser irradiation (647.1 nm, Coherent Radiation System, USA) was delivered through a Zeiss slit lamp with a handheld cover-slide serving as a contact lens. Lesions (500 μm diameter) were delivered at 750 mW intensity for 0.2 s duration. Seven to nine overlapping lesions were performed in DN or VT quadrants. For total retinal lesions, up to 20 overlapping laser lesions were targeted in a full circle around the optic disk. The distance between lesions and the optic disk was approximately one disk diameter; pilot studies indicated that we destroyed all retinal ganglion cells at the laser site and axotomised all retinal ganglion cells peripheral to the lesion (Fig. 2.1). We excluded the possibility that retinal ganglion cells in intact retinal regions of the experimental eye or in the opposite eye were non-functional due to secondary damage (Levkovitch-Verbin *et al.*, 2001; Levkovitch-Verbin *et al.*, 2003): immediately pre-surgery and at weekly intervals thereafter orienting responses were present to food items presented (Milner & Taylor, 1990) monocularly to the intact region of the experimental retina or to the opposite eye. No responses were detected *via* the experimental eye in the Total group (data not shown).

Collicular ablations

In guinea-pig, midline commisurotomy prevents intercollicular transfer in sub-adult animals, (Withington & McCrossan, 1996). However, in adult rat, a lateral approach was necessary to avoid damage to the superior sagittal sinus and collicular ablations were therefore undertaken to prevent intercollicular transfer. Following optic nerve section to trigger collicular up-regulation of ephrin-A2 (Rodger *et al.*, 2001), the skin was cut along the midline and retracted laterally; the animal was placed on its side with the temporal ridge facing upwards. A bone flap, centred on the temporal ridge and extending rostrally from lambda, was removed. The *superior colliculus* was exposed by aspirating lateral, and thus auditory, cortex. Further aspiration removed the *superior colliculus* up to the midline, which was identified by the ventricle. Sterile gelfoam was used to stem the minimal bleeding, the boneflap replaced and the skin closed with 6/0 sutures. During the procedure, we could not exclude the possibility that some visual areas, in particular V1, were aspirated. However, aspiration of visual areas would not have influenced the outcome since visual corticotectal projections are ipsilateral (Sefton *et al.*, 2004).

Anterograde tracing

At one month, CTβ (5 µl, 1% phosphate buffered saline (PBS), pH 7.2) was injected into the experimental eye using a 10 µl micro-dissection syringe (Hamilton, U.S.A). Twenty-four hours were allowed for anterograde transport before rats were terminally anaesthetized, transcardially perfused with 0.9% sodium chloride followed by 4% paraformaldehyde.

Tissue preparation

Retinae

For wholemounts, retinae were air-dried onto Superfrost® Plus glass slides and stained with 0.5% cresyl violet (Humphrey & Beazley, 1985); retinae were mounted in Depex (BDH, England) and examined by light microscopy. For immunohistochemistry, the cornea and lens were removed and the eyes post-fixed in 4% paraformaldehyde for 6 h and cryoprotected in 15% sucrose in PBS, (pH 7.2) overnight. Left and right eyes were cryosectioned horizontally at 25 µm to expose the naso-temporal axis. Sections were collected on Superfrost® Plus glass slides and stored at –70°C.

Brains

Brains were prepared for immunohistochemistry by post-fixation in 4% paraformaldehyde (6 h) and cryoprotected in 15% sucrose in PBS overnight. Right and left hemispheres were cryosectioned sagittally to expose the rostro-caudal axis. Alternate sections were collected for CTβ analysis (30 µm; collected free floating in PBS containing 0.1% sodium azide), GFAP and ephrin-A2 analysis (20 µm mounted on Superfrost® Plus glass slides and stored at –70°C).

In situ *hybridization*

A 301 base pair sequence was amplified from rat cDNA using the following ephrin-A2 specific primers: forward 5'-CTATACCGTGGAGGTGAGCA-3'; reverse 5'-CAGGTGCTCCAACCCTCCAC-3' (designed to rat sequence in GenBank AF131912). The sequence was cloned into pBluescript II SK vector (Stratagene, CA). Digoxygenin (DIG)-labelled ephrin-A2 cRNA probes, message complementary (antisense) and noncomplementary (sense), were generated from completely linearized cDNA template using the appropriate RNA polymerase (T3, Promega, WI; T7, Ambion, TX). The labelling efficiency of the cRNA probes was checked by comparing serial dilutions with

a standard of known concentration on a dot blot (Schaeren-Wiemers & Gerfin-Moser, 1993).

Non-radioactive *in situ* hybridization was carried out based on previous methods (Giger *et al.*, 1996). Sections were air dried at room temperature (RT) for 1 h, incubated 2 × 5 min with DEPC-treated PBS and postfixed in 4% paraformaldehyde for 20 min at RT. Following a rinse in diethyl pyrocarbonate (DEPC) -treated water, tissue was acetylated 2 × 5 min in 0.25% acetic anhydride in 0.1 M triethanolamine and washed 2 × 5 min in DEPC-treated PBS, followed by 2 × 5 min in 2× saline sodium citrate (SSC; 1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2). Tissue was prehybridized for 6 h at RT in hybridization mixture (40% formamide, 10% dextran suphate, 0.2 mg/ml RNAse free bovine serum albumin (BSA; Roche, Australia), 4× SSC, 10 mM dithiothreitol (DTT; Roche, Australia), 1 mg/ml yeast tRNA (Roche, Australia), 1 mg/ml fish sperm DNA (Roche, Australia)). DIG-labelled RNA probe (1 µl of 500 ng/µl) in 500 µl of hybridization solution was denatured at 80°C for 5 min and immediately placed on ice. Probe (150 µl) was applied to each section, covered with parafilm and incubated overnight in a humidified chamber (4× SSC/50% formamide) at 55˚C.

For immunological detection, slides were washed in 2× SSC for 5-10 min at RT to remove parafilm, then transferred to fresh 2× SSC and washed for 30 min at RT. Washes of increasing stringency were then carried out (2× SSC, 0.1× SSC, 1 h each at 65°C) before blocking in Tris buffered saline, pH 7.5 (TBS: 100 mM Tris/150 mM NaCl; pH 7.5) + 2% normal horse serum + 0.1% Triton X-100. The antibody (anti-DIG-AP Fab fragment; Roche, Australia) was diluted 1:1000 in blocking solution and applied overnight at 4°C in TBS. The following day, sections were washed 2 × 15 min in TBS, incubated for 10 min with detection buffer (100 mM Tris/50 mM $MgCl_2$/100 mM NaCl; pH 9.5) and developed with 200 µl NBT/BCIP solution in the dark (20 min up to a maximum of 24 h without shaking). Colour reaction was stopped by incubating the slides in stop buffer (10 mM Tris/1 mM EDTA; pH 8.1). To remove non-specific precipitate, slides were incubated in 95% ethanol for 1 h in the dark, rinsed in water for 15 min and mounted in Fluoromount-G™ aqueous based mounting medium (SouthernBiotech, AL) and sections were allowed to dry in the dark before observation and analysis. Sections hybridized with sense probe were negative (Fig. 2.3F).

Immunohistochemistry

Sections (retinae and brains) were rinsed in PBS and endogenous peroxidases inhibited with 0.3% H_2O_2 in PBS. To detect CTβ, sections were blocked in 5% BSA and incubated overnight at 4ºC in goat anti- CTβ (1:4000; Sapphire Biosciences, Australia). To detect GFAP, ephrin-A2 and EphA5, sections were treated with PBS containing 0.2% TritonX100 for 10 minutes and incubated overnight at 4ºC in anti-G-A-5 (1:500 Sigma, NSW, Australia), rabbit anti-ephrin-A2 (1:500 Santa Cruz Biotechnology, CA) or rabbit anti-EphA5 (1:100 Santa Cruz Biotechnology). Sections were washed in PBS and signal detected with a biotin-conjugated secondary antibody (CTβ: anti-goat-biotin (Sigma); G-A-5, ephrin-A2 and EphA5: anti-mouse/rabbit-biotin (DAKO, CA)) and peroxidase-conjugated streptavidin (DAKO, CA). Signal was visualised with diaminobenzidine-metal concentrate (Pierce, IL) applied for 2 minutes. Slides were rinsed in PBS, dehydrated in increasing alcohol concentrations, cleared in xylene and mounted in DEPEX.

Antibody specificity

We have previously characterized the antibodies to ephrin-A2 and EphA5 used in the present study using western blotting and immunohistochemical controls (Rodger *et al.*, 2001; Rodger *et al.*, 2004). The antibody to ephrin-A2 has been used by others to label to human, mouse, rat, gerbil and chick tissue *in vivo* and *in vitro* (Bianchi & Gale, 1998; Brantley *et al.*, 2002; Davenport *et al.*, 1998; Hattori *et al.*, 2000). In our hands, the antibody detected a doublet at roughly 28 kDa in rat *superior colliculus* homogenates (Rodger *et al.*, 2001), and did not cross react with ephrin-A5 protein in a dot-blot assay (Rodger *et al.*, 2004), supporting the Manufacturer's statement that no cross-reactivity has been observed with ephrin-A family members other than ephrin-A2. We have performed western blots using the antibody to EphA5 (Rodger *et al.*, 2001), the results being similar to data provided by the manufacturers (www.scbt.com.). The staining patterns for the antibodies to ephrin-A2 and EphA5 are compatible with recombinant receptor and ligand staining (collicular ephrin-As, Knoll et al., 2001; retinal EphAs, Feldheim et al., 2000) and *in situ* hybridisation (retinal EphA5, Brown et al., 2000). We performed additional immunohistochemical controls for both antibodies as previously described (Rodger *et al.*, 2001): sections were negative when the primary antibody was pre-adsorbed with a blocking peptide, was omitted, or when the antibody was replaced by normal rabbit serum (Figs. 2.4Ai and 2.6G).

Quantification and analysis

Extent of retinal lesions

Lesioned regions were defined as those lacking retinal ganglion cells, both within and peripheral to the lesion. Images were captured using a JVC video camera attached to an Olympus light microscope and the extent of the lesions (% total retinal area) measured using Adobe Photoshop.

Extent of superior colliculus denervation

For *superior colliculus* sections, we confirmed complementary staining patterns for CTβ and GFAP in adjacent sections (see Results): denervated regions were negative for CTβ and positive for GFAP, with the converse for intact regions. Lack of an overlap between CTβ and GFAP staining indicated that an absence of CTβ was a true indicator of the extent of denervation (Figs. 2.2A-D). However, CTβ provided a more distinct boundary between denervated and intact regions compared to GFAP and was used for quantitation. CTβ-stained sections (n = 4 per *superior colliculus*) spanning the medio-lateral axis were used to reconstruct a three dimensional representation using Adobe Photoshop. Percentage denervation for each *superior colliculus* was calculated from a dorsal view by measuring the surface of denervated areas and dividing by the total *superior collicular* area.

Densitometry

Semi-quantitative measures of ephrin-A2 and EphA5 immunoreactivity were obtained to allow comparison between animals with different lesions (Rodger *et al.*, 2001; Symonds *et al.*, 2001). Images were captured using a JVC video camera attached to an Olympus light microscope. Image Pro Plus software was used to determine levels of immunoreactivity for ephrin-A2 across the rostro-caudal axis of the *superior colliculus* and for EphA5 in retinal ganglion cells across the naso-temporal retinal axis. Ephrin-A2 immunolabelling of the *superior colliculus* was detected within the *stratum griseum superficiale* as a dense fibrous network (Rodger *et al.*, 2001), presumably representing localization in neurons, as in mouse *superior colliculus* and in chick and goldfish tectum (Davenport *et al.*, 1998; King *et al.*, 2003). To measure the gradient of ephrin-A2 immunoreactivity across the rostro-caudal axis of the *superior colliculus*, sections were photographed at 40× magnification. Digital images (n = 4-8 per group) were imported into Image Pro Plus, and a line drawn manually within the *stratum griseum superficiale* spanning the full rostral to caudal extent of the *superior colliculus*. The

software sampled the intensity of immunoreactivity at 100 equidistant points along the line; these values were exported to Microsoft Excel for analysis. *In situ* hybridization was quantified using the same procedure. Care was taken to sample only sections that included denervated regions of the *superior colliculus*. Since DN lesions frequently spared far-medial *superior colliculus* and VT lesions spared far-lateral *superior colliculus*, sections were taken from mid-*superior colliculus* (roughly 50% of the collicular surface) to ensure consistent comparisons between groups. Nevertheless, we did not observe any qualitative differences between the spared and denervated regions. Furthermore, the variation in the extent of the spared region (medial for DN and lateral for VT) precluded statistical comparison of spared *vs* denervated regions.

To control for variation between sections and allow semi-quantitative comparison, densitometry measurements were normalized against values taken from the cerebellum, a region expressing ephrin-A2 (Rogers *et al.*, 1999) and not affected by retinal lesion. A maximum dark value (D) was estimated within a 50 × 50 μm^2 area, sampled in the strongly immunopositive molecular layer of lobule VIII of the rostral cerebellum and a minimum light value (L) from the same area sampled in the immunonegative granular layer (Fig. 2.4H). Values were normalized using the equation: Normalized value = (raw value-L)/(D-L). Each *superior collicular* measurement was normalized against cerebellum values taken from the same section. In this way, we took into account changes that were due to variation in intensity of specific labelling (dark values) and in background "non-specific" labelling (light values). We also verified that ephrin-A2 expression levels did not vary across the medio-lateral axis of lobule VIII ($p > 0.05$).

Within the retina, the majority of retinal ganglion cells were EphA5-positive. Analysis was confined to retinal ganglion cells, identified by their large cross-sectional soma area (greater than 15 μm in diameter (Perry, 1979)) and characteristic morphology (Fukuda, 1977; Perry, 1979; Rodger *et al.*, 2001). The criteria includes Types I and II but excludes Type III and IV cells, which are thought to be displaced amacrine cells (maximum diameter 11.4 μm (Fukuda, 1977; Perry, 1979; Rodger *et al.*, 2001)). Only sections encompassing the optic disk were measured to ensure optimal assessment of graded expression along the naso-temporal axis and consistency between animals (n = 3 sections per retina; 1000× magnification). For normal animals and the unlesioned opposite retinae of DN, VT and Total experimental groups, we quantified intracellular immunoreactivity by drawing around the cytoplasm of all retinal ganglion cells within

four 100 μm long strips located in far-nasal, central-nasal, central-temporal and far-temporal retina (average 4.64 $\pm$ 1.11 retinal ganglion cells per location). In experimental retinae of DN and VT experimental groups, only areas outside the lesioned region were measured: far- and mid-temporal retina for the DN group and far- and mid-nasal retina for VT group. The lesioned retina of the Total group was not assessed due to the absence of retinal ganglion cells.

To control for variation between sections, densitometry measurements were normalized against background labelling (light values; L) within a 50×50 μm^2 in the outer nuclear layer: Normalized value = (raw value–L)/L. Every retinal ganglion cell measurement was normalized against values taken from the same section. Control dark values were not obtained since immunoreactivity for EphA5 was restricted to retinal ganglion cells.

Statistical analysis

For each experimental group, values were compared to those in normal rats. To assess changes in ephrin-A2 expression, levels of immunoreactivity in rostral (average of the 10 rostral-most values), mid (average of 10 values from mid-*superior colliculus*) and caudal (average of 10 caudal-most values) *superior colliculi* were compared to equivalent levels in normal rats using ANOVA (lesion type and hemisphere as factors; Dunnett's *post-hoc* test). To test for significance between rostral and caudal *superior collicular* regions within a group, we used a paired *t*-test. To analyse gradient steepness, a linear regression line equation was calculated for the 100 points and the slope of the regression line compared between normal and experimental groups using ANOVA (lesion type and hemisphere as factors; Dunnett's *post-hoc* test).

For EphA5 expression, levels of immunoreactivity were compared to equivalent locations in normal animals using ANOVA (lesion type as the factor; Dunnett's *post-hoc* test). Presence of graded expression was assessed using a paired *t*-test to compare immunoreactivity at the far-nasal and the far-temporal locations within groups.

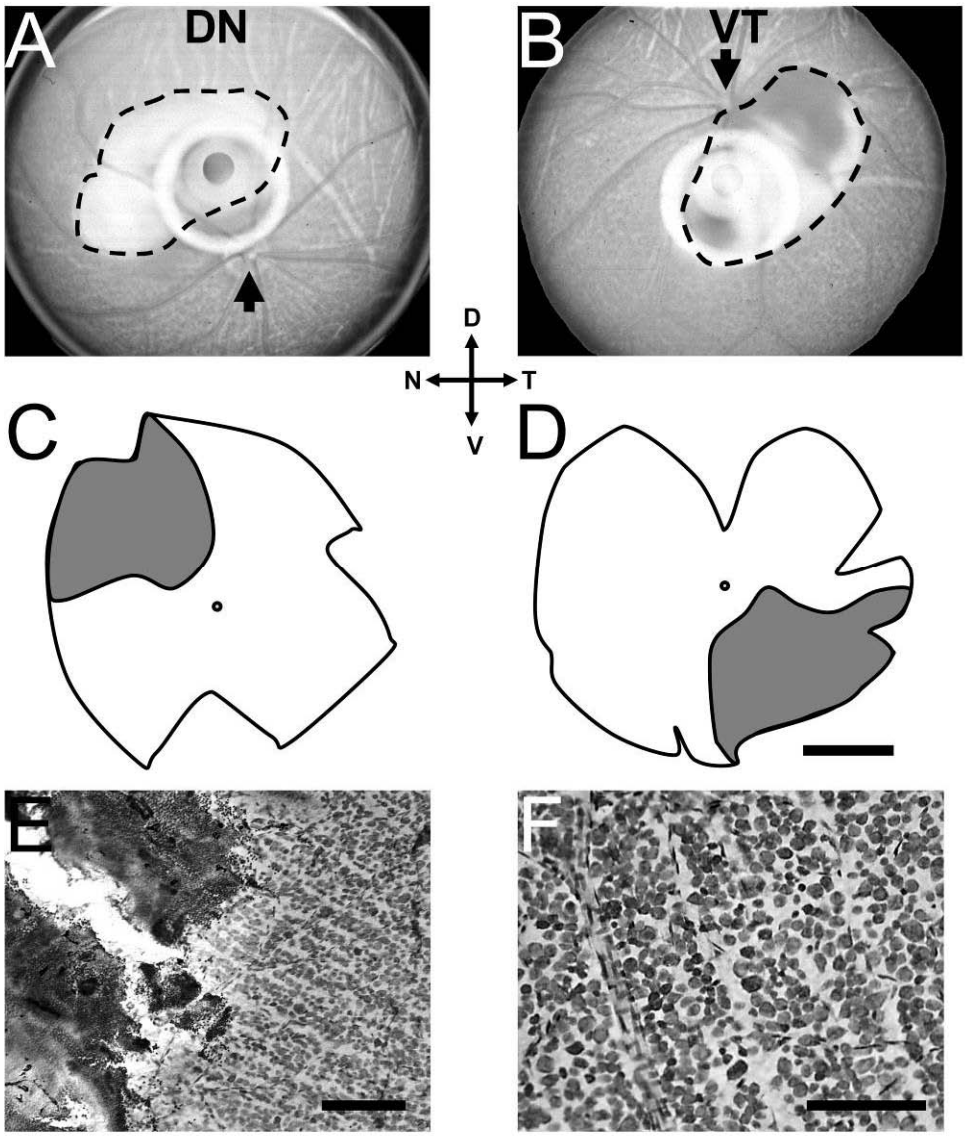

Figure 2. 1: (A and B) Fundus photographs of the experimental (left) retina immediately following dorso-nasal (A) or ventro-temporal ablations (B). The optic disk is indicated with an arrow; the photograph in A shows predominantly dorsal retina and that in B predominantly ventral retina. The white circle is an artefact of the camera lens; lesioned areas appear as pale areas due to haemorrhage and are outlined with dotted lines. (C and D) Diagram of retinal wholemounts after dorso-nasal (C) or ventro-temporal (D) ablations showing intact (white) and axotomised areas (grey). (E and F) Photomicrograph of the cresyl violet stained retinal wholemount in C showing lesioned (E) and intact (F) areas. Scale bars, 2 mm (C and D); 250 μm (E) and 100 μm (F). D, Dorsal; V, ventral; N, nasal; T, temporal.

Results

Retinal lesions

The location of lesions was confirmed immediately by fundus photography (Figs. 2.1A and B). Analysis of cresyl violet stained wholemounts at 1 month confirmed the restriction of lesions to DN or VT quadrants (Figs. 2.1C and D). The area in which retinal ganglion cells had been axotomised was readily identified by the absence of such cells in a sector extending from the lesion to the retinal periphery. Cells in the inner and outer nuclear layers were mostly intact except where the laser had penetrated the pigment epithelium. Blood vessels appeared normal within retinal ganglion cell-free areas with no apparent neovascularisation. The areal extent of affected retina was similar for DN and VT groups (unpaired *t*-test: p = 0.09), representing 28.58% (SD 5.07) of total retinal area in DN and 23.5% (SD 3.54) in VT animals. For total lesions, no retinal ganglion cells were observed in cresyl violet stained wholemounts or cryosections. Retinal ganglion cell densities were normal in the unaffected areas of the experimental eye in the DN and VT groups and in the opposite eye of all groups (Figs. 2.1E and F).

Extent of superior colliculus denervation

Normal animals

Anterograde tracing with CTβ and immunolabelling for GFAP matched previous descriptions for the retinocollicular projection (Harvey *et al.*, 1993; Sefton *et al.*, 2004). Contralateral CTβ staining was restricted to the *stratum opticum* and *stratum griseum superficiale*, and was uniform across the medio-lateral and rostro-caudal axes (Fig. 2.2A). Ipsilaterally, staining was restricted to a small rostro-medial patch in the deep *stratum griseum superficiale* (Fig. 2.2A)**.** GFAP immunoreactivity was sparsely distributed in glial cells throughout the *stratum opticum* and *stratum griseum superficiale* with immunopositive processes most commonly observed at the pial surface and frequently associated with blood vessels.

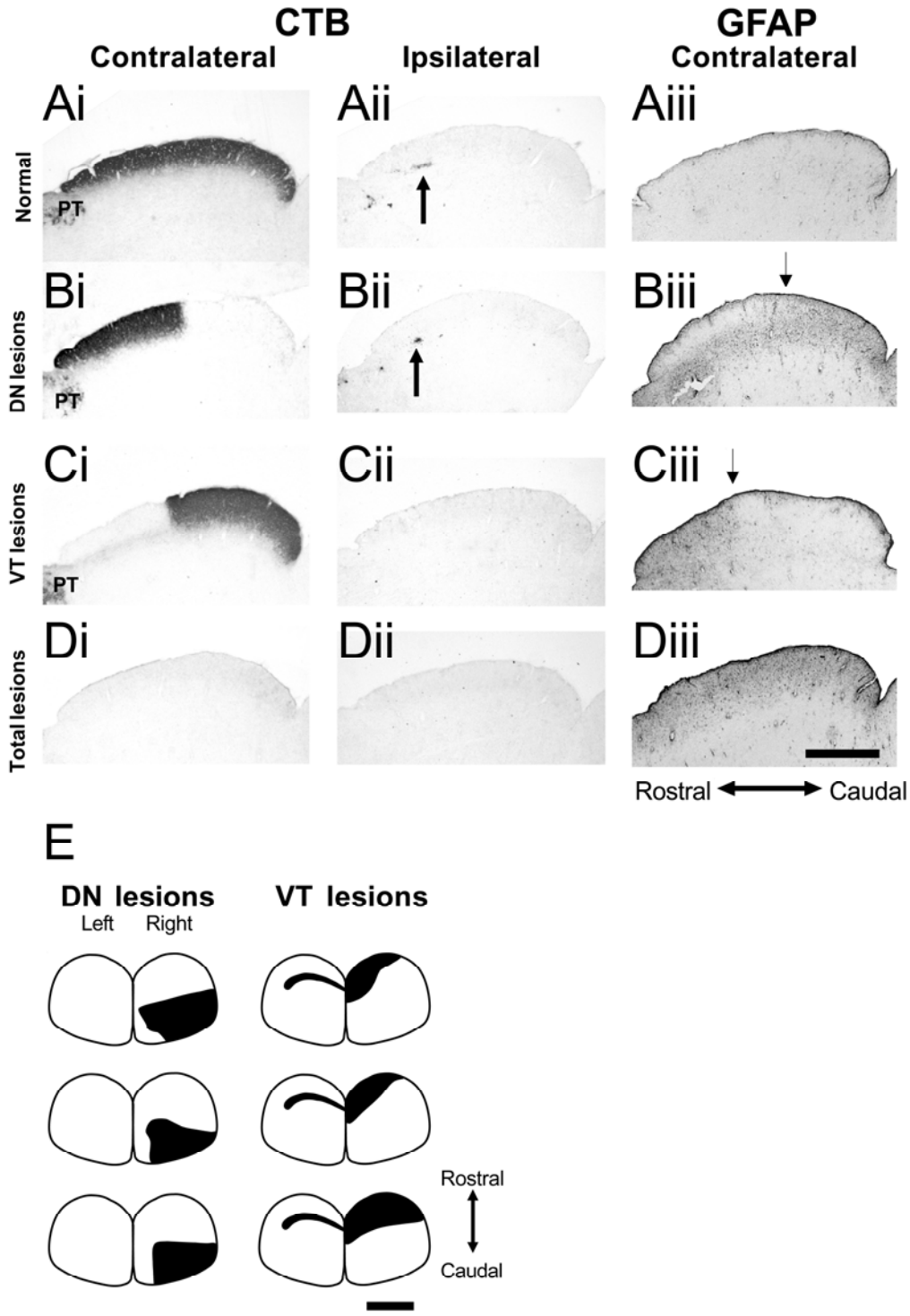

Figure 2. 2: (A-D) Sagittal sections of the *superior colliculus* immunolabelled for the anterograde tracer CTβ (i: contralateral; ii: ipsilateral) or the GFAP (iii: contralateral) in normal animals (A) and DN (B), VT (C) and total lesion (D) groups. (E) Reconstructions of three representative brains from DN and VT groups showing a dorsal view of the extent of denervation (in black) across the contralateral and ipsilateral *superior colliculus*. Total lesions are not shown since the entire *superior colliculus* was denervated. PT (Ai; Bi; Ci): pretectum; thick arrows (Aii; Bii) indicate the small ipsilateral projection labelled with anti-CTβ. Thin arrows (Biii; Ciii) indicate the limit of GFAP immunoreactivity within the *stratum griseum superficiale*. Scale bars, 1mm.

Retinal lesion animals

In the DN group, CTβ immunoreactivity was present contralaterally in all but caudo-medial *superior colliculus*; a small patch of labelling was present ipsilaterally in rostro-medial *superior colliculus* (Fig. 2.2B). In the VT group, immunoreactivity was detected contralaterally in all but rostro-medial *superior colliculus*; CTβ label was absent from ipsilateral *superior colliculus* (Fig. 2.2C). Reconstructions confirmed that the area denervated in contralateral *superior colliculus* was comparable in individual animals and between groups (21.33% + 9.87 in DN animals; 23.33% + 8.39 in VT animals; Fig. 2.2E). No CTβ labelling was detected for the Total group (Fig. 2.2D).

Staining patterns for GFAP were complementary to those with CTβ. As previously reported (McLoon, 1986; Schmidt-Kastner *et al.*, 1993), GFAP immunopositive processes in the *stratum griseum superficiale* appeared abnormally numerous, densely labelled and of greater length in the denervated regions as defined by CTβ tracing (Figs. 2A-D). Moreover, in the contralateral *superior colliculus* of the DN group, GFAP immunoreactivity was elevated in the *stratum opticum* rostrally as well as caudally, presumably corresponding to degeneration of retinal ganglion cell axons of passage projecting to caudal *superior colliculus* (Fig. 2.2B). In the Total group, GFAP immunoreactivity was intense throughout the contralateral *stratum opticum* and *stratum griseum superficiale* (Fig. 2.2D). The technique was, however, was not sufficiently sensitive to detect the small denervated region of ipsilateral *superior colliculus* in the VT and Total groups.

Ephrin-A2 expression

In situ hybridization and immunohistochemistry revealed similar changes (Figs. 2.3, 2.4 and 2.5).

In situ *hybridization*

Ephrin-A2 mRNA expression appeared as an increasing rostro-caudal gradient in the *superior colliculus* of normal animals as previously reported (Rodger *et al.*, 2001). Expression was increased caudally in DN and total groups but appeared unchanged in VT lesioned animals (Figs. 2.3A-D). Changes were observed bilaterally (Figs. 2.3A and B). mRNA levels were decreased to a flat expression level in the remaining colliculus following collicular ablation (Fig. 2.3E).

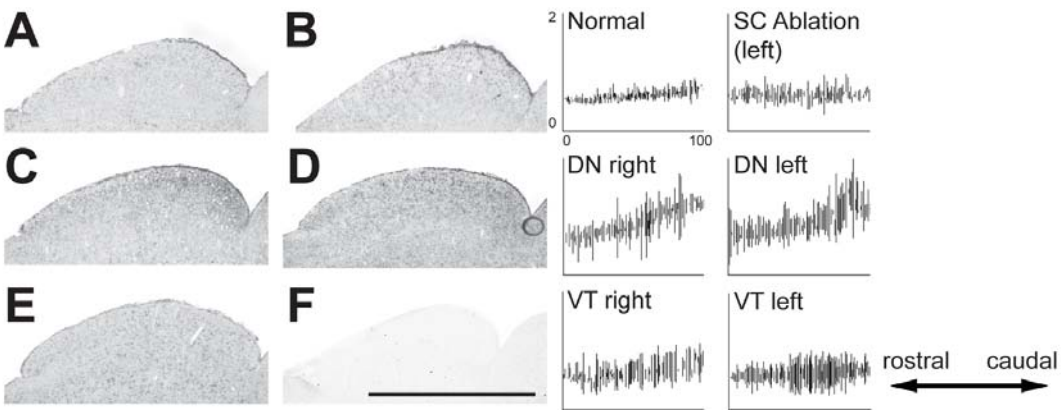

Figure 2. 3: Photomicrographs of ephrin-A2 *in situ* hybridisation in the SC. (A) normal; (B) VT lesion contralateral *superior colliculus*; (C and D) DN lesion (C, contralateral; D, ipsilateral SC); (E) collicular ablation; (F) antisense probe control on DN tissue. Densitometry plots showing ephrin-A2 expression across the rostro-caudal SC axis in normal, DN, VT and SC ablation groups. Plots are averaged from five animals using 3-5 sections per animal. Levels of ephrin-A2 immunoreactivity in the SC were normalized against those in the cerebellum. Scale bar, 1mm.

Table 2. 1: Ephrin-A2 immunoreactivity in the *superior colliculus*.

	Rostral	Mid	Caudal
Normal	79.74 ± 13.03	85.02 ± 14.28	99.76 ± 7.14
DN lesion			
Contralateral	89.11 ± 25.62	93.54 ± 28.35	159.57 ± 32.00*
Ipsilateral	81.23 ± 11.13	93.51 ± 31.64	135.31 ± 16.85*
VT lesion			
Contralateral	74.31 ± 16.54	81.42 ± 7.09	95.42 ± 12.60
Ipsilateral	65.89 ± 12.08	65.60 ± 9.81	83.59 ± 10.89
Total lesion			
Contralateral	83.40 ± 7.96	106.71 ± 11.58	160.46 ± 10.51*
Ipsilateral	96.23 ± 7.58	108.61 ± 3.39	143.43 ± 9.38*
Colliculus ablation			
Ipsilateral	48.80 ± 19.85*	61.40 ±12.20*	51.01 ± 23.44*

Values are normalized to dark and light values measured in the cerebellum as described in methods.

*$P < 0.05$ compared with those at the equivalent location in normal animals.

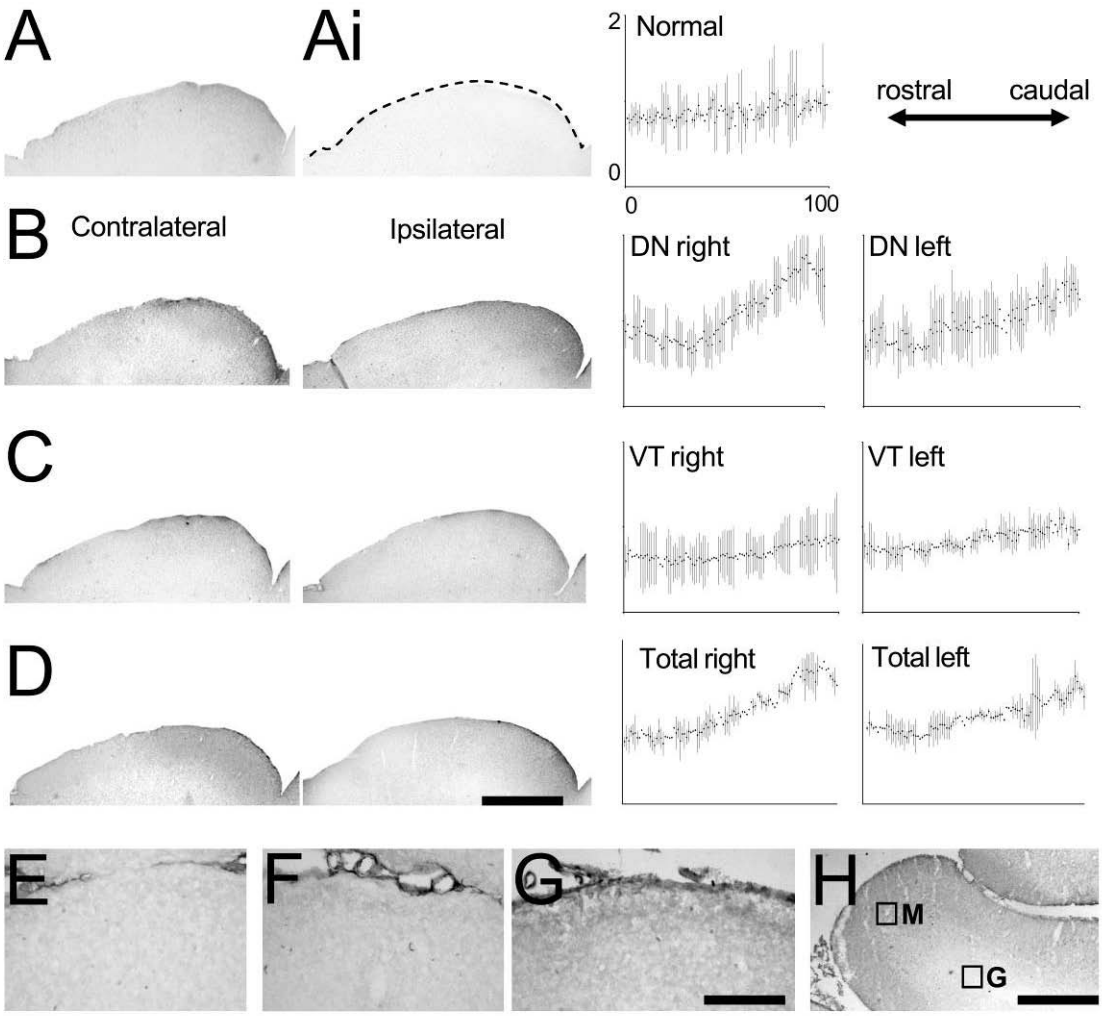

Figure 2. 4: Photomicrographs of ephrin-A2 immunoreactivity in sagittal sections of normal SC (A) and contralateral and ipsilateral *superior colliculus* from DN (B), VT (C) and Total lesion (D) animals. Sections were not stained when incubated with rabbit serum instead of anti-ephrin-A2 (shown here for normal animals; Ai). High power photomicrographs of the *superior colliculus* are shown for VT rostral (E), VT caudal (F) and DN caudal (G) regions. Densitometry plots showing ephrin-A2 expression across the rostro-caudal axis of the *superior colliculus* in normal, DN, VT and total lesion groups. Plots are averaged from 5 animals using 3-5 sections per animal. Levels of ephrin-A2 immunoreactivity in the *superior colliculus* were normalised against those in the cerebellum (H). Expression was high in the granular layer (boxed G) and absent from the molecular layer (boxed M). Scale bars, 1 mm (A-D); 100 μm (E-G); 200 μm (H).

Immunohistochemistry

Intensity of ephrin-A2 immunoreactivity is summarized in table 2.1.

Normal animals. There was no significant difference in ephrin-A2 immunoreactivity in the *stratum griseum superficiale* between hemispheres with shallow rostral^{low} to caudal^{high} gradients of expression (Table 2.1). Nevertheless levels were significantly higher caudally than rostrally ($p < 0.05$) with a slope of 0.22 ± 0.17 (Fig. 2.4A). Values

are similar to those previously reported (Rodger *et al.*, 2001). Our initial study detected a strong trend rather than a significant difference between rostral and caudal *superior colliculus*, presumably reflecting the greater number of animals studied here (n = 5 present study; n = 3 previously).

Retinal lesion animals. Changes in ephrin-A2 expression in the *superior colliculus* were superimposed on persistent rostral^{low} to caudal^{high} gradients in all groups (rostral vs caudal: $p < 0.05$,). Initial statistical analysis indicated that changes in expression were affected by lesion type and hemisphere. *Post-hoc* (Dunnett's) analysis confirmed a significant difference between experimental groups. Changes in ephrin-A2 expression were only seen in groups (DN and Total) in which caudal tectum had been denervated and furthermore were confined to this region. The similarity of expression between tectal hemispheres in experimental compared to normal animals showed that changes were bilateral throughout.

In the DN group, levels of immunoreactivity did not differ from normal in either rostral ($p > 0.05$) or mid-*superior colliculus* ($p > 0.05$; Table 2.1). By contrast, values were increased caudally in both hemispheres compared to normal ($p < 0.05$; Table 2.1) and the gradient was steeper than normal (contralateral: 1.01 ± 0.29, $p < 0.05$; ipsilateral: 0.64 ± 0.01, $p < 0.05$; Figs. 2.4B and G)

In the VT group, levels of immunoreactivity were not significantly different from normal for rostral, mid or caudal *superior colliculus* in either hemisphere ($p > 0.05$; Table 2.1). At each collicular level and for both *superior colliculi*, slopes were not significantly different from normal (contralateral: 0.31 ± 0.02, $p > 0.05$; ipsilateral: 0.20 ± 0.18, $p > 0.05$; Figs. 2.4C, E and F).

In the Total group, immunoreactivity was similar to normal rostrally (both hemispheres, $p > 0.05$) and in mid *superior colliculus* (both hemispheres, $p > 0.05$). However, as previously reported (Rodger *et al.*, 2001), values were increased caudally in both hemispheres ($p < 0.05$; Table 2.1). As a result, the gradient was steeper than normal (contralateral: 0.97 ± 0.23, $p < 0.05$; ipsilateral: 0.73 ± 0.29, $p < 0.05$; Fig. 2.4D).

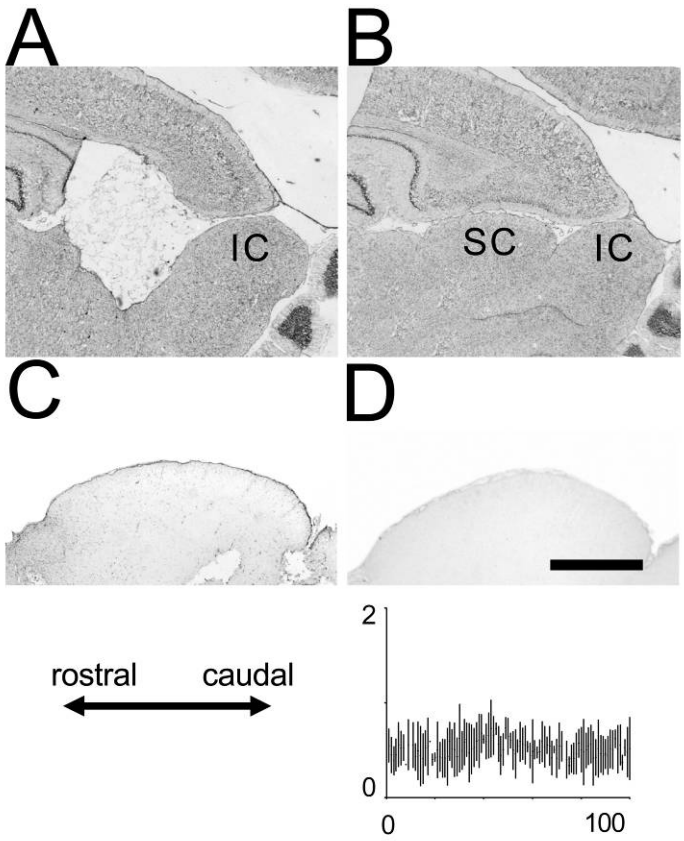

Figure 2. 5: Photomicrographs of the remaining *superior colliculus* after unilateral collicular ablation. (A and B) Cresyl violet stained section showing (A) the ablation site and (B) the opposite (unlesioned) hemisphere. (C) GFAP-stained section showing intact tissue contralateral to the ablation with no evidence of glial hypertrophy. (D) Ephrin-A2 expression across the SC was lower than normal and was not expressed as a gradient. Scatterplot shows semi-quantitative analysis of ephrin-A2 expression in the *superior colliculus* contralateral to the ablation. Scale bar, 1 mm (A-C).

Collicular ablation animals. Cresyl violet staining confirmed complete unilateral collicular ablation with the inferior colliculus remaining intact (Fig. 2.5A). GFAP staining in the remaining *superior colliculus* was negative confirming an absence of direct damage (Fig. 2.5B). Ephrin-A2 expression was lower than normal across the entire *superior colliculus* (Table 2.1) and lacked a gradient (Figs. 2.5C and D) (0.03 ± 0.40; $p > 0.05$).

EphA5 expression

The intensity of EphA5 immunoreactivity is summarized in table 2.2.

Normal animals

As described previously (Rodger *et al.*, 2001), EphA5 immunolabelling was cytoplasmic and axonal and dendritic processes often defined. A significant nasal^{low} to temporal^{high} expression gradient was present (temporal: 7.35 ± 2.0; nasal 26.16 ± 2.67, $p < 0.05$; Figs. 2.6A and B).

Table 2. 2: EphA5 immunoreactivity in the retina.

	Far nasal	Mid nasal	Mid-temporal	Far temporal
Normal	26.16 ± 2.67	30.81 ± 3.93	32.01 ± 2.77	37.35 ± 2.07
DN lesion				
Lesioned retina	NA	NA	42.26 +/- 2.12	42.13 +/- 2.03
Unlesioned retina	40.71 ± 4.82*	41.28 ± 8.45	43.49 ± 11.96	42.98 ± 7.24
VT lesion				
Lesioned retina	35.69 ± 1.53*	38.34 ± 2.95	NA	NA
Unlesioned retina	39.90 ± 5.06*	40.20 ± 6.04	43.10 ± 2.48	43.16 ± 4.00
Total lesion				
Lesioned retina	NA	NA	NA	NA
Unlesioned retina	38.39 ± 4.88*	38.87 ± 8.03	40.14 ± 5.13	39.43 ± 3.07

Values are normalized to dark and light values measured in the cerebellum as described in methods.
*$P < 0.05$ compared with those at the equivalent location in normal animals

Retinal lesion animals

In DN and VT experimental groups, EphA5 immunoreactivity was absent from the lesioned area as a result of cell loss (Fig 2.6C; Table 2.2) and, as expected, in the Total group, EphA5 immunostaining was absent throughout (Table 2.2). Changes in EphA5 expression were only seen nasally and were thus confined to the remaining nasal retina of the experimental eye in the VT group. Changes were also bilateral with EphA5 being up-regulated in nasal retina of the opposite eye in all groups.

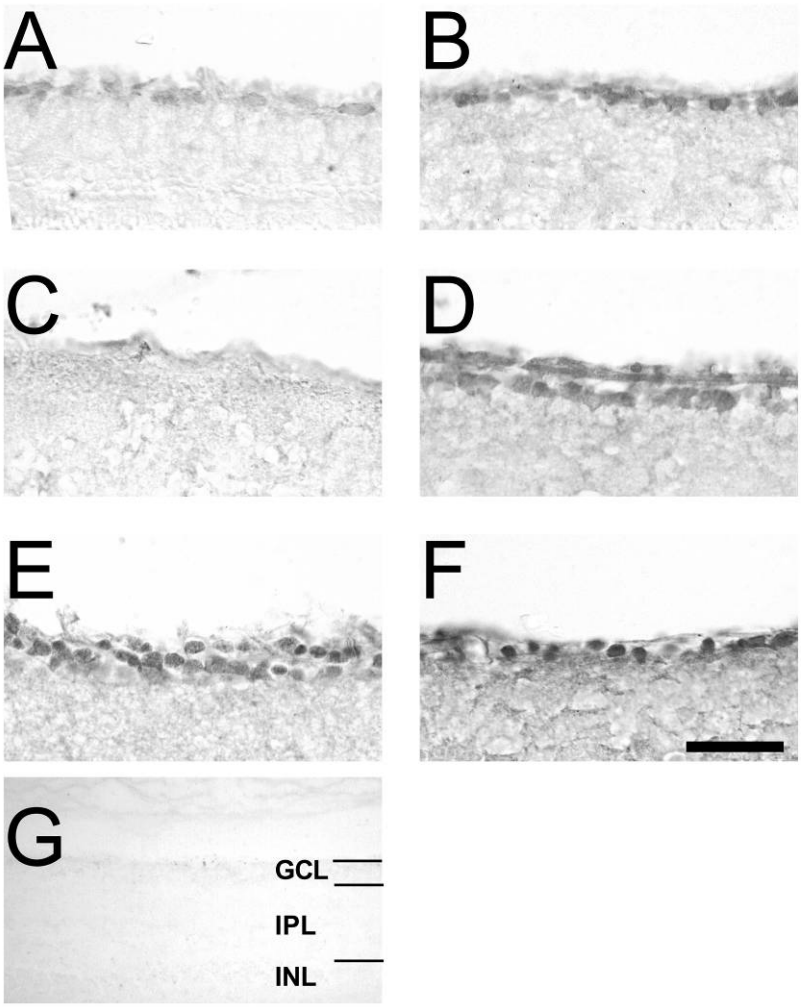

Figure 2. 6: Photomicrographs of EphA5 immunoreactivity in normal animals (left retina: A and B) and DN groups (left retina: C and D; right retina: E and F). A, C and E are taken in far-nasal and B, D and F in far-temporal retina. Sections were not stained when incubated with rabbit serum instead of anti-EphA5 (shown here for normal animals, temporal retina; G). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar, 50 µm.

In the experimental retina of the DN group, EphA5 expression was normal in the remaining mid- ($p > 0.05$) and far-temporal ($p > 0.05$) regions (Table 2.2; Fig. 2.6D). In the experimental retina of the VT group, EphA5 expression was increased compared to normal in the far-nasal region ($p > 0.05$) but was normal mid-nasally ($p < 0.05$; Table 2.2). The opposite retina of all groups followed a similar expression pattern, with far-nasal increased compared to normal ($p < 0.05$; Table 2.2), and all other regions at normal levels ($p > 0.05$). As a consequence, the nasal^{low} to temporal^{high} EphA5 gradient was replaced by a uniform naso-temporal distribution (Figs. 2.6E and F).

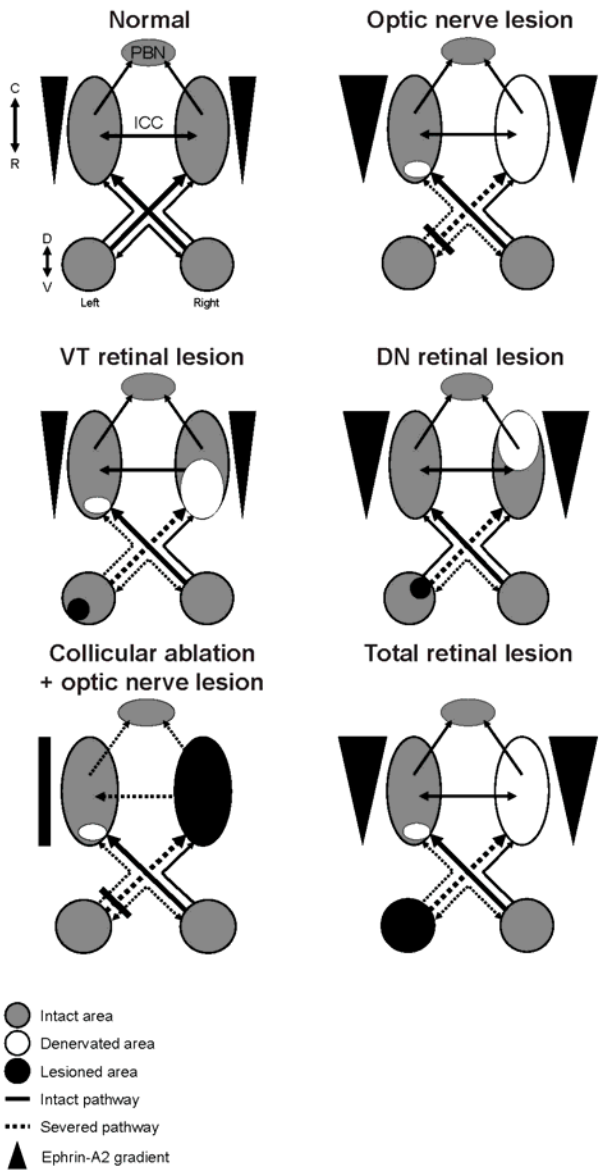

Figure 2. 7: Diagram summarising lesions, predicted denervation patterns and ephrin-A2 expression in the *superior colliculus*. Normal animals have a major contralateral projection, a minor ipsilateral one and a minimal retino-retinal pathway; the two *superior colliculi* are linked *via* the intercollicular commissure, and caudal *superior colliculus* projects ipsilaterally to the parabigeminal nucleus. There is an ascending rostro-caudal gradient of ephrin-A2 (black triangles). Unilateral optic nerve lesion (black line at lesion site and dotted lines along pathways) results in denervation (white zones in *superior colliculus*) of the contralateral, and a small region of the rostral ipsilateral *superior colliculus*; intercollicular and superior collicular-to-parabigeminal pathways remain intact. The rostro-caudal ephrin-A2 gradient is up-regulated compared to normal and equally in both *superior colliculi*, despite only minimal denervation ipsilaterally. VT retinal lesions (small black zone in retina) result in the denervation of the rostral half of the contralateral, and a small region of the rostral ipsilateral, *superior colliculus*; intercollicular and *superior collicular*-to-parabigeminal pathways remain intact. The rostro-caudal ephrin-A2 gradient does not differ from normal. DN retinal lesions (small black zones in retina) result in denervation of the caudal half of the contralateral *superior colliculus*, but no denervation ipsilaterally; intercollicular and *superior collicular*-to-parabigeminal pathways remain intact. Rostro-caudal ephrin-A2 gradients are up-regulated in both *superior colliculi* and to the same extent as that observed after optic nerve lesion despite the ipsilateral *superior colliculus* remaining intact. Optic nerve lesion and contralateral collicular ablation (black zone in *superior colliculus*) results in denervation of a small region in the ipsilateral *superior colliculus*, and the removal of the intercollicular and *superior collicular*-to-parabigeminal pathways. Ephrin-A2 expression declines below normal values and a rostro-caudal gradient is not observed. Total retinal lesions (black zone in retina) result in denervation of the contralateral, and a small region of the ipsilateral, *superior colliculus*; intercollicular and *superior collicular*-to-parabigeminal pathways remain intact. Similar to optic nerve lesion alone and to DN retinal lesions, the rostro-caudal ephrin-A2 gradient is up-regulated equally in both *superior colliculi* despite only minimal denervation ipsilaterally.

Discussion

In summary, we show that denervation in caudal *superior colliculus* is required to trigger caudal up-regulation of ephrin-A2, resulting in a steeper rostro-caudal gradient. In addition, caudal up-regulation is always observed bilaterally. Taken together, we conclude that the mechanism of ipsilateral up-regulation cannot be direct denervation since ipsilateral projections are restricted to rostral *superior colliculus* which does not up-regulate ephrin-A2 following denervation. Rather, we show, using collicular ablation, that intercollicular pathways play an important role in regulating and maintaining bilaterally equivalent ephrin-A2 expression in the *superior colliculus* (summarized in Fig. 2.7). The polysynaptic aspect of guidance cue regulation is further collicular axis and may trigger the differential ephrin-A2 up-regulation we describe. Changes emphasized by changes in EphA5 expression in both the experimental and opposite "control" retina.

Differential up-regulation of ephrin-A2 expression across the rostro-caudal axis of the superior colliculus

We report that ephrin-A2 expression is up-regulated in the *superior colliculus* following removal of retinal ganglion cell input, but only in caudal *superior colliculus* (DN and Total groups) with no detectable changes in rostral *superior colliculus* (VT, Total groups).

After *superior collicular* denervation, key developmental signalling and/or transcriptional pathways are presumably reactivated differentially across the rostro-caudal superior colliculus axis and may trigger the differential ephrin-A2 up-regulation we describe. Changes occur at the transcriptional level and therefore candidates are members of the Fgf family of secreted morphogens and one of their targets, the transcription factor engrailed, previously shown to be expressed as a rostro-caudal gradient in the developing *superior colliculus* and known to directly regulate ephrin-A2 and -A5 expression (Friedman & O'Leary, 1996; Itasaki & Nakamura, 1996; Lee *et al.*, 1997). Future studies will address the regulation of such developmentally important regulator molecules following injury in the adult central nervous system.

As an alternative, or in addition to secreted morphogens, recent studies have highlighted the possibility that the constitutive shallow ephrin-A2 gradient seen in normal animals, could itself be providing graded signalling information to *superior collicular* cells and

therefore underlie the observed differential up-regulation (Rodger *et al.*, 2004). In goldfish, regenerating retinal ganglion cell axons trigger an increased gradient of ephrin-A2 when they reach the tectum (Rodger *et al.*, 2000). However, the regeneration-induced up-regulation is prevented by blocking interaction between EphAs and ephrin-As on retinal ganglion cell axons and tectal cells (Rodger *et al.*, 2004). The result suggests that EphA/ephrin-A interactions feedback to regulate tectal ephrin-A2 expression. The implication is that a shallow initial ephrin-A gradient provides a substrate from which a steeper gradient is sculpted (Rodger *et al.*, 2004). Consistent with the possibility, in adult rat, thalamic neurons, known to sprout into denervated *superior colliculus* (Arce *et al.*, 1995), express Eph receptors (Gao *et al.*, 1998; Mann *et al.*, 2002) and may therefore, in our model, transform the weak ephrin-A2 gradient present in normal *superior colliculus* by up-regulating ephrin-A2 more in caudal than in rostral *superior colliculus*. It is possible that rostral increases are present in the present study but increments are insufficient to be revealed by our procedures. However, the hypothesis is not consistent with the up-regulation observed in the ipsilateral *superior colliculus* where no sprouting or plasticity is occurring. We can only speculate that intercollicular signalling (discussed below) can regulate ephrin-A2 expression *via* a distinct mechanism in order to match levels in both *colliculi*.

The up-regulation of EphA5 expression observed following retinal lesions contrasts with our finding of decreased expression following optic nerve crush (Rodger *et al.*, 2001). The difference is presumably due to the nature of the lesions. A laser lesion affects the retina directly, immediately destroying retinal ganglion cells at the ablation site and causing rapid apoptotic death of retinal ganglion cells axotomised near their somata; by contrast, an optic nerve crush induces a slower rate of retinal ganglion cell apoptosis. As a result, it is likely that, compared to optic nerve crush, retinal lesions induce a greater inflammatory response within the retina. The up-regulation of EphA5 we observe is therefore consistent with the up-regulation of EphA and EphB receptors in glial cells and oligodendrocytes associated with the inflammatory response at spinal cord lesion sites(Willson *et al.*, 2002; Willson *et al.*, 2003) and in hippocampal neurons that survive excitotoxic damage (Moreno-Flores & Wandosell, 1999). Although EphA5 expression was detected only in retinal ganglion cells, it is possible that glial cells in the retina indirectly affect expression through secreted molecule- or contact-dependent signalling.

Injury to the brain, spinal cord and neuromuscular contacts consistently triggers changes in the expression of one or more Eph/ephrins (Lai *et al.*, 2001; Moreno-Flores & Wandosell, 1999; Willson *et al.*, 2002; Willson *et al.*, 2003). However, regulation of Eph/ephrin expression has not always been detected following optic nerve lesions. Significant increases have been detected in rat (Rodger *et al.*, 2001) (replicated in the present study) and goldfish (Rodger *et al.*, 2000) but not in mouse (Knoll & Drescher, 2002), frog (Bach *et al.*, 2003) or zebrafish (Becker *et al.*, 2000). A possible explanation is that the species react differently to injury. Alternatively, different experimental methods may explain the contrasting results. Eph/ephrin mRNA expression was shown to be stable by *in situ* hybridisation following optic nerve lesion in zebrafish (Becker *et al.*, 2000). However, the study did not involve protein detection methods and therefore we cannot rule out the possibility that post-transcriptional changes, such as increased RNA stability and/or translation, result in increased protein levels. The studies in mouse and frog visual pathways used primarily receptor and ligand binding (Bach *et al.*, 2003; Knoll & Drescher, 2002), a method that cannot differentiate between individual Eph and ephrin proteins due to the promiscuous binding within families. It is therefore not possible to conclude that individual Ephs and/or ephrins were not regulated following injury despite the total protein complement remaining stable. Receptor-ligand binding also faces methodological problems of whether Ephs and ephrins can be detected if they are bound to each other (Sobieszczuk & Wilkinson, 1999). Moreover, consistent with our reports in rat of low levels of protein expression, in mouse, *in situ* hybridisation failed to detect ephrin-A2 and ephrin-A5 expression in the *superior colliculus* (Knoll & Drescher, 2002).

Bilateral changes

Our finding of changed ephrin-A2 expression in the remaining *superior colliculus* after ablation of the other supports the hypothesis that expression of at least this guidance molecule is regulated by feedback between the two *superior colliculi*. The intercollicular commissure and the parabigeminal system are topographically ordered pathways that link the *superior colliculi* on the two sides of the brain (Jen & Au, 1986; Linden & Perry, 1983; Withington & McCrossan, 1996; Yamasaki *et al.*, 1984). The pathways have been implicated in instigating other molecular, as well as functional, changes in one *superior colliculus* following interventions to the other, although the molecular mechanisms underlying these have not been determined. For example, although monocular enucleation in adult guinea pig results in bilateral disruption of the

collicular auditory map, commissural section prevents the ipsilateral effects (Withington & McCrossan, 1996). Similarly in frog the isthmic nucleus, homologue of the mammalian parabigeminal nucleus, regulates substance P expression in the ipsilateral *superior colliculus* following contralateral application of a NMDA antagonist (Tu *et al.*, 2000). One or both intercollicular pathways are presumably responsible for the bilateral expression changes reported here and described following optic nerve section in goldfish and adult rat (King *et al.*, 2003; Rodger *et al.*, 2000; Rodger *et al.*, 2001). In accord with the possibility, ephrin-A2 was up-regulated only unilaterally in rat with retinal ganglion cell axotomy performed at neonatal stages before the intercollicular connections are fully formed (Linden & Perry, 1983; Symonds *et al.*, 2001).

The functional significance of communicating ephrin-A expression between the two *superior colliculi* is unknown. However, it is likely that during development and throughout adulthood, similar levels of activity in both *superior colliculi* are required to maintain the alignment and synchronous function of somatosensory, auditory and visual maps within and between *colliculi* (Hyde & Knudsen, 2001; King *et al.*, 1998; Knudsen, 1982). In support of the possibility, bilateral denervation of the *superior colliculus* induces smaller changes in gene expression compared to unilateral denervation (Fujiyama *et al.*, 2003; Zilles *et al.*, 1989). Furthermore, bilateral enucleation in rat slightly reduces serotonin-1 receptor density in the *superior colliculus* whereas unilateral enucleation leads to significant increases (Boulenguez *et al.*, 1993). The different outcomes of bilateral and unilateral lesions described above could explain why, in mouse, bilateral optic nerve crush results in unchanged ephrin-A expression in the *superior colliculus* (Knoll *et al.*, 2001), whereas in rat unilateral optic nerve crush leads to a significant bilateral increase (Rodger *et al.*, 2001).

There is extensive evidence that, as in the present study, a lesion to one eye results in bilateral retinal changes. The young Braille became blind in both eyes because of the "fellow eye" effect, losing sight in the uninjured eye as a result of sympathetic ophthalmia. Similar to the results described, here, the condition is thought to activate the expression of various molecules including integrins and cell adhesion molecules, which may play a role in the pathogenesis of intraocular inflammation (Kuppner *et al.*, 1993). Although the damaging effects of this disease mainly operate through the immune system, there could still be a role for a direct retino-retinal lesion signal. Bilateral effects have also been reported for the activation of non-neuronal retinal cells

following unilateral optic nerve crush in rat (Bodeutsch *et al.*, 1999). Intraocular signalling for changes in expression may involve the direct retino-retinal projection (Muller & Hollander, 1988) although the pathway incorporates less than 100 retinal ganglion cells in adult rat (Muller & Hollander, 1988). As an alternative, the signal for changed expression may involve the crossed retinocollicular projection, an intercollicular pathway and a retrograde signal to the opposite retina as suggested by anterograde transneuronal labelling by pseudorabies virus (Smith *et al.*, 2000). The extensive and complex connectivity within the visual system would also allow other combinations of trans-synaptic anterograde and retrograde signals (Sefton *et al.*, 2004).

Irrespective of the circuitry involved, the present study illustrates that signals for expression of guidance cues are relayed beyond mono-synaptic pathways. Furthermore, the findings highlight the importance of using appropriate controls; many studies inappropriately use the unoperated side of the visual system as a control, potentially overlooking bilateral changes. Such bilateral effects emphasize the requirement for normal animals to be included in any experimental design.

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Chapter 3:

EphA5 and ephrin-A2 expression in the peripheral nerve graft model of adult mammalian central nervous system regeneration

Introduction

During development of the visual system, topographic connections between the retina and the *superior colliculus* are established using guidance molecules. The EphA family of tyrosine kinase receptors and their ephrin-A ligands are important for establishing topography between the temporo-nasal axis of the retina and the rostro-caudal axis of the *superior colliculus*. In rodents, EphA5 is constitutively expressed in the retina throughout life in a temporal^{high} to nasal^{low} gradient (Feldheim *et al.*, 1998; Rodger *et al.*, 2001), while ephrin-A2 and ephrin-A5 are expressed in complementary rostral^{low} to caudal^{high} gradients in the *superior colliculus* (Frisén *et al.*, 1998). Ephrin-As and EphAs are also expressed as counter gradients in the retina and *superior colliculus* respectively (Hornberger *et al.*, 1999; Rashid *et al.*, 2005), further contributing to the topographic organization of the retinocollicular projection (Hornberger *et al.*, 1999; Klein, 2005; Marquardt *et al.*, 2005).

Following unilateral optic nerve transection, greater than 90% of retinal ganglion cells die by 14 days (Berkelaar *et al.*, 1994), and further cell loss occurs over the following months, with less than 5% of retinal ganglion cells surviving at one year post injury (Villegas-Perez *et al.*, 1993). The surviving retinal ganglion cells, as for other neurons in the central nervous system, fail to spontaneously regenerate axons to their targets in the brain (Ramon y Cajal, 1991) In addition, the surviving retinal ganglion cells down-regulate EphA5 expression to a uniform low level (Rodger *et al.*, 2001). Interestingly, the non-experimental partner eye down-regulates to a similarly uniform low level of EphA5 expression (Rodger *et al.*, 2001).

It has been shown in this and other laboratories that, after optic nerve transection, there is a topographically appropriate gradient of ephrin-A expression in the *superior colliculus* (Knoll *et al.*, 2001; Rodger *et al.*, 2001). However, due to the lack of spontaneous regeneration of retinal ganglion cell axons, this topographically appropriate gradient is not utilised and long term denervation leads to gross cellular reorganisation of remaining connections within the *superior colliculus* (Lund & Lund, 1972).

Research more than two decades ago by So & Aguayo (1985) demonstrated that retinal ganglion cells can be encouraged to survive and regenerate their axons after optic nerve section if a segment of peripheral nerve is attached to the back of the eye. Unlike the central nervous system, the peripheral nervous system promotes survival of neurons and

the regeneration of their axons. Peripheral nerve grafting encourages approximately 10% of retinal ganglion cells to survive up to at least three months post surgery (Cui *et al.*, 2003; Villegas-Perez *et al.*, 1988). It is not known whether these surviving retinal ganglion cells retain or re-express topographically appropriate levels of guidance molecules such as EphA5 and ephrin-As after the introduction of a peripheral nerve graft.

Approximately 1,000 of the 10,000 retinal ganglion cells that survive after peripheral nerve grafting regenerate an axon into the graft (Cui *et al.*, 2003; Villegas-Perez *et al.*, 1988). The distal end of the peripheral nerve graft can be inserted into the brain, a favoured site being lateral aspect of the *superior colliculus* to allow regenerating axons to re-enter their main visual target (Sauvé *et al.*, 1995; Vidal-Sanz *et al.*, 1987). In approximately one-third of animals undergoing peripheral nerve graft surgery, regenerating retinal ganglion cell axons robustly reinnervate the *superior colliculus* (Aviles-Trigueros *et al.*, 2000). Electrophysiological recordings from the *superior colliculus* after peripheral nerve grafting, show that the regenerated projection does not form a precise topographic map (Sauvé *et al.*, 2001). However, superimposed on this apparent disorganization is a small, but significant tendency for regenerating retinal ganglion cell axons to array themselves in a topographically appropriate rostro-caudal location with respect to their cell soma position along the temporo-nasal axis of the retina (Sauvé *et al.*, 2001).

The topographically appropriate expression of ephrin-A2 observed after optic nerve transection, if retained after peripheral nerve graft surgery, may be responsible for guiding regenerating axons in the *superior colliculus*, and giving rise to the small topographic tendency (Sauvé *et al.*, 1995). If the topographic gradient of ephrin-As is utilized by the regenerating retinal ganglion cell axons, it is perplexing that the regenerated projection shows only a small tendency for topography (Sauvé *et al.*, 2001). One possibility is that the peripheral nerve graft surgery modifies the expression of ephrin-A2 in some way, which prohibits the regenerating axons from fully utilizing the topographic gradient. Insertion of the distal end of the peripheral nerve graft into the lateral aspect of the *superior colliculus* is equivalent to a penetrating injury. Such trauma to the adult central nervous system has been shown to up-regulate other guidance molecules at the injury site (Pasterkamp *et al.*, 1999), and a similar response might be anticipated for ephrin-A2.

Sciatic nerve transection is a required part of peripheral nerve graft surgery. While the expression of EphA5 and ephrin-A2 in the adult peripheral nervous system has not been studied, other guidance molecules have been shown to be up-regulated in the sciatic nerve after its transection (Scarlato *et al.*, 2003). Increased expression of these guidance molecules in the sciatic nerve graft may additionally regulate retinal ganglion cell regeneration.

Therefore, in this study I investigated the expression of EphA5 and ephrin-A2 in the peripheral nerve graft model of adult mammalian central nervous system regeneration. Topographically appropriate EphA5 expression is retained in retinal ganglion cells of the experimental eye by attachment of a peripheral nerve graft. Furthermore, I provide evidence that at one month post surgery ephrin-A2 is expressed in a topographically appropriate gradient across the *superior colliculus*. The exception is at the insertion site of the peripheral nerve graft, where expression is at ectopically high levels. These high levels implicate ephrin-A2 as an inhibitory molecule preventing axon regeneration into the *superior colliculus*; a similar inhibition has already been demonstrated for EphA4 in the spinal cord (Goldshmit *et al.*, 2004). Finally, ephrin-A2 mRNA is expressed at very low levels in adult sciatic nerve, but after transection and grafting, expression is increased one month post surgery. The results of this study provide a molecular basis to better understand the electrophysiological data that have been reported in the peripheral nerve graft model.

Materials and methods

Animals and anaesthesia

Adult female rats were sourced from the Animal Resource Centre, Perth, Australia (PVG/c hooded, 180 g) and from University College London, UK (Lister hooded, 180-200 g). Animals underwent peripheral nerve graft surgery after either unilateral (PVG/c animals) or bilateral (Lister animals) optic nerve transection. For surgery in Perth, PVG/c rats were anaesthetised by intraperitoneal injection of 10 mg/kg xylazine (Ilium Xylazil, Troy Laboratories, NSW, Australia) combined with 50 mg/kg ketamine (Ketamil, Troy Laboratories). All PVG/c rats were terminally anaesthetized by intraperitoneal injection of 120 mg/kg pentobarbitone (Valabarb, Jurox, NSW, Australia). Procedures were approved by the Animal Ethics and Experimentation

Committee of the University of Western Australia. For surgery in Utah, Lister rats were anaesthetised by intraperitoneal injection of 300 mg/kg 0.25% tribromoethanol (Avertin®; Sandorfi Winthrop Laboratories, NY). All Lister rats were terminally anaesthetised with an overdose of tribromoethanol administered by intraperitoneal injection. Procedures were approved by the Animal Ethics and Experimentation Committee of the University of Utah.

Pre-crush of sciatic nerve

One week prior to peripheral nerve graft surgery, the left sciatic nerve was exposed, and crushed close to the spinal cord with No.5 fine jewellers forceps. To ensure all axons were transacted, the forceps were pressed together firmly for 5 seconds, and immediately after a clear gap could be seen through the nerve sheath. Pre-crushing initiates Wallerian degeneration and has previously been shown to increase regeneration of retinal ganglion cells into the peripheral nerve graft (You *et al.*, 2002). After surgery, animals were placed on a heating pad to recover and were monitored. The eyes were covered with a moist tissue to prevent drying.

Peripheral nerve graft surgery

Peripheral nerve graft surgery was performed on deeply anaesthetized rats as previously described (Sauvé *et al.*, 1995; Vidal-Sanz *et al.*, 1987; Whiteley *et al.*, 1998). The left common peroneal nerve was exposed, and an approximately 3 cm length removed and stored in sterile PBS (pH 7.4) on ice while the animal was prepared for autologous grafting. The orbit of the right eye was exposed to access the optic nerve. A longitudinal incision of the dura was made, and the optic nerve transected approximately 0.5 mm from the eye, taking care to avoid damage to the ophthalmic artery (Vidal-Sanz *et al.*, 1987). Transection of the optic nerve close to the eye has previously been shown to increase regeneration into the peripheral nerve graft (You *et al.*, 2000). The peripheral nerve graft was anastomosed to the optic nerve stump with two 10-0 sutures (Ethilon; Ethicon, Johnson & Johnson, GA). The length of peripheral nerve was briefly placed in the orbit to prevent drying, whilst the contralateral *superior colliculus* was exposed. The lateral-most aspect of the left *superior colliculus*, delineated by a 'V' of blood vessels, was exposed by removal of a small bone flap and aspirating the overlying cortex. The length of peripheral nerve was then placed across the skull and the distal end inserted close to the apex of the 'V' of blood vessels. After

graft insertion the skin overlaying the skull was sutured together, taking care not to dislodge the graft.

A separate series of animals underwent bilateral optic nerve transection. The non-experimental optic nerve was transected on completion of peripheral nerve graft surgery. After surgery animals were placed on a heating pad to recover and were monitored. The eyes were covered with a moist tissue to prevent drying and a subcutaneous injection of the analgesic buprenorphine (0.02 mg/kg, Temgesic; Reckitt & Colman, UK) administered.

Four of the single nerve transaction series were used for analysis of retinae and peripheral nerve grafts at one month post surgery. Twenty-two of the bilateral transaction series were used for analysis of brains at one (n = 7) and six (n = 11) months post surgery. Four normal rats of each series did not undergo surgery and were used as controls.

Tissue preparation

At one or six months post surgery rats were terminally anaesthetised, exsanguinated by transcardial perfusion with PBS containing 0.9% sodium chloride, followed by fixation with fresh filtered 4% paraformaldehyde. The eye, peripheral nerve graft and brain were dissected from the head. Special care was taken to avoid dislodging the distal tip of the graft from the *superior colliculus.*

Retinae

The cornea and lens were removed and both eyes postfixed for 1 h with gentle agitation, before cryoprotection in 15% followed by 30% sucrose in PBS at 4°C overnight. Left and right eyes were covered in Tissue Tek optimal cutting temperature (OCT) compound (Sakura, Japan), frozen on dry ice and cryosected (Leica CM1900) horizontally (14 µm) to expose the temporo-nasal axis. Sections were collected on Superfrost® Plus glass slides (Menzel Glaser, Germany) and stored at -80°C.

Brains

Brains were post fixed for 6 h before cryoprotection in 15% followed by 30% sucrose in PBS (pH 7.4) at 4°C overnight. Brains were trimmed before covering in Tissue Tek OCT compound, frozen on dry ice and cryosected, either coronally (16 µm) to expose

the peripheral nerve graft insertion site (n = 4 at 1 month; n = 8 at 6 months), or sagittally (20 µm) to expose the rostro-caudal axis (n = 3 at each time point). Sections were collected on Superfrost® Plus glass slides and stored at -80°C.

Peripheral nerve graft

Nerve grafts were post fixed for 1 h with gentle agitation, before cryoprotection in 15% followed by 30% sucrose in PBS (pH 7.4) at 4°C overnight. Nerve grafts were trimmed before covering in Tissue Tek OCT compound, frozen on dry ice and cryosected longitudinally (16 µm). Sections were collected on Superfrost® Plus glass slides and stored at -80 °C.

In situ *hybridization*

Non-radioactive *in situ* hybridization was carried out as described in Chapter 2. An EphA5 cRNA probe was used on retinal sections, and an ephrin-A2 cRNA probe on peripheral nerve graft and brain sections. The two probes were amplified from rat cDNA using specific primers. EphA5 primers gave a 196 base pair sequence (forward primer: 5'-CGGCGTCTTCAGTCGACGG-3'; reverse primer: 5'-GGGCAACAGCGCACAGGG-3'; designed to rat sequence in GenBank X78689). The sequence was cloned into pGem®-T Easy Vector (Promega, WI). Digoxygenin (DIG)-labelled EphA5 cRNA probes, message complementary (antisense) and noncomplementary (sense), were generated from completely linearized cDNA template using the appropriate RNA polymerase (SP6, Promega, WI; T7, Ambion, TX). The details of the ephrin-A2 probe are described in Chapter 2. The EphA5 probe was hybridized at 65°C and the ephrin-A2 probe at 55°C overnight in a humidified chamber. Immunological detection was carried out as described in Chapter 2.

Immunohistochemistry

Immunoperoxidase histochemistry

Sections (retinae, grafts and brains) were air dried for 1 h at RT, incubated 2 × 5 min with PBS and endogenous peroxidases quenched by incubation in 0.3% H_2O_2 in methanol for 30 min at RT. Following 3 × 10 min washes in PBS, sections were blocked in PBS containing 5% normal goat serum (Sigma, NSW, Australia) and 0.1% Tween-20 (Sigma, NSW, Australia; blocking solution) for 1 h at RT. Primary antibody was diluted in blocking solution, applied to sections and incubated overnight at 4°C in a humidified chamber. Primary antibodies used were rabbit anti-EphA5$^{C\text{-}16}$ (1:500; Santa

Cruz, CA) on retinal sections, and rabbit anti-ephrin-$A2^{L-20}$ (1:500; Santa Cruz, CA)) on brain sections. The specificity of EphA5 and ephrin-A2 antibodies is described in Chapter 2. For immunological detection, slides were washed 3 × 5 min in PBS plus 0.1% Tween-20 before antibody binding was visualised using a biotin-avidin-peroxidase system (VECTASTAIN® ABC, Vector Laboratories, CA) and diaminobenzidine-metal complex (Pierce, IL). Slides were rinsed in PBS, water and then dehydrated through an increasing alcohol concentrations, cleared in xylene and mounted in DEPEX (BDH Laboratory Supplies, UK).

Immunofluorescence histochemistry

Immunofluorescence followed similar steps to the immunoperoxidase histochemical protocol except that the incubation step with H_2O_2 in methanol was omitted. Various primary antibodies were used and are described in the cell phenotypes section below to determine the identity of cells present across the *superior colliculus*, particularly those expressing ephrin-A2 mRNA. Secondary antibodies were, Alex Fluor 488 nm (1:400) and 546 nm (1:400) goat anti-mouse or goat anti-rabbit, depending on the primary antibodies used. Secondary antibodies were diluted in PBS plus 0.1% Tween-20 and were either applied separately or in combination to sections, incubating for 2 h at RT in the dark. Hoechst-33342 (Sigma, NSW, Australia) was also added to the secondary antibody solution (final concentration 1 µg/ml) to fluorescently stain all cell nuclei in the brain. Sections were washed 3 × 10 min in PBS in the dark and mounted in Fluoromount-G™ aqueous based mounting medium (SouthernBiotech, AL) and sections were allowed to dry in the dark before observation and analysis.

Cell phenotypes of ephrin-A2 expressing cells

To determine which cells were expressing ephrin-A2 mRNA, *in situ* hybridization was combined with immunohistochemistry either prior to (immunoperoxidase) or after (immunofluorescence) labelling mRNA. For immunoperoxidase histochemistry prior to *in situ* hybridization, modifications to the protocol were made to reduce degradation of mRNA transcripts within the tissue. All PBS was treated with DEPC and RNase inhibitor (RNasin, Promega, CA) was added to primary antibody blocking solution. Various primary antibodies were used for histochemistry to determine cell phenotype in the brain: mouse anti-NeuN (neurons; 1:100; Chemicon, CA). rabbit anti-GFAP (astrocytes; 1:500; DAKO, CA), mouse anti-G-A-5 (astrocytes; 1:500; Sigma, NSW, Australia), mouse anti-fibronectin (meningeal cells, fibroblasts; 1:500; Sigma, NSW,

Australia) and mouse anti-vimentin (meningeal cells, fibroblasts; 1:500; DAKO, CA). An antibody to chondroitin sulphate proteoglycan (Extracellular matrix; 1:500; Sigma, NSW, Australia) was also used. NeuN gave the best signal using an immunoperoxidase protocol and GFAP using immunofluorescence. It has previously been reported that the histochemical protocol used can determine the quality of signal achieved with an antibody, when combined with *in situ* hybridization (Pasterkamp *et al.*, 1999). NeuN and GFAP double labelling was also performed on brain tissue without *in situ* hybridization, to study the distribution of neurons and astrocytes after peripheral nerve graft surgery. The other antibodies, despite using a range of protocols and antigen retrieval methods, did not give successful immunolabelling.

Quantification and analysis

Microscopy and image collection

After processing for *in situ* hybridization, either alone or combined with immunoperoxidase histochemistry, sections were digitally imaged (DP70 camera; Olympus, Japan) using a bright-field microscope (BX52, Olympus) at a resolution of 2040 × 1536 dpi. Immunofluorescently labelled sections (brain and graft) were digitally imaged (CoolSNAP*cf*, Photometrics, AZ) using a Leitz Diaplan fluorescent microscope (Leica Microsystems, NSW, Australia) connected to a computer with Image-Pro Plus software (MediaCybernetics, MD) at a resolution of 1392 × 1040 dpi. In addition, laser confocal microscopy (Bio-Rad MRC 1000) with z-axis drive attached to an upright microscope (Optiphot-2, Nikon, Japan) was used to image brain sections fluorescently immunolabelled for NeuN and GFAP.

Densitometry/ Eph-A5 cell intensity analysis

Semi-quantitative measures of EphA5 immunoreactivity in retinal ganglion cell somas were obtained to allow comparisons between experimental, non-experimental and normal eyes (Rodger *et al.*, 2001; Rodger *et al.*, 2004), and was carried out as described in Chapter 2. Previous studies have shown that after unilateral optic nerve transection, EphA5 expression in retinal ganglion cells of the non-experimental eye is also affected (Chapter 2; Rodger *et al.*, 2001). For this reason, EphA5 cell intensity analysis was carried out on retinal sections from animals that had received unilateral optic nerve section prior to peripheral nerve graft surgery. In normal, experimental and non-experimental retinae, intracellular immunoreactivity was quantified by outlining retinal ganglion cell somas within four 100 μm long strips located in far-nasal, central-nasal,

central-temporal and far-temporal retina (Normal and non-experimental: average 4.64 ± 1.11 retinal ganglion cells per location; experimental: average 3.25 ± 1.37).

Ephrin-A2 expression strength analysis

Semi-quantitative ephrin-A2 mRNA expression strength analysis was carried out based on a previous method (Carson *et al.*, 2005) with minor modifications. Transcripts labelled by non-radioactive in situ hybridization cause deposits of dye in the cells in which they are expressed. Because the quantity of dye precipitate increases with the number of detected transcripts, to facilitate analysis, the levels of ephrin-A2 mRNA were classified into four categories: cells with no detectable precipitate (-), weakly expressing cells with scattered minute particles of deposit (+), moderately expressing cells partially filled with precipitate (++), and strongly expressing cells filled with dye precipitate (+++) (Carson *et al.*, 2005).

Previous studies investigating topography of the regenerated projection have used bilateral optic nerve sections (Sauvé *et al.*, 1995, 2001). For this reason, expression strength analysis was carried out on brain sections from rats that had received bilateral optic nerve section prior to peripheral nerve graft surgery.

Signal strength analysis was compared between inserted and non-inserted sides of the brain within the same animal. It was important to make comparisons within the same animal because each peripheral nerve graft is different, with small variations in the location of the insertion site. Comparing between the inserted and non-inserted sides of the brain controlled for bilateral changes that take place in the *stratum griseum superficiale* layer of the *superior colliculus* after unilateral or bilateral nerve transection (Knoll *et al.*, 2001; Rodger *et al.*, 2001). In this way, it was possible to investigate if direct injury from the insertion of the peripheral nerve graft resulted in increased ephrin-A2 expression, additional to that resulting from nerve transection alone.

Images used for expression strength analysis were taken at 80× magnification to give a sample frame of 400 µm × 320 µm. Three sample areas for each *superior colliculus*, both inserted and non-inserted, were analysed at the midline, mid and lateral locations across coronal sections. The lateral location in the inserted *superior colliculus* corresponded to the peripheral nerve graft insertion site. For each animal, at least five sections were analysed.

Cell counts

Ephrin-A2 immunopositive cell counts were made from brain sections using a BX-2 bright field microscope (Olympus, Japan). Counts were made at a magnification of 1000× under oil immersion. For counts of sagittally sectioned brains, two locations in rostral and caudal *superior colliculus* were analysed in 3-4 sections per animal. Sections were chosen from the middle of the medio-lateral axis, where the rostro-caudal length was greatest. For counts of coronally sectioned brains, four locations across each colliculus were taken, spaced equally along the medio-lateral axis from close to the midline to laterally. These coronal sections were analysed in n = 2 animals at each time point to reconstruct three-dimensional profiles.

Statistical analysis

Values in experimental animals for both EphA5 intensity and ephrin-A2 immunoperoxidase cell counts were compared to those obtained for normal animals. For EphA5 expression, levels of immunoreactivity were compared to equivalent locations in normal animals using ANOVA (treatment group as the factor; Scheffe's *post-hoc* test). To assess changes in ephrin-A2 immunopositive cell counts at rostral or caudal locations in the *superior colliculus*, were compared to counts at equivalent locations in normal animals using ANOVA (treatment group and collicular location as factors, Scheffe's *post-hoc* test). A paired *t*-test was used to test for significance between rostral and caudal collicular regions within a group. For expression strength analysis, each location in the experimental colliculus was compared the equivalent location (midline, mid or lateral) in the non-inserted colliculus using ANOVA (treatment group and collicular location as factors, Scheffe's *post-hoc* test).

Bioinformatics

It has previously been shown that a 3'-untranslated region (3'-UTR) DNA sequence can influence local protein translation in the growth cone (Brittis *et al.*, 2002; Richter, 1999). The appropriate 3'-UTR cytoplasmic polyadenylation element consensus sequence has been identified in the EphA2 gene and shown to have a role in local protein synthesis in spinal commissural neuronal growth cones during development (Brittis *et al.*, 2002). To investigate the 3'-UTR of EphA5, the EphA5 sequence was first obtained from the Rat Genome Database (http://rgd.mcw.edu). The 3'-UTR was then manually analysed for the presence of the cytoplasmic polyadenylation element consensus sequence: UUUUUAU.

Results

EphA5 expression in the retina

In situ hybridization and immunohistochemistry revealed similar expression of EphA5 mRNA and of protein across the naso-temporal axis of the retina (Fig. 3.1).

In situ *hybridization*

Normal animals. EphA5 mRNA was expressed in a smooth increasing naso-temporal gradient in retinal ganglion cells (Figs. 3.1A and B), with expression also seen in the outer nuclear layer (data not shown).

Experimental right eye. As expected, in the experimental right eye, retinal ganglion cells were sparse in the ganglion cell layer compared to normal animals due to extensive cell death (Villegas-Perez *et al.*, 1988). However, in the experimental eyes that received a peripheral nerve graft, more retinal ganglion cells survived, compared to those after optic nerve transection alone (Fig 4.1 & 4.3, Chapter 4; Rodger *et al.*, 2001; Villegas-Perez *et al.*, 1988). The surviving retinal ganglion cells in experimental eyes expressed EphA5 mRNA in an increasing naso-temporal gradient. The gradient observed was similar to that seen in normal retinae, but less prominent due to a lower retinal ganglion cell density (Figs. 3.1C and D).

Non-experimental left eye. EphA5 mRNA expression was increased in nasal retinal ganglion cells compared to normal. As a consequence, the increasing naso-temporal gradient was replaced by a uniform naso-temporal distribution of high EphA5 mRNA expression (Figs. 3.1E and F). The uniform EphA5 expression level was similar to the highest expression level observed normally in temporal retina.

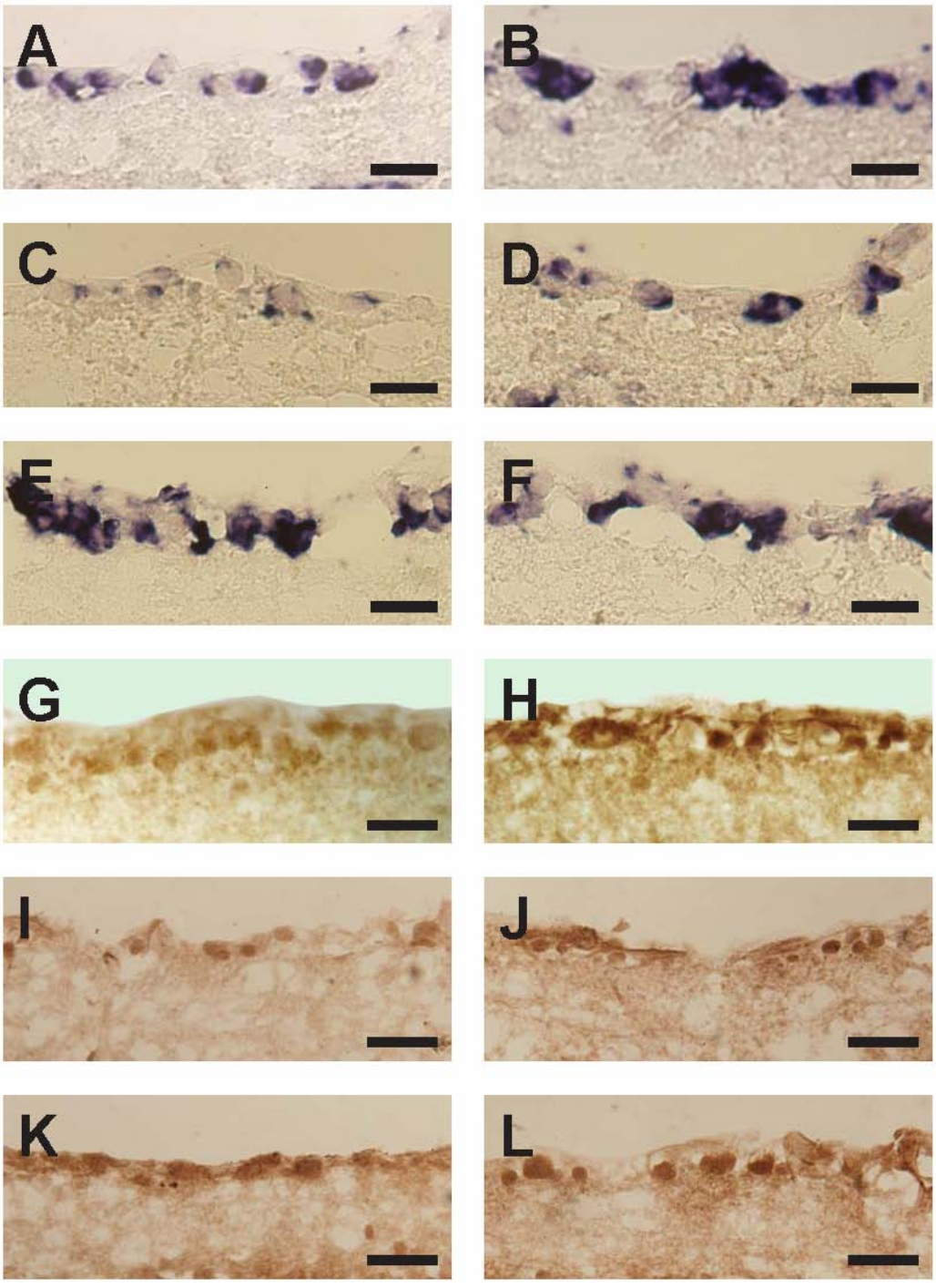

Figure 3. 1: Photomicrographs of EphA5 mRNA (A-F) and immunoreactivity (G-L) in retinal ganglion cells of normal animals (A, B, G and H), the experimental right eye which had a peripheral nerve graft attached (C, D, I and J) and the non-experimental left eye (E, F, K and L). A, C, E, G, I and K are taken in far-nasal and B, D, F, H, J and L in far-temporal retina. Scale bars, 20 µm (A-F); 50 µm (G-L).

Immunohistochemistry

The intensity of EphA5 immunoreactivity is summarized in table 3.1.

Normal animals. As described previously (Rodger *et al.*, 2001), EphA5 immunoreactivity labelling was cytoplasmic with axonal and dendritic processes often defined. The intensity of EphA5 immunoreactivity was significantly higher in far-temporal (34.21 ± 2.92) than in far-nasal (27.10 ± 2.72) retinal ganglion cells ($p < 0.05$), consistent with a nasal^{low} to temporal^{high} gradient (Table 3.1; Figs. 3.1G and H).

Experimental right eye. EphA5 immunoreactivity in the experimental eye followed a similar expression pattern to normal in surviving cells. Immunoreactivity values were significantly higher in far-temporal (31.52 ± 3.15) than in far-nasal (27.64 ± 3.12) retinal ganglion cells ($p < 0.05$), similar to that seen in normal retina (Table 3.1; Figs. 3.1I and J).

Non-experimental left eye. Unlike the experimental eye, EphA5 immunoreactivity was increased in far-nasal retinal ganglion cells compared to normal (36.01 ± 2.88; $p < 0.05$), resulting in a uniform temporo-nasal intensity of EphA5 (Table 3.1; Figs. 3.1K and L).

Table 3. 1: EphA5 immunoreactivity in the retina

	Far nasal	Mid nasal	Mid temporal	Far temporal
Normal	27.10 ± 2.72	30.89 ± 2.93	31.88 ± 2.89	34.21 ± 2.92
Right experimental eye	27.64 ± 3.12	28.78 ± 3.82	29.89 ± 2.45	31.52 ± 3.15
Left non-experimental eye	36.01 ± 2.88*	33.57 ± 4.05	34.74 ± 3.07	35.50 ± 3.55

Data are shown as mean ± SEM. *$P < 0.05$ compared with those at the equivalent location in normal animals.

EphA5 gene contains a 3'-UTR capable of modulating protein synthesis in the growth cone

Analysis of the EphA5 3'-UTR showed that it contains a cytoplasmic polyadenylation element consensus sequence, 294 base pairs downstream of the end of translation (Fig. 3.2). This cytoplasmic polyadenylation element is approximately 600 base pairs upstream of the polyadenylation signal.

Ephrin-A2 expression in the superior colliculus

In situ hybridization and immunohistochemistry both reveal similar changes of ephrin-A2 expression at the insertion site (Figs. 3.3, 3.4 and 3.6). Additionally, immunohistochemistry on sagittal sections demonstrated the expression pattern of ephrin-A2 across the rostro-caudal axis of the colliculus (Figs. 3.5 and 3.6).

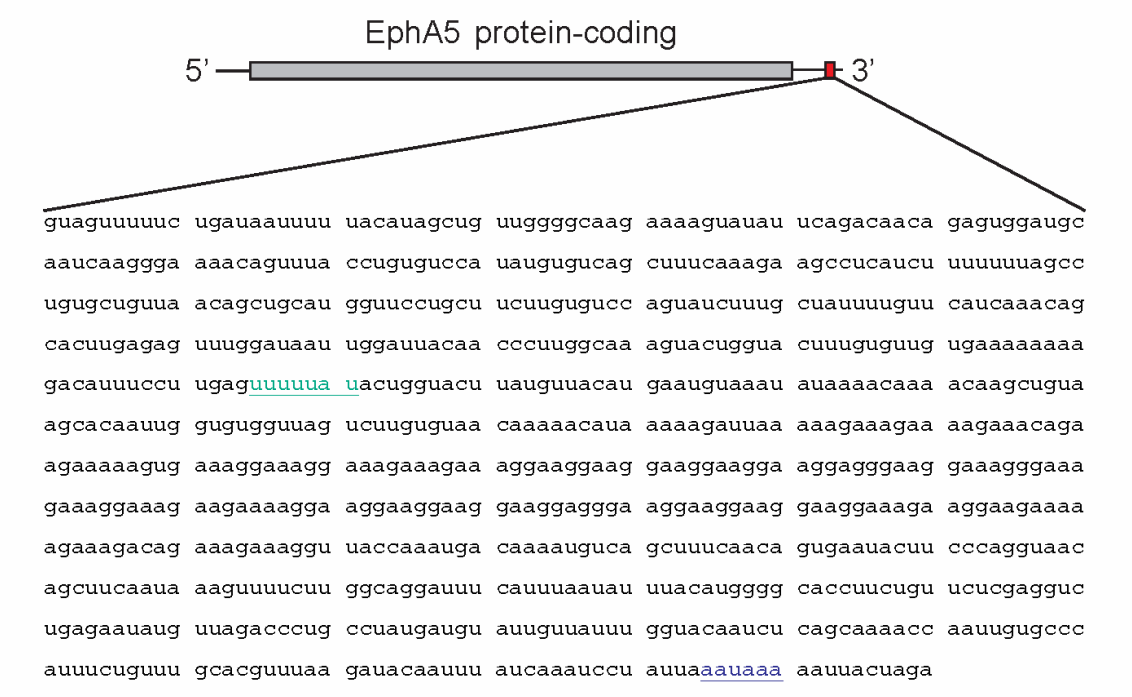

Figure 3. 2: The EphA5 mRNA 3'-untranslated region contains a cytoplasmic polyadenylation element (CPE) consensus (green, underlined) which has been shown to allow modulation of protein expression in axonal processes. This CPE consensus is 600 base pairs upstream of a hexanucleotide polyadenylation sequence (blue, underlined).

In situ *hybridization*

One month animals. At the insertion site of the peripheral nerve graft significantly more cells expressed very high levels of ephrin-A2 mRNA (+++) than at the equivalent location in the non-inserted side of the brain (Scheffe's *post-hoc* test; Figs. 3.3B, C and 3.4). These highly expressing cells were in addition to cells expressing lower levels of ephrin-A2 mRNA. The number of cells expressing lower levels of ephrin-A2 mRNA (+ and ++) was not significantly different between inserted and non-inserted *superior colliculi* at lateral locations (Scheffe's *post-hoc* test; Figs. 3.3B, C and 3.4). There was no significant difference in ephrin-A2 mRNA expression between inserted and non-inserted sides of the *superior colliculi* at locations distant from the insertion site (Scheffe's *post-hoc* test; Figs. 3.4B and C).

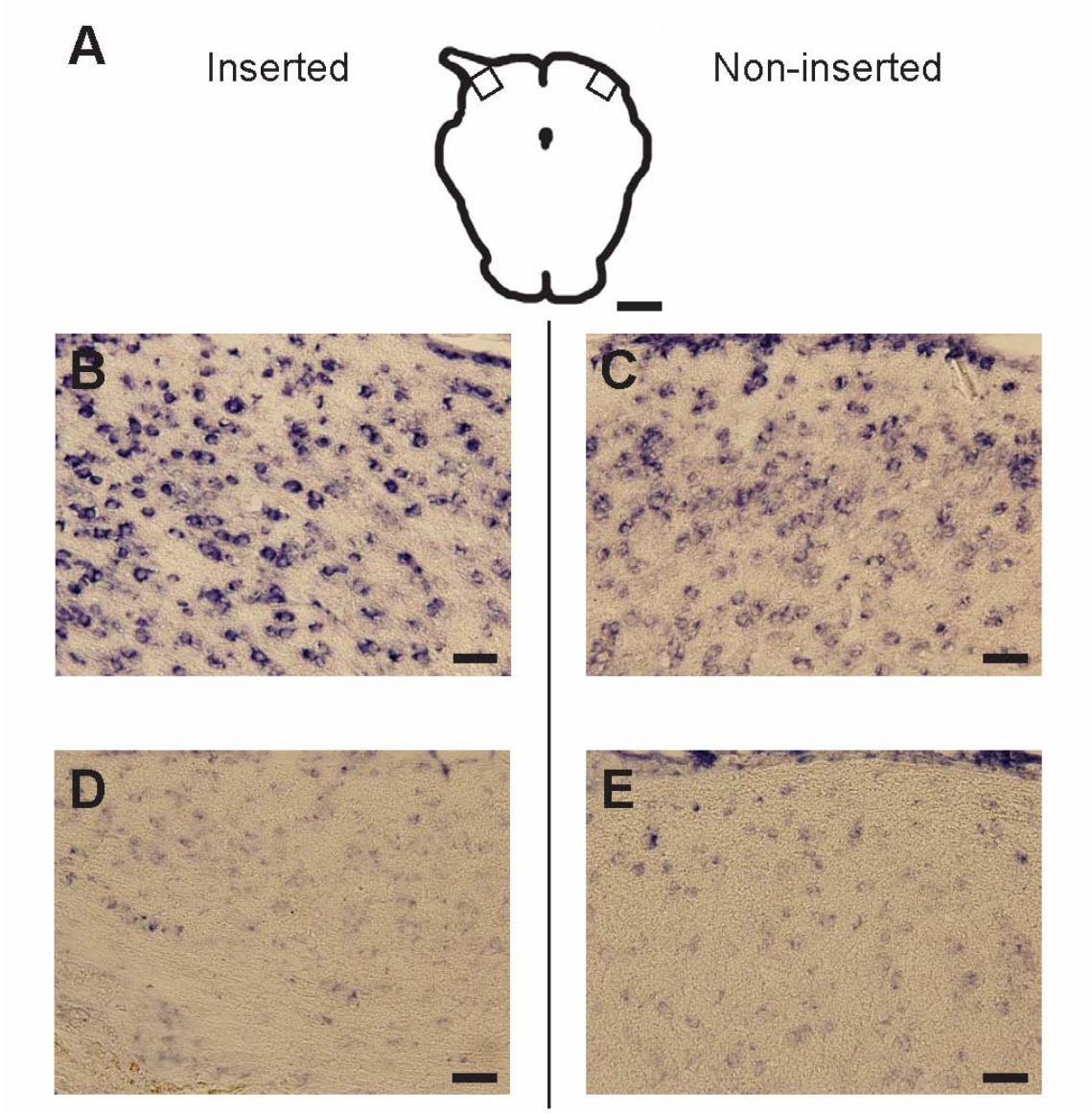

Figure 3. 3: Coronal brain sections showing ephrin-A2 mRNA at lateral locations (A) on the inserted (B and D) or non-inserted (C and E) side of the brain at one month (B and C) and six months (D and E) post surgery. Scale bars, 1 mm (A); 50 µm (B-E).

Six month animals. Ephrin-A2 mRNA expression in these brain was very low and was not quantified (Figs. 3.3D and E). The low signal was not due to tissue degradation, because in 6 month brain sections with the distal portion of the graft attached, ephrin-A2 mRNA expression was at similar levels to those observed in one month animals (data not shown).

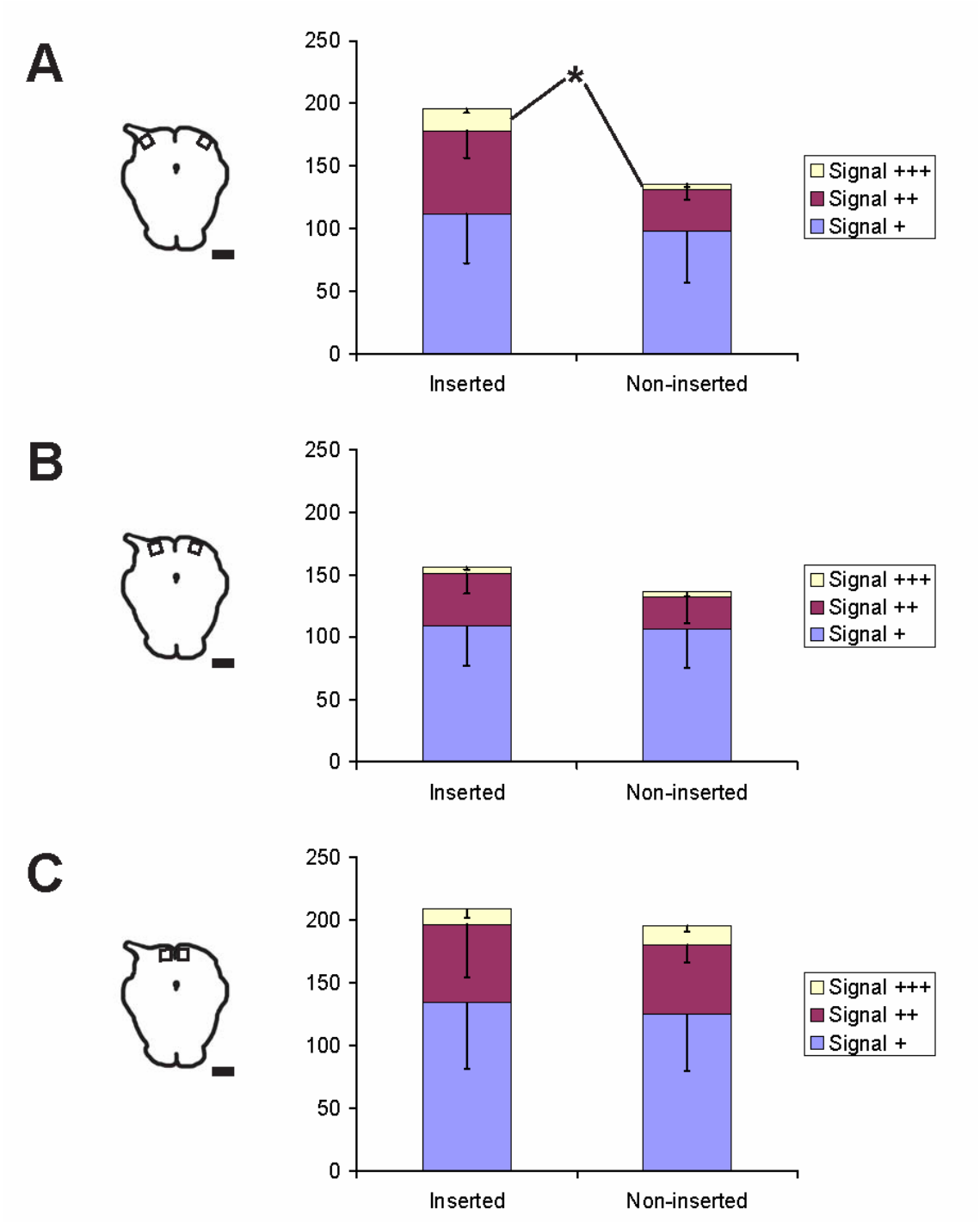

Figure 3. 4: Ephrin-A2 expression strength analysis for different locations in the *superior colliculus*. (A) There are significantly more cells strongly expressing ephrin-A2 (+++) at the insertion site than the corresponding lateral location in the non-inserted *superior colliculus*. Expression strength analysis at mid (B) and medial (C) locations showed no significant difference in the number of cells expressing ephrin-A2 mRNA at any of the three signal strengths.

Immunohistochemistry

Normal animals. Cell counts at rostral and caudal locations in the *superior colliculus* not differ significantly in normal animals ($p > 0.05$; Fig. 3.5).

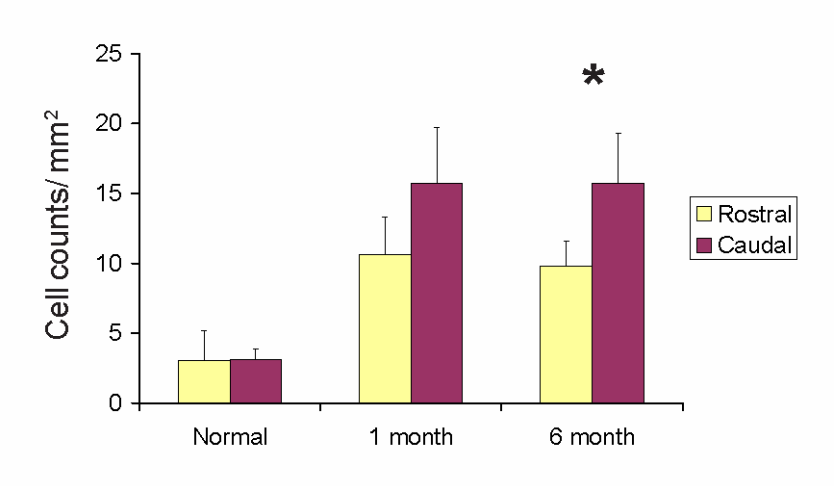

Figure 3. 5: Ephrin-A2 immunopositive cell counts at rostral and caudal locations in the contralateral *superior colliculus* of normal animals and at one month and six months post surgery. *$P < 0.05$.

One month animals. Cell counts, away from the graft insertion site, at rostral and caudal locations showed a strong trend towards a rostral^{low} and caudal^{high} difference, but these counts were not significantly different ($p > 0.05$; Fig. 3.5). At the insertion site, midway along the rostro-caudal axis of the *superior colliculus*, there was an increase in the number of ephrin-A2 positive cells (Fig. 3.6A).

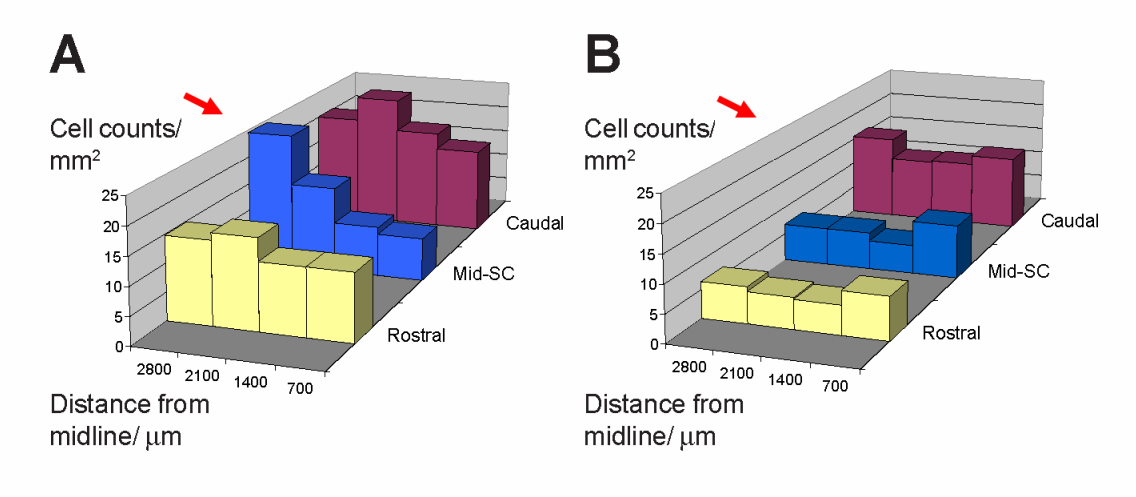

Figure 3. 6: Ephrin-A2 immunopositive cell counts of representative animals at one month (A) and 6 months (B) post surgery at locations across both the rostro-caudal and medio-lateral axis. The high cell counts laterally, midway along the rostro-caudal axis correspond to the peripheral nerve graft insertion site (red arrow) at one month (A), but these high counts are not seen in the six month animals (B).

Six month animals. Cell counts at rostral and caudal locations showed a significant rostral^{low} to caudal^{high} difference ($p < 0.05$; Fig. 3.5). At the insertion site, there was no difference in ephrin-A2 cell counts compared to other areas of the *superior colliculus* (Fig. 3.6B).

Phenotype of ephrin-A2 expressing cells

At all locations throughout *superior colliculi*, in both those rats with inserted or non-inserted grafts, greater than 95% of cells expressing ephrin-A2 mRNA co-localized with the neuronal nuclear marker, NeuN (Fig. 3.7C). The exception was the area immediately adjacent to the lateral insertion site, in which NeuN immunolabelling was low compared to the equivalent location in the non-inserted *superior colliculus* (Figs. 3.7B and 3.8D). NeuN co-localization with cells expressing ephrin-A2 mRNA occurred, but to a lesser extent (Fig. 3.7B).

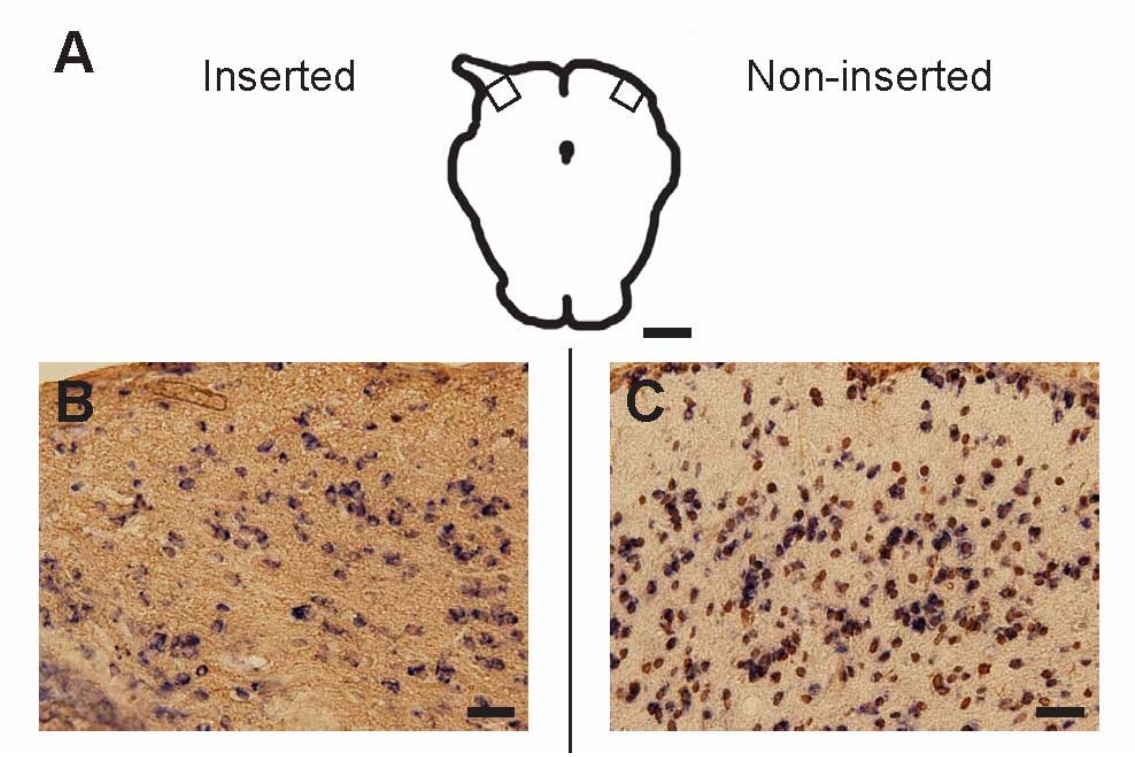

Figure 3. 7: Coronal brain sections showing ephrin-A2 mRNA (purple) combined with NeuN immunoreactivity (brown; B and C) at lateral locations (A) on the inserted (B) and non-inserted (C) side of the brain at one month. Scale bars, 1 mm (A); 50 µm (B and C).

After optic nerve section alone a band of GFAP positive hypertrophic astrocytes was seen parallel to the dorsal pial surface of the *superior colliculus*. This band of GFAP positive hypertrophic astrocytes is at a depth of 50-100 µm, corresponding to the *stratum griseum superficiale* layer (Fig. 3.8C) (Chapter 2) (Rodger *et al.*, 2005). In addition to this band of astrocytes, GFAP expression at the insertion site was greatly increased (Fig. 3.8B). At the insertion site, GFAP-positive processes were seen to surround ephrin-A2 mRNA positive cell somas (data not shown).

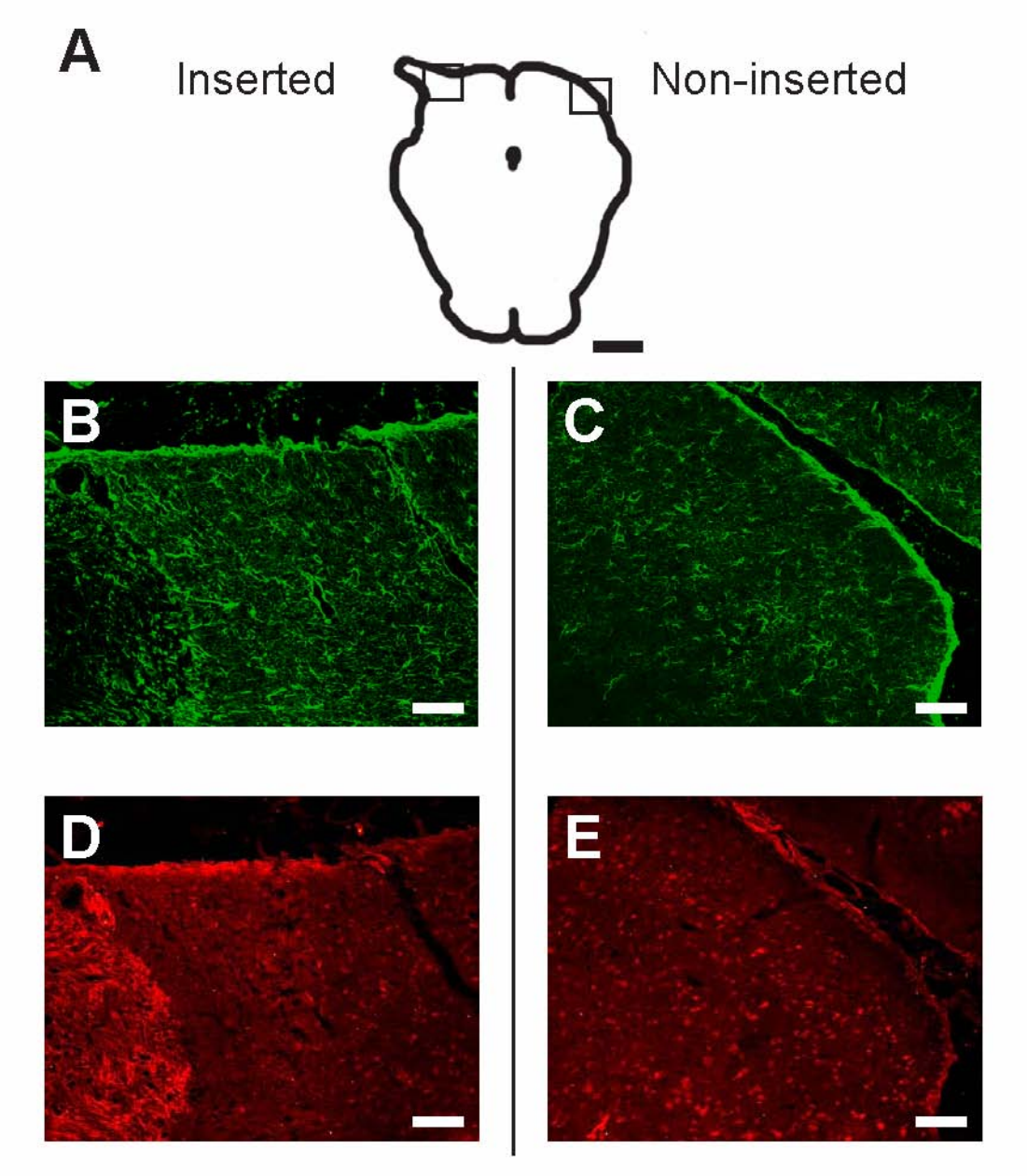

Figure 3. 8: Coronal brain sections showing GFAP (B and C) and NeuN (D and E) immunoreactivity at lateral locations (A) on the inserted (B and D) and non-inserted (C and E) side of the brain at one month. Scale bars, 1 mm (A); 100 µm (B-E).

Ephrin-A2 expression in the peripheral nerve graft

In situ *hybridization*

Normal sciatic nerve. Ephrin-A2 mRNA expression was detected only in epineurial fibroblasts (Fig. 3.9B).

Grafted peripheral nerve. Strong ephrin-A2 mRNA was detected throughout the 3 cm length of nerve grafted between the eye and brain. Abnormally high expression was seen in epineurial fibroblasts (Fig. 3.9C) with strong expression also in perineurial and Schwann cells throughout the length and width of the graft (Figs. 3.9 A, C-F).

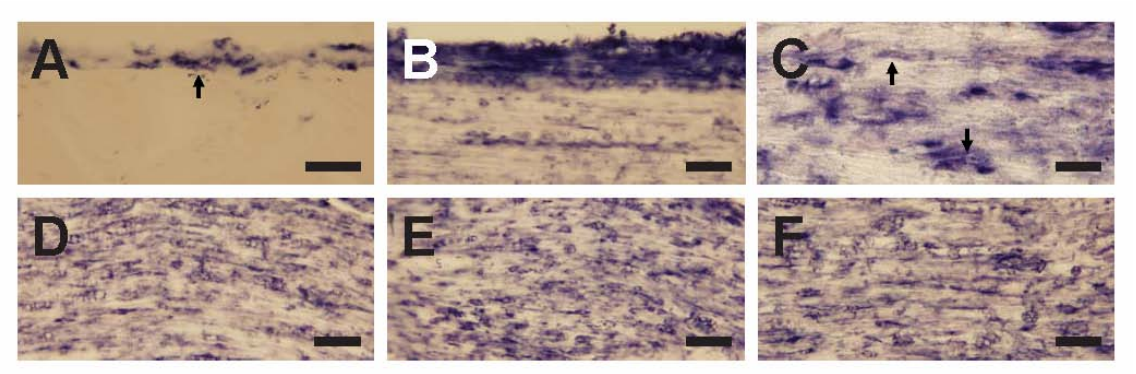

Figure 3. 9: Longitudinal sections of normal sciatic nerve (A) and peripheral nerve graft (B-F) one month post surgery showing ephrin-A2 mRNA. In normal animals only epineurial cells expressed ephrin-A2 (arrow, A). After peripheral nerve grafting epineurial cells ephrin-A2 mRNA expression was increased from normal (B). High-power photomicrograph (C) showing perineurium (downwards arrow), and Schwann cells expressing ephrin-A2 mRNA after peripheral nerve grafting (upwards arrow). which were not seen in normal (A). Ephrin-A2 expression does not differ along the 3 cm length of nerve from proximal to the eye (D) to mid graft (E) to distal to the eye (F). Scale bars, 20 µm (C); 50 µm (A, B and D-F).

Discussion

In summary, my results demonstrate that expression of EphA5 and ephrin-A2 is increased in the rat visual system following optic nerve crush and peripheral nerve grafting. The expression patterns are not a simple recapitulation of developmental ones, but have the potential both to guide regenerating retinal ganglion cell axons to topographically appropriate locations in the *superior colliculus*, and to prevent them from reinnervating the brain.

Retina

The topographically appropriate EphA5 gradient in surviving retinal ganglion cells after peripheral nerve grafting is unlike the uniform, low expression of EphA5 observed after optic nerve transection alone (Rodger *et al.*, 2001). Attaching a length of peripheral nerve to the back of the eye increases the supply of trophic factors to retinal ganglion cells, thus making the environment more conducive to survival (David & Aguayo, 1981; So & Aguayo, 1985). These data suggest that such an environment is sufficient to rescue the nasal^{low} to temporal^{high} EphA5 gradient in surviving retinal ganglion cells after unilateral optic nerve transection.

Although expression was examined in the RGC cell bodies, the expression of EphA5 in the growth cone is important in determining the response of growing axons during development and axon regeneration (Carmeliet & Tessier-Lavigne, 2005). It has been shown that protein expression levels of EphA2 in the growth cone of spinal commissural neurons are mirrored in the cell bodies (Brittis *et al.*, 2002). It is likely that a similar mechanism plays a role for the related EphA5 protein, and therefore that the immunoreactivity quantified in this study reflects the protein expression level in the growth cone.

Expression levels of EphA2 protein in spinal commissural neurons have been shown to be regulated locally in the growth cone, by a mechanism which relies on a sequence in the 3'-UTR of the EphA2 gene (Brittis *et al.*, 2002). I have shown that the EphA5 gene contains the appropriate 3'-UTR, similar to EphA2, to respond to local signals at the growth cone. The dynamic regulation of EphA5 in the growth cone of regenerating retinal ganglion cell axons may play an important role in determining whether or not they form successful connections in the *superior colliculus*. The environment of adult central nervous system which regenerating axons must re-enter to form connections in the brain may contain stimuli that modulate EphA5 expression in different ways from those operating during development. If the adult central nervous system environment and/or injury site contain cues that impact negatively on EphA5 expression in the growth cone, this may prevent large numbers of regenerating axons from entering the *superior colliculus*.

Ephrin-A2 expression throughout the superior colliculus

When the insertion site is excluded from analysis, cell counts reveal a topographically appropriate rostral^{low} to caudal^{high} gradient of ephrin-A2, similar to that present after optic nerve section alone (Knoll *et al.*, 2001; Rodger *et al.*, 2001). The gradient is maintained for up to six months and has the potential to guide regenerating RGC axons to appropriate locations. Unlike many previous studies, we examined both mRNA and protein levels and show a correlation. We also show that ephrin-A2 is expressed by neurons in the rat *superior colliculus*, as shown for chick and mouse (Davenport *et al.*, 1998). Presumably, it is these neurons that increase ephrin-A2 expression following denervation of the *superior colliculus* (Present study; Rodger *et al.*, 2001). The increase is presumably linked to the reorganization of collicular circuitry in response to denervation and the very minimal reinnervation observed in the peripheral nerve graft model (Chapter 2; Lund & Lund, 1973).

Local changes at the graft insertion site in the superior colliculus

As discussed above, the graded ephrin-A2 expression pattern away from the graft insertion site appears favourable to axon regeneration and restoration of topography. However, superimposed on the gradient, the high levels of ephrin-A2 observed at the insertion site might mediate a more negative outcome. During development, ephrin-A2 repels retinal ganglion cells *in vitro* (Hansen *et al.*, 2004; Nakamoto *et al.*, 1996) and *in vivo* (Nakamoto *et al.*, 1996), and a similar repulsive effect has been suggested on adult cells (Wizenmann *et al.*, 1993). We therefore propose that the localised up-regulation of ephrin-A2 at the insertion site acts as a barrier, preventing regenerating retinal ganglion cell axons from exiting the graft. A similar effect has previously been suggested for the guidance cue semaphorin-3A (Pasterkamp *et al.*, 1999). As for ephrin-A2, semaphorin-3A guides axons during development by mediating growth cone repulsion (Pasterkamp *et al.*, 1998; Pasterkamp *et al.*, 1998). Following a stab wound in the adult central nervous system, an injury similar to insertion of a peripheral nerve graft, semaphorin-3A is ectopically expressed in high levels and may contribute to failure of repair (Pasterkamp *et al.*, 1999).

Further support for an inhibitory role for ephrin-A2 comes from the localisation of its mRNA in astrocytes within the glial scar associated with the graft insertion procedure (Sauvé *et al.*, 2001; Vidal-Sanz *et al.*, 1987). Beyond the insertion site, ephrin-A2 is expressed only by neurons. However, at the insertion site, neuronal cells were rarely

observed, presumably due to neuronal cell death after injury (King *et al.,* 1997), and ephrin-A2 mRNA was associated with glial cells. In addition to a role for ephrin-A2 in simple repulsion, as has been described for other repulsive guidance molecules (Pasterkamp *et al.*, 1999), it is possible that Eph-ephrin signalling may contribute to the formation of the scar itself. Recent work has shown that EphA4 is up-regulated in astrocytes after spinal cord injury and not only inhibits axonal growth but also contributes to the formation of the glial scar (Goldshmit *et al.,* 2004). Our results, therefore, suggest a number of mechanisms whereby ephrin-A2 may prevent regenerating retinal ganglion cell axons from reinnervating the *superior colliculus*.

The highest levels of ephrin-A2 at the insertion site are observed one month after surgery, a time when the fastest regenerating axons are reaching the end of the peripheral nerve graft. However, at six months post surgery, expression is not detected at the insertion site and the topographically appropriate gradient of ephrin-A2 persists, across the *superior colliculus*. It might be possible to take advantage of the differential ephrin-A2 expression to increase the number of regenerating retinal ganglion cell axons that can successfully reinnervate the *superior colliculus*. The peripheral nerve graft could be inserted several months prior to optic nerve transection, allowing ectopic ephrin-A2 expression at the insertion site to return to background levels before the arrival of regenerating axons. Additional treatment to reduce the inhibitory nature of the glial scar, such as chondroitinase ABC (Fouad *et al.*, 2005; Tropea *et al.*, 2003), might further facilitate reinnervation.

Bilateral and unilateral changes

In the present study, a gradient of EphA5 is restored in the experimental eye but not in the non-experimental one. Moreover, the uniform, high expression of EphA5 in the non-experimental eye contrasts with the low uniform levels observed following optic nerve transection alone (Rodger *et al.*, 2001). These data suggest that the non-experimental eye may only partially mirror the experimental eye in retaining EphA5 expression. The reasons for the nasal increase in EphA5 expression in the non-experimental eye after unilateral optic nerve transection and peripheral nerve grafting, however, remain to be determined.

In the *superior colliculus*, up-regulation of graded expression is observed bilaterally, whereas the strong up-regulation of ephrin-A2 at the insertion site is strictly unilateral.

The difference could be due to different triggers for the up-regulation. We have previously shown that collicular denervation triggers bilateral up-regulation of ephrin-A2, presumably due to plasticity and reorganisation of intra-collicular pathways (Rodger *et al.*, 2005; Rodger *et al.*, 2001). However, the up-regulation observed at the insertion site is presumably caused by direct damage and may be mediated by inflammatory mechanisms (Goldshmit *et al.*, 2004). The results suggest that ephrin-A2 expression can be regulated by at least two signals. One of these is likely to be associated with neuron-specific expression leading to polysynaptic changes in expression. The other may be restricted to glial cells and remain locally associated with inflammation.

Effect of global and local ephrin-A2 expression on regenerating axons

This study suggests that the glial scar formed at the insertion site and the associated high levels of ephrin-A2, are a significant barrier to regenerating axons. The few axons that reinnervate the *superior colliculus*, therefore, must regenerate through both the ephrin-A2 barrier described here, in addition to other molecules known to be associated with the glial scar. Presumably, these few axons are capable of such reinnvervation due to an intrinsic insensitivity to these inhibitory molecules. In support of this concept, it has been shown that a small number of regenerating axons can grow through the glial scar formed after spinal cord injury (Campbell *et al.*, 1999; Matthews *et al.*, 1979; Meier & Sollmann, 1978; Reier, 1985).

There is an apparent contradiction. In order to reinnvervate the *superior colliculus*, regenerating axons must overcome 'local' high levels of ephrin-A2, presumably by insensitivity to it, but to form topographic connections these same axons must utilize the 'global' ephrin-A2 gradient. Ephrin-A2 could plausibly be involved in both inhibition and guidance if EphA5 receptor expression in the regenerating axon growth cone is modulated in response to different concentrations of ephrin-A2; the modulation may relate to a local protein translation mechanism involving the 3'UTR (Brittis *et al.*, 2002). To compensate for ectopically high levels of ephrin-A2 at the insertion site may result in down-regulation of EphA5 in the growth cone, allowing some axons to regenerate through it, but once these axons have passed the insertion site and entered the *superior colliculus,* the more physiologically lower ephrin-A2 levels my trigger up-regulation of EphA5 back to topographically appropriate levels. The few axons that can

reinnervate the *superior colliculus* presumably have a growth promoting intrinsic state, so that ectopic levels of ephrin-A2 alone may not prevent re-entry.

Alternatively, other guidance molecules that participate in the rostral-caudal patterning of the *superior colliculus* may be involved, and may have a different response to direct physical injury. One candidate is Engrailed-2 which is expressed in a rostral^{low} to caudal^{high} gradient, similar to ephrin-A2, and has recently been shown to guide retinal ganglion cells (Brunet *et al.*, 2005). Further work is required to determine the contribution that ephrin-A2 has on regenerating axons on both the local and global scale.

If ephrin-A2 is shown to influence regenerating axons that are capable of reinnervating the *superior colliculus*, then the presence of the ephrin-A2 global gradient combined with the retinal EphA5 gradient suggests that the regenerating axon should locate a topographically correct location. However, the overall organisation of the regenerated projection is low, with only a small, but significant, tendency towards topography (Sauvé *et al.*, 2001). An additional contributor to the overall disorder of the regenerated projection could be the physical characteristics of surgery.

The peripheral nerve graft is inserted laterally, orthogonal to the normal entry point of retinal ganglion cells during development (Simon & O'Leary, 1992). During development, topographically correct termination zones along the rostro-caudal axis are not formed by the leading axon growth cones, but rather by interstitial branches. These interstitial branches depend on leading growth cones overshooting their target along the rostro-caudal axis. The axonal overshoot allows the growth cones to explore fully the rostral-caudal extent of the *superior colliculus*, before topographically appropriate branching occurs, directed in part by ephrin-A2 (Rashid *et al.*, 2005; Yates *et al.*, 2001). After peripheral nerve grafting, however, the lateral insertion relocates the entry point of regenerating axons by 90°. The rotation combined with the limited outgrowth of reinnervating axons into the *superior colliculus* (Sauvé *et al.*, 1995), suggests that the guidance mechanisms of ephrin-A2 may be severely compromised. Some adaptation of these mechanisms must be possible, however, as evidenced by the small tendency towards topography (Sauvé *et al.*, 2001). Although guidance could also involve other molecular cues, such as engrailed-2 (Brunet *et al.*, 2005) these guidance systems would presumably be under similar constraints.

Ephrin-A2 expression in peripheral nerve graft

Up-regulation of ephrin-A2 expression in the grafted peripheral nerve one month after surgery is similar to that previously reported for two other repulsive guidance molecules, semaphorin-3A and -3F, after sciatic nerve crush alone (Scarlato *et al.*, 2003). Semaphorin-3A and -3F mRNA expression were reported to be most intense immediately distal to the crush site and intermediate at greater distances (Scarlato *et al.*, 2003). The results showed ephrin-A2 to be uniformly intense along the length of grafted peripheral nerve, presumably reflecting the required dual transection of both proximal and distal ends to facilitate grafting, compared to only a single crush site in the previous study (Scarlato *et al.*, 2003).

A proposed role for semaphorins in the injured sciatic nerve is to guide regenerating axons by restricting their growth into well defined fascicles (Scarlato *et al.*, 2003). Ephrin-As have also been implicated in fasciculation of axons in the peripheral nervous, and visual systems (Caras, 1997; Eberhart *et al.*, 2000), and therefore, may work synergistically with semaphorins in guiding regenerating axons in the peripheral nerve.

The parallels between the up-regulation of ephrin-A2 and semaphorin-3A in both the stab wound and sciatic nerve injury models, points against another possibility for up-regulation of ephrin-A2 at the insertion site at 4 weeks, namely, the influence of the invading regenerating axons themselves. There are so few of these regenerating axons (less that 100), that a role in influencing collicular ephrin-A2 expression seems unlikely.

Conclusion

Topographically appropriate patterns of EphA5 in surviving retinal ganglion cells and of ephrin-A2 in the denervated *superior colliculus* suggest that Ephs and ephrins can be reset to developmental expression patterns if cells receive appropriate survival factors. The result would be promising for possible regenerative therapies if it were not for the emerging evidence of a close association between developmental guidance molecules and astrocytic gliosis caused by physical injury such as a stab wound (Pasterkamp *et al.*, 1999) or more general injury to the central nervous system (Goldshmit *et al.*, 2004). Future experiments might combine the use of chondroitinase ABC in EphA or ephrin-A knock out mice with PN grafting experiments. I predict that under these conditions, reinnervation of the *superior colliculus* would be improved, but the regenerated

projection would lack topography. I conclude that peripheral nerve graft surgery is an excellent model in which to study the response of retinal ganglion cells to injury and test the outcomes of neuroprotective and neuroregenerative strategies. However, the results clearly show that the physical and molecular limitations of the model render it unsuited to studying the restoration of topography in regenerated projections.

Chapter 4:

Metallothionein-I/II increases regeneration of adult rat retinal ganglion cell axons

Introduction

After optic nerve transection, retinal ganglion cells do not spontaneously regenerate axons to their target (Ramon y Cajal, 1991), and by two weeks post injury more than 95% of retinal ganglion cells have died (Berkelaar *et al.*, 1994). In order to restore function, cell death must be minimised and axons encouraged to regenerate to visual brain centres.

Two main strategies have been used to increase survival and regeneration in the visual system. One approach is to use the existing optic nerve pathway combined with exogenous intravitreal administration of survival and/or regeneration factors to encourage axons to regenerate through the lesion site and back to the brain. From these studies, it has become clear that survival and regeneration operate via different pathways and that surviving retinal ganglion cells do not regenerate axons by default (Goldberg *et al.*, 2002; Klocker *et al.*, 2001).

Intravitreal injection of factors such as BDNF, NT-4/5, CNTF and erythropoietin transiently increase retinal ganglion cell survival, but do not stimulate regeneration (Mansour-Robaey *et al.*, 1994; Peinado-Ramon *et al.*, 1996; Weishaupt *et al.*, 2004; Zhang *et al.*, 2005). Regeneration of retinal ganglion cell axons beyond the lesion site after optic nerve transection has been reported in only a small number of studies after various treatments (Berry *et al.*, 1996; Fischer *et al.*, 2000; Lehmann *et al.*, 1999; Leon *et al.*, 2000; Lucius *et al.*, 1998; Yin *et al.*, 2003). In most of these studies, however, axon regeneration was not observed beyond 1 mm distal to the lesion, and only one study reported regeneration beyond the optic chiasm (Fischer *et al.*, 2001).

An alternative approach for increasing survival and regeneration is to provide a 'reconstructed pathway' from the eye to the brain (Vidal-Sanz *et al.*, 1987). A length of peripheral nerve is grafted to the optic nerve stump behind the eye, and the distal end inserted into the *superior colliculus* (Vidal-Sanz *et al.*, 1987). Proliferating Schwann cells in the peripheral nerve graft secrete trophic factors which support both survival and regeneration of retinal ganglion cells (Hall & Berry, 1989; So & Aguayo, 1985; Villegas-Perez *et al.*, 1988). Approximately 10% of the original retinal ganglion cell population of approximately 110,000 cells (Danias *et al.*, 2002) survive after peripheral nerve graft surgery and approximately 10% of these surviving cells regenerate axons within the graft (1000 axons) (Cui *et al.*, 2003; Villegas-Perez *et al.*, 1988).

Survival and regeneration in the peripheral nerve graft model can be augmented by the exogenous administration of survival and regeneration factors. BDNF increases survival, but not regeneration (Mansour-Robaey *et al.*, 1994), whereas CNTF alone does not increase survival, but increases regeneration (Cho *et al.*, 1999). CNTF, potentiated by elevated cAMP levels, has an even greater regenerative effect whilst also increasing survival (Cui *et al.*, 2003).

The most impressive increases in survival and regeneration in the visual system result from lens damage after optic nerve transection and peripheral nerve grafting (Cui *et al.*, 2003; Fischer *et al.*, 2001). Both these paradigms involve the release of multiple endogenous factors, suggesting that a 'cocktail' of exogenous factors will be required to achieve optimal survival and regeneration of retinal ganglion cells (Cui *et al.*, 2003). For this reason it is important to examine as many molecules as possible with putative protective and regenerative effects. It will then be possible to optimize the combination of factors and to understand the signalling pathways activated by them.

Further to this aim, metallothionein-I/II (MT-I/II) is a promising molecule that may impact on both the survival and regeneration of retinal ganglion cells. MTs are low molecular weight, heavy metal binding proteins, whose precise function in the body is still unclear (Palmiter, 1998). MT has a preference for zinc binding and can carry between one and seven zinc molecules (Palmiter, 1998). MT-I and MT-II are two of the four isoforms found in the body. The expression of these two isoforms is similar, both spatially and chronologically, and they are often considered together as one molecule, termed MT-I/II (Chung *et al.*, 2003), which is ubiquitously expressed throughout the body (Palmiter, 1998)

A potential dual role for MT-I/II in neuroprotection and neuroregeneration after injury to the visual system has been suggested by recent investigations. Mice over-expressing MT-I have significantly reduced apoptotic cell death in two models of induced neurodegeneration in the brain (Palmiter, 1998; Penkowa *et al.*, 2003; Penkowa *et al.*, 2002). Additionally, recent *in vitro* evidence has shown that exogenous administration of MT-I/II increases neurite elongation (Chung *et al.*, 2003). Furthermore, MT-I/II is up-regulated in two *in vitro* models which have successful outgrowth in the presence of myelin-associated glycoprotein and myelin, both of which usually inhibit outgrowth (Siddiq & Filbin, 2005). Building on this previous work, we have investigated the

protective and regenerative effect of a bolus dose of MT-I/II on retinal ganglion cells after injury to the visual system.

We show that four weeks after optic nerve transection, a bolus dose of MT-I/II did not have a survival effect on retinal ganglion cells in either optic nerve transection or peripheral nerve grafting models. However, the dose promoted retinal ganglion cell axon growth into and through the lesion site. No regeneration into the lesion site was seen in controls. When optic nerve transection was combined with peripheral nerve grafting, no significant difference was seen between the MT-I/II and control injected groups. However there was a strong trend toward higher axon numbers in the nerve graft of the MT-I/II group at 2 mm behind the eye.

Materials and methods

Animals and anaesthesia

Adult female rats (PVG/c hooded, 180 g) were sourced from the Animal Resource Centre, Perth, Australia. A bolus intravitreal injection of MT-I/II (A kind gift from A. West, University of Tasmania) or vehicle only (PBS) was administered to animals immediately after either optic nerve transection alone, or peripheral nerve graft surgery. For all surgery, animals were anaesthetised by intraperitoneal injection of 10 mg/kg xylazine (Ilium Xylazil, Troy Laboratories, NSW, Australia) combined with 50 mg/kg ketamine (Ketamil, Troy Laboratories). All animals were terminally anaesthetized by intraperitoneal injection of 120 mg/kg pentobarbitone (Valabarb, Jurox, NSW, Australia). Procedures were approved by the Animal Ethics and Experimentation Committee of the University of Western Australia.

Optic nerve transection and peripheral nerve graft surgery

Animals received either optic nerve transection alone or peripheral nerve graft surgery as described in Chapter 3.

Intravitreal injections

Intravitreal injections were performed immediately on completion of surgery, while animals were still anaesthetised. Each experimental retina received two injections, one temporal and one nasal, to provide even delivery across the retina. Each injection delivered 1.25 µl of solution into the vitreal chamber; a total of 2.5 µl to each experimental eye. A puncture was made in the eye, using a 30 G needle (Becton Dickinson, NJ), just behind the ora serrata. The location of the puncture prevented cataract formation in the cornea while minimizing damage to peripheral retina. A blunt 32 G needle attached to a 10 µl Hamilton syringe (Hamilton, NV) was inserted into the hole at an angle of 45°, to a depth of 1 mm, using a micromanipulator. The depth of 1 mm avoided damage to the lens, a procedure that has been shown to stimulate release of neuroprotective and -regenerative factors for retinal ganglion cells (Fischer *et al.*, 2001; Yin *et al.*, 2003). The syringe was held in place with a stereotaxic syringe clamp and the required volume delivered manually by slowly and steadily plunging over ten seconds. To prevent leakage, the needle was left in the eye for a further ten seconds, before being carefully removed. After injections, the retinal vasculature was checked by placing a cover slip on the cornea with PBS, and observing the blood flow using an operating microscope. Animals were placed on a heating pad to recover and were monitored. The eyes were covered with a moist tissue to prevent drying and a subcutaneous injection of the analgesic buprenorphine (0.02 mg/kg, Temgesic; Reckitt & Colman, UK) administered.

Metallothionein-I/II experimental and control groups

On completion of surgery, animals were immediately assigned randomly to experimental or control group and the appropriate solution was injected into the vitreous chamber of the eye.

Optic nerve transection

For this surgical model, there were two experimental and three control groups with four animals in each group. The two experimental groups consisted of a high and low dose of MT-I/II solution. The high dose group received 2.5 µg of MT-I/II to their experimental eye (2.5 µl of 1 mg/ml solution) and the low dose group 0.25 µg of MT-I/II (2.5 µl of 0.1 mg/ml solution). One control group received optic nerve transection only without an intravitreal injection. A second control group received an injection of PBS. The final control group received 80 ng of zinc sulphate ($ZnSO_4$; 2.5 µl of 32

µg/ml solution). The zinc control was necessary because there is some evidence that zinc can act as a neuroprotective molecule (DiSilvestro & Carlson, 1994; Itoh *et al.*, 1997).

Peripheral nerve graft

In peripheral nerve grafted animals, the experimental group received the MT-I/II low dose described above (0.25 µg; 2.5 µl of 0.1 mg/ml solution). The control group received PBS injections.

Retrograde tracing

At 25 d post surgery, peripheral nerve grafted animals were anaesthetised and regenerating retinal ganglion cells were retrogradely labelled using the fluorescent dye FluroGold (FG; Fluorochrome, CO). The peripheral nerve graft was exposed where it overlaid the temporal ridge. Approximately 1 cm from the back of the eye, a 10-0 suture (Ethilon; Ethicon, Johnson & Johnson, GA) was positioned either side of the selected incision point. The sheath was opened using microscissors to form a small longitudinal incision, taking care not to damage the nerve and a small FG crystal inserted. The 10-0 suture was tied, bringing the cut ends of the sheath together. After surgery, animals were placed on a heating pad to recover and were monitored. Their eyes were covered with a moist tissue to prevent drying. Three days were allowed for transport, as this has been shown to be optimal for FG retrograde labelling (Cui *et al.*, 2003).

Tissue preparation

At 28 d post surgery, all rats were terminally anaesthetized, exsanguinated by transcardial perfusion with PBS containing 0.9% sodium chloride, followed by fixation with fresh filtered 4% paraformaldehyde. Animals were decapitated and the retina and optic nerve or peripheral nerve graft carefully dissected.

Retinae

Retinae were transferred to a 24 well plate and post fixed at 4°C overnight, before further processing for immunofluorescence.

Optic nerves and peripheral nerve grafts

Optic nerves and nerve grafts were post fixed for 1 h with gentle agitation, before cryoprotection in 15% followed by 30% sucrose in PBS (pH 7.4) at 4°C overnight. Nerves were trimmed before covering in Tissue Tek OCT compound (Sakura, Japan), frozen on dry ice and cryosected (Leica CM1900, NSW, Australia) longitudinally (16 µm). Sections were collected on Superfrost® Plus glass slides (Menzel Glaser, Germany) and stored at -80°C.

Immunofluorescence histochemistry

Retinae

After post-fixation, retinae were washed 3 × 10 min in PBS before incubating with PBS plus 0.1% Triton X-100 (BDH Chemicals, VIC, Australia) for 2 h at RT. Primary antibodies were diluted in PBS plus 0.1% Triton X-100, applied to retinae and incubated overnight at 4°C. Two primary antibodies were used on each retina, mouse anti-βIII-tubulin (TUJ1; 1:500; Covance, NJ), and rabbit anti-ferritin (1:500; DAKO, CA). TUJ1 is a marker for viable retinal ganglion cells in retinal flat mounts (Cui *et al.*, 2003). Anti-ferritin is a marker for microglia and macrophages (King *et al.*, 2001) and was used to confirm that no damage to the retina had occurred due to intravitreal injection. The following day, retinae were washed 3 × 10 min in PBS before application of fluorescent secondary antibodies. Secondary antibodies were diluted in PBS, applied to retinae and incubated for 2 h at RT with gentle agitation in the dark. Alexa Fluor secondary antibodies (Molecular Probes, Invitrogen, VIC, Australia) were used; goat anti-mouse 488 nm (1:400) and goat anti-rabbit 546 nm (1:400). Retinae were washed 3 × 10 min in PBS in the dark, before flat mounting on Superfrost® Plus glass slides and cover slipping using Fluoromount-G™ aqueous based mounting medium (SouthernBiotech, AL). Flat mounts were allowed to dry in the dark before observation and analysis.

Optic nerves and peripheral nerve grafts

Sections were air dried for 1 h at room temperature (RT), incubated 2 × 5 min with PBS before blocking in PBS plus 5% normal goat serum (Sigma, NSW, Australia and 0.1% Tween-20 (Sigma, NSW, Australia; blocking solution) for 1 h at RT. Primary antibody was diluted in blocking solution, applied to sections which were incubated overnight at 4°C in a humidified chamber. Primary antibodies used were mouse anti-βIII-tubulin

(1:500; TUJ1 labels regenerating axons in nerve sections) (Yin *et al.*, 2003), mouse anti-RT-97 (200 kDa neurofilament-medium chain; 1:500; Chemicon International, CA) and mouse anti-SMI-32 (Dephosphorylated neurofilament medium and heavy chain; 1:1000; Sternberger, MD). The following day, sections were washed 3 × 10 min with PBS plus 0.1% Tween-20 before application of fluorescent secondary antibody. The secondary antibody, Alex Fluor goat anti-mouse 488 nm (1:400) was diluted in PBS plus 0.1% Tween-20, applied to sections and incubated for 2 h at RT in the dark. Hoechst-33342 (Sigma, NSW, Australia) was also added to the secondary antibody solution (final concentration, 1 µg/ml) to fluorescently stain all cell nuclei. Sections were washed 3 × 10 min in PBS in the dark and mounted in Fluoromount-G™ aqueous based mounting medium (SouthernBiotech, AL) and sections were allowed to dry in the dark before observation and analysis.

Quantification and analysis

Flat mount cell counts

TUJ1 positive retinal ganglion cell counts were made double-blinded as previously described (Kermer *et al.*, 2001; Villegas-Perez *et al.*, 1988) using a fluorescent Leitz Diaplan microscope (Leica Microsystems, NSW, Australia). FG positive retinal ganglion cells were also counted in the peripheral nerve grafted retinae. Sample frames of 235 × 235 μm^2 were analysed for TUJ1 positive cells (Leitz Ploemopak filter block I2/3, exciting filter BP 450-490); in peripheral nerve graft retinae this analysis was followed by counts of FG positive ganglion cells in the sample frame (Leitz Ploemopak filter block A, exciting filter BP 340-380). Counts were made at 12 locations in each retina. For each retinal quadrant (dorsal, nasal, ventral and temporal) three counts were taken at 1, 2 and 3 mm from the optic disc. The cell density per mm^2 at each point was calculated and averaged over the 12 points to give a mean value of retinal ganglion cells per retina (Villegas-Perez *et al.*, 1988). To measure retinal area, flat mounts were digitally imaged (CoolSNAP*cf*, Photometrics, AZ) at low power using a bright field microscope (Leitz Diaplan) connected to a computer with Image-Pro Plus software (MediaCybernetics, MD) at a resolution of 1392 × 1040 dpi. Retinal area was measured by drawing around flat mounts in Image-Pro Plus.

Scoring regeneration in the optic nerve

Due to branching and fasciculation of individual axons, no attempt was made to determine the absolute number of axons at or beyond the lesion site. The lesion site is a

complicated structure and it would be uncertain if counts were of separate axons, branches of the same axons, or of those with tortuous trajectories (Borgens & Bohnert, 1997; Dunlop *et al.*, 2002). The overall density of axons in the optic nerve was scored on a scale of zero to three using a double-blinded protocol. No TUJ1 labelled axons scored zero, while the maximum number of axons, often seen in all animals at the proximal sampling area, scored three. Three locations for each nerve were analysed: 200 µm distal to the optic nerve head, 200 µm proximal to the lesion site and 100 µm distal to the lesion site.

Axon counts in peripheral nerve graft

Axon counts were made blinded at 500 µm intervals from the proximal end of the graft. Three or four sections per graft were counted, spaced approximately 200 µm apart. The location for counting was measured from the proximal end of the nerve graft using an eye piece graticule viewed for Hoechst staining (Leitz Ploemopak filter block A, exciting filter BP 340-380). Magnification was then increased to 40× with oil immersion, for axon counts of RT-97 positive axons (Leitz Ploemopak filter block I2/3, exciting filter BP 450-490). The procedure avoided biased counts towards high or low areas of axon labelling in the graft. A linear eye piece graticule was used for axon counts, positioned orthogonal to the course of axons in the longitudinal plane of section. RT-97 positive axon was counted if they intersected the linear graticule. The thickness of the nerve at each interval was also measured to calculate the average number of RT-97 positive axons at each interval along the nerve.

The axon counting method does not distinguish between *de novo* axon growth, axon branching or looping, because counts were made of the number of axons intercepting a linear graticule. To investigate these possibilities further, a confocal laser scanning microscope (MRC 1000, Bio-Rad, CA), attached to an upright Optiphot-2 microscope, (Nikon, Japan) with z-axis drive was used to image nerve sections fluorescently immunolabelled for RT-97 positive axons.

Statistical analysis

Optic nerve transection model

For the MT-I/II experimental groups, cell survival was compared to those of vehicle and zinc sulphate injected control animals. To assess changes in cell survival in retinal flat mounts, average retinal ganglion cell densities for each animal were compared by

ANOVA (Injection group as the factor, Scheffe's *post-hoc* test). Average scores for each animal were compared by ANOVA (Injection group and scoring location as the factors, Scheffe's *post-hoc* test) at each sampling area.

Peripheral nerve graft model

The MT-I/II experimental group was compared to those of vehicle injected control animals. Average retinal ganglion cell densities for survival (TUJ1) and regeneration (FG) for each animal were compared between groups by *t*-test (null hypothesis: no difference between MT-I/II and control means; unpaired; two-tailed). Average axon counts per nerve graft for each animal were compared by ANOVA (Injection group as the factor, Scheffe's *post-hoc* test) for each interval along the nerve graft).

Results

Limited injury to the retina from injection protocol

The double injection protocol used in this study caused little damage to the retina, as evidenced by the lack of ferritin immunoreactivity. The small amounts of damage to peripheral retina that were seen in a few retinae were localized to the injection site, and counting near these areas was avoided.

Optic nerve transection model

Survival

In vehicle injected animals, the average density of TUJ1 positive retinal ganglion cells at four weeks was 226 ± 56 cell/mm^2 (mean ± SEM), which was not significantly different from optic nerve transection alone, 99 ± 13 cell/mm^2 (Using a five treatment ANOVA, Scheffe's *post-hoc* test; Fig. 4.1). Although there is no significant difference between these two control groups, the large difference in mean shows a similar trend to a previous study that used three control groups: optic nerve section only, vehicle injection or puncture only (Mansour-Robaey *et al.*, 1994). The large variation in means may be due to the low number of surviving retinal ganglion cells after injury, combined with the small percentage of the retinal area sampled. However, although not significant, there seems to be a strong tendency for any intravitreal injection to increase cell survival in the retina. The injection protocol presumably stimulates the release of neuroprotective factors (Fischer *et al.*, 2000; Yin *et al.*, 2003). Zinc sulphate injected control animals had a density of 238 ± 27 cell/mm^2, which is also not significantly

different from vehicle or optic nerve transection only experimental groups (Using a five treatment ANOVA, Scheffe's *post-hoc* test; Fig. 4.1). The density of TUJ1 positive retinal ganglion cell assessed from retinal flat mounts in both MT-I/II injected groups (high dose: 175 ± 37 cell/mm^2; low dose: 227 ± 34 cell/mm^2) were also not significantly different from controls (Using a five treatment ANOVA, Scheffe's *post-hoc* test; Fig. 4.1).

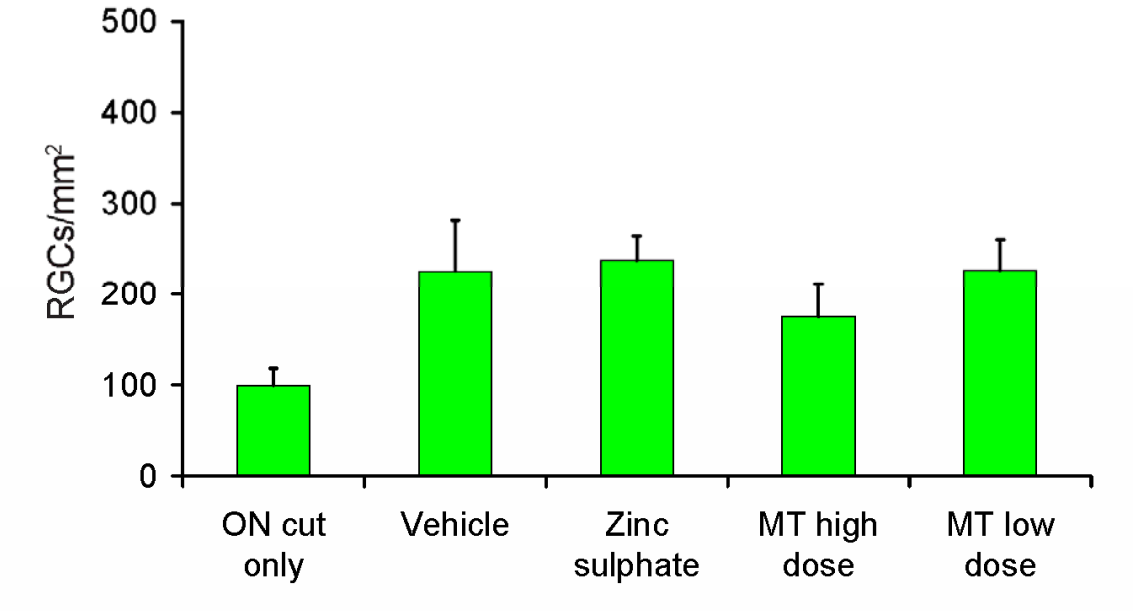

Figure 4. 1: Density of TUJ1 positive retinal ganglion cell per mm^2 under different experimental treatments. There was no significant difference between any of the treatment groups in a five treatment ANOVA.

Regeneration

Results for the scoring of axonal density in the optic nerves of all groups are summarized in table 4.1.

In optic nerves of vehicle injected control animals after optic nerve transection, no axons were seen at locations in or beyond the injury site (Figs. 4.2A and B). Regenerating axons distal to the injury site were seen, however, in the optic nerves of MT-I/II group animals (high dose 2 of 6 animals, Figs. 4.2C and D; low dose 3 of 4 animals, Figs. 4.2E and F). These regenerating axons were seen as RT-97 or TUJ1 positive profiles. Regenerating axons distal to the injury site were also seen in 4 of 6 zinc sulphate control animals (Figs. 4.2G and H). All the zinc sulphate optic nerves with axons in the distal optic nerve had lower axon densities compared to the low dose

Table 4. 1: Scoring of TUJ1 positive axonal regeneration into the optic nerve four weeks after transection

Treatment group and animal number	Analysis location		
	Proximal 1	Proximal 2	Distal
Vehicle 1	2	0.5	0
Vehicle 2	2	1	0
Vehicle 3	1.5	1	0
Vehicle 4	2.5	2	0
Vehicle 5	3	2	0
Vehicle 6	3	3	0
Vehicle average ± SEM	**2.3 ± 0.6**	**1.6 ± 0.9**	**-**
MT high 1	1.5	1	0
MT high 2	3	2	1.5
MT high 3	2	1.5	0
MT high 4	1	1	0
MT high 5	3	2	0.5
MT high 6	3	1	0
MT high average ± SEM	**2.25 ± 0.9**	**1.4 ± 0.5**	**0.3 ± 0.6**
MT low 1	3	2	1
MT low 2	3	2.5	1.5
MT low 3	3	2	0.5
MT low 4	2.5	1	0
MT low average ± SEM	**2.9 ± 0.3**	**1.9 ± 0.6**	**0.75 ± 0.6**
$ZnSO_4$ 1	3	2	0.5
$ZnSO_4$ 2	3	2.5	0.5
$ZnSO_4$ 3	2	1.5	0
$ZnSO_4$ 4	2	0	0
$ZnSO_4$ 5	1.5	0.5	0.5
$ZnSO_4$ average ± SEM	**2.3 ± 0.7**	**1.3 ± 1.0**	**0.3 ± 0.3**

MT-I/II injected optic nerves, although the difference was not significant (Table 4.1; Figs. 4.2C-H).

All animals showed similar axonal labelling proximal to the injury site, with a higher axon density closer to the eye, at proximal location 1, compared to proximal location 2, which was closer to the injury site (Table 4.1; Fig. 4.2).

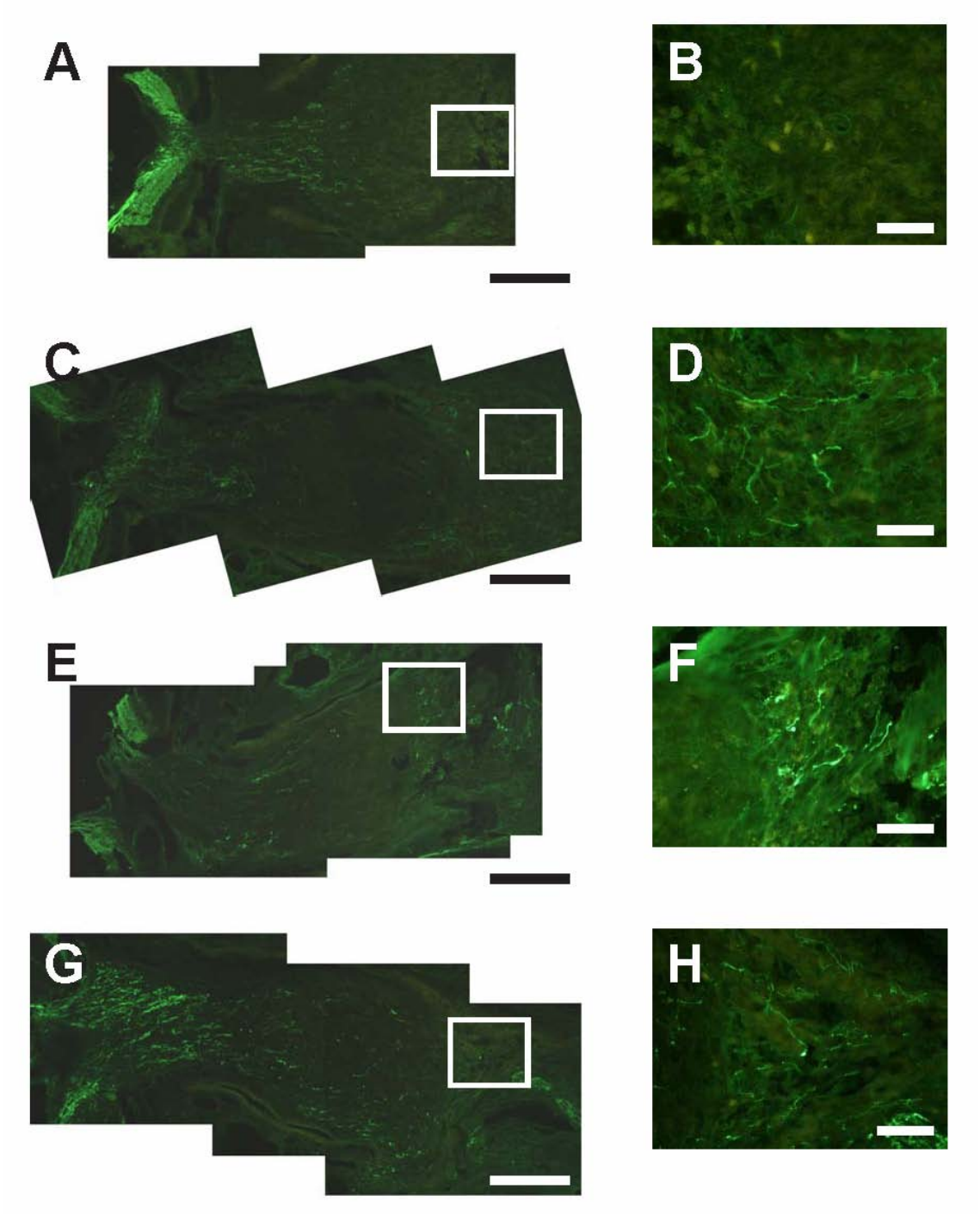

Figure 4. 2: Photomicrographs showing the optic nerves after different experimental treatments following optic nerve transection alone. A, C, E and G show low power montages of the nerve, while B, D, F and H show high power photos of the nerve distal to the injury site. No TUJ1 positive axons are seen distal to the injury site in the vehicle injected group (A and B), while axons are seen in both MT-I/II treatment groups (high dose, C and D; low dose E and F) and to a lesser degree in the zinc sulphate control group (G and H). Scale bars, 200 μm (A, C, E and G); 50 μm (B, D, F and H).

Peripheral nerve graft model

Survival

The average density of TUJ1 positive retinal ganglion cell counts from retinal flat mounts in the low dose MT-I/II injected group was 388 ± 35 cell/mm^2, which was not significantly different from vehicle injected controls, 431 ± 21 cell/mm^2 (*t*-test, $p > 0.05$; Figs. 4.3A, B and E).

Regeneration

FG retrograde labelling in the retina. The average density of FG positive retinal ganglion cell counts from retinal flat mounts in the low dose MT-I/II injection group was 71 ± 20 cell/mm^2, which was not significantly different from vehicle injected controls, 79 ± 20 cell/mm^2 (*t*-test, $p > 0.05$; Figs. 4.3C, D and E).

Axonal counts in the nerve. There was no significant difference between MT-I/II and vehicle injected animals in the average number of RT-97 positive retinal ganglion cell axon profiles at any location along the nerve graft (ANOVA, Scheffe's *post-hoc* test; Fig. 4.4C). However, at 2 mm behind the eye there was a strong trend towards greater axons number in MT-I/II animals. This location corresponds to the distance regenerating axons may travel if regenerating at a slightly faster rate than the adult growth rate of 50 μm/day (Goldberg & Barres, 2000). A similar trend for increased axon numbers at 2 mm behind the eye was seen using TUJ1 and SMI-32 (data not shown).

Axonal profile morphology by laser scanning confocal microscopy

Peripheral nerve graft sections from MT-I/II and vehicle injected groups were compared 2 mm behind the eye to study the morphology of axonal profiles. At this location there is a trend towards a greater number of RT-97 positive axons in the MT-I/II group compared to the vehicle injected animals, an appearance which could be due to *de novo* axon growth, axon branching, axon looping or a combination of these possibilities.

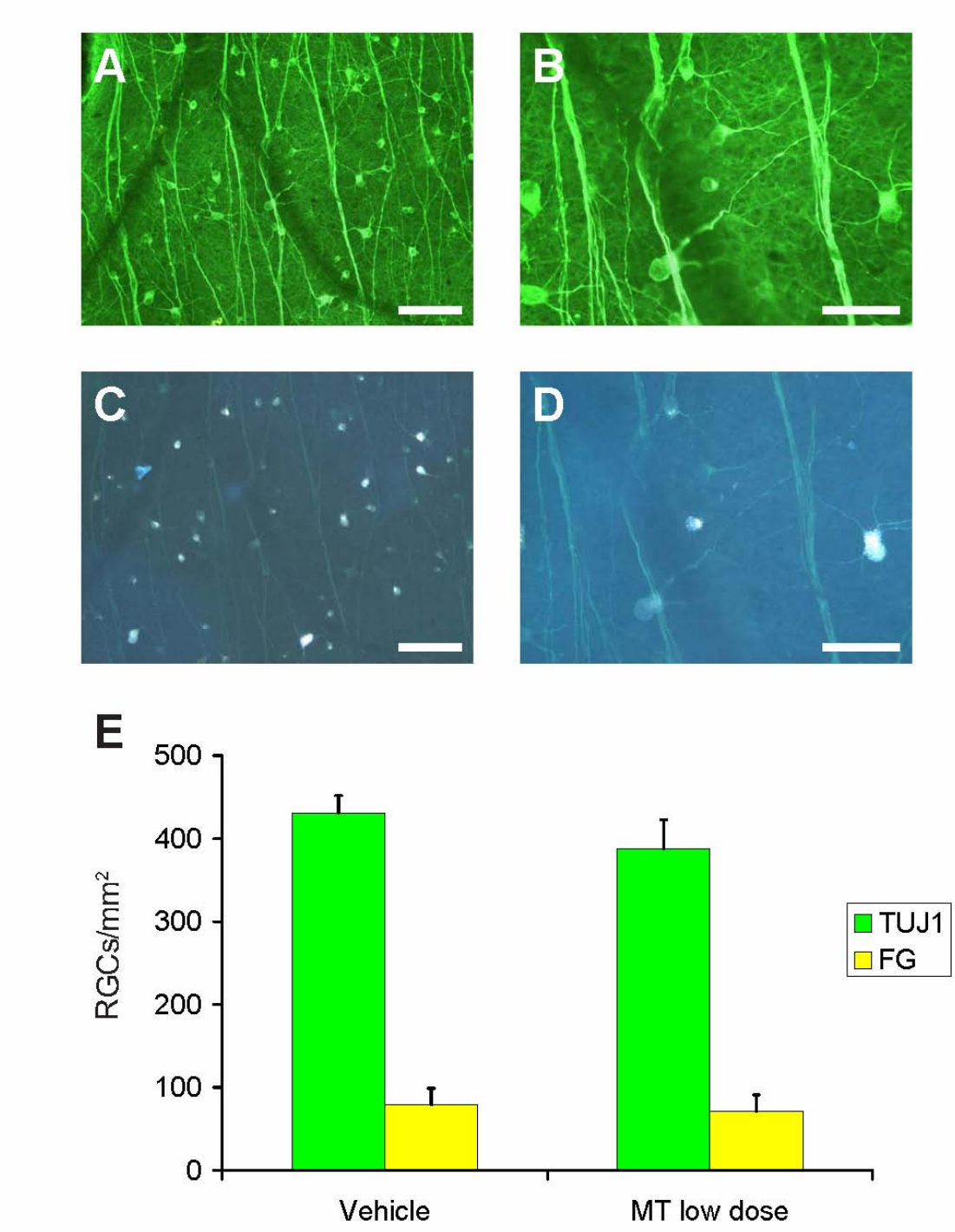

Figure 4. 3: Photomicrographs of TUJ1 (A and B) and FG (C and D) positive flat mount retinal ganglion cells from a peripheral nerve grafted animal, four weeks after surgery and MT-I/II treatment. Photos are taken at an eccentricity half way between central and peripheral retina. A and C are low magnification photos of the same frame, while B and D are higher magnifications also of the same frame. There was no significant difference between either TUJ1 positive cell counts and FG positive cell counts in MT-I/II and vehicle injected groups (E). Scale bars, 100 µm (A and C); 50 µm (B and D).

Using laser scanning confocal microscopy, there was no evidence for axon branching in the peripheral nerve graft of either treatment group (Figs. 4.4A and B). However, in the MT-I/II injected peripheral nerve, there were signs that axons may have looped within the z-series of optical sections at 2 mm behind the eye (Fig. 4.4A), a feature not seen in vehicle injected animals (Fig. 4.4B).

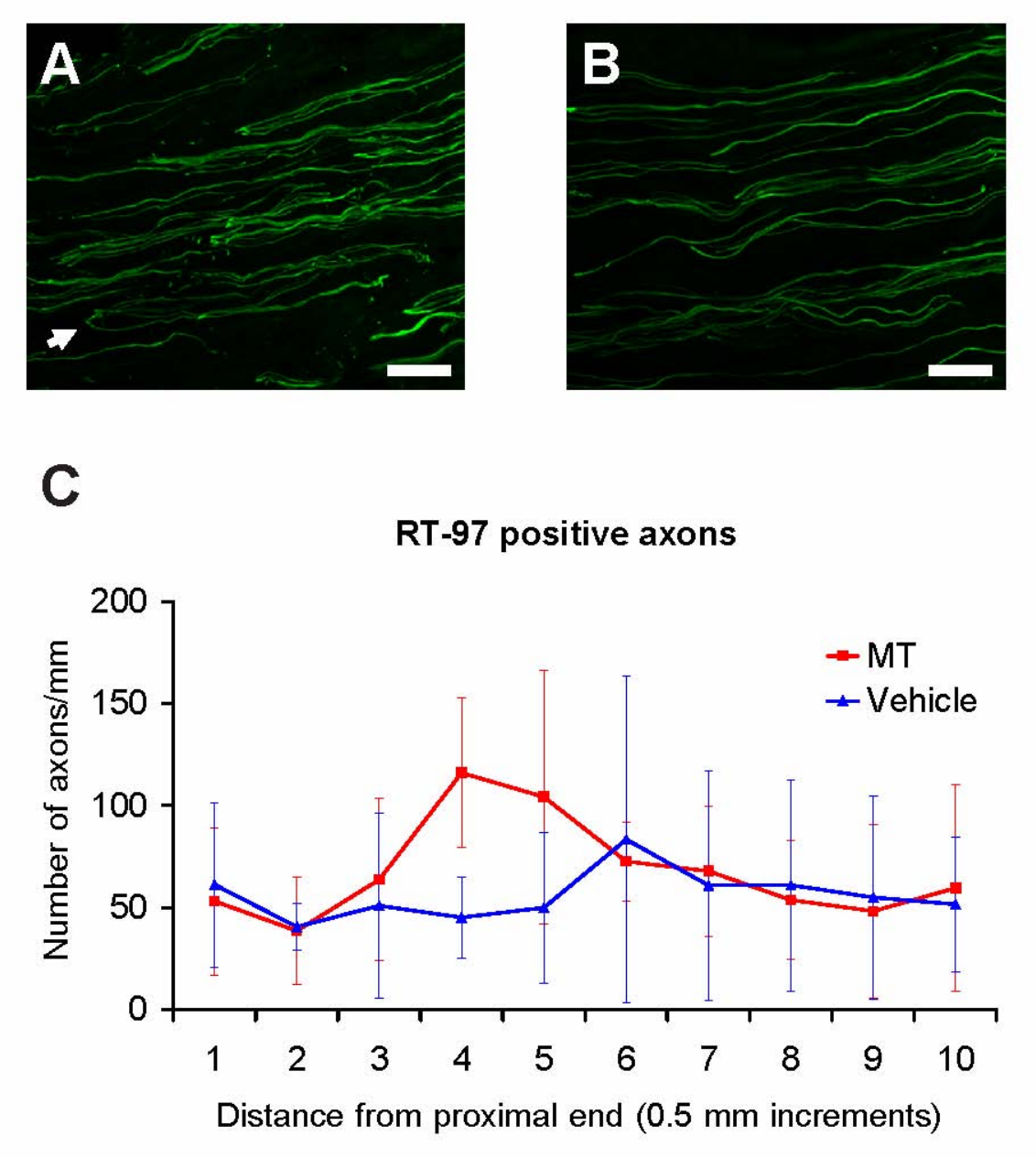

Figure 4. 4: Laser scanning confocal microscope images of peripheral nerve grafts at 2 mm behind the eye in MT-I/II (A) and vehicle (B) treatment groups. Arrow in A shows possible looping of regenerating axons. There was no significant difference in RT-97 positive axon number in the nerve graft at any location behind the eye between MT-I/II and vehicle treatment groups (C). Scale bars, 50 µm (A and B).

Discussion

This is the first study of the protective and regenerative potential of MT-I/II after injury to the visual system. MT-I/II acted as a neuroregenerative but not as a neuroprotective molecule in the optic nerve transection model and shown a trend towards increasing axon numbers in the peripheral nerve graft animals. This initial study provides the basis for further experiments to assess fully the potential of MT-I/II as a protective and regenerative factor after injury to the visual system *in vivo*.

Axonal regeneration after optic nerve transection

Exogenous administration of MT-I/II promotes a subset of surviving retinal ganglion cells to regenerate axons through the lesion site after optic nerve transection. MT-I/II application to cultured cortical neurons has been shown to increase neurite extension (Chung *et al.*, 2003). In addition, MT-I/II can overcome inhibition by MAG and myelin in cultured cortical neurons (Siddiq & Filbin, 2005). In two models of regeneration, cAMP application *in vitro* or its elevation *in vivo*, overcomes the three main myelin-based growth inhibitory proteins, MAG, NOGO, and OMgp (Cai *et al.*, 2001; Cai *et al.*, 1999; Siddiq & Filbin, 2005). In both of these successful regeneration models, MT-I/II is transcriptionally up-regulated (Siddiq & Filbin, 2005). The myelin-based growth inhibitory proteins signal through a p75 receptor complex resulting in Rho activity and inhibition of neurite growth (Domeniconi *et al.*, 2005; Yamashita *et al.*, 2005). The p75 receptor transduces the inhibitory signal to the cell by cleavage of an intracellular domain. This cleavage is carried out by α- and γ-secretases (Domeniconi *et al.*, 2005). It has been hypothesized that the role of MT-I/II is to modulate the activity of these secretases (Siddiq & Filbin, 2005). This mechanism is a likely candidate for stimulating retinal ganglion cell axonal growth into and through the glial scar at the lesion site, which has high levels of myelin-based growth inhibitory proteins (Ferraro *et al.*, 2004).

Consistent with our results, most studies that have reported retinal ganglion cell axonal growth beyond the lesion site, did not observe much axonal regeneration beyond 1 mm distal to the lesion (Berry *et al.*, 1996; Fischer *et al.*, 2000; Lehmann *et al.*, 1999; Leon *et al.*, 2000; Lucius *et al.*, 1998; Yin *et al.*, 2003). Only one study reported regeneration past the optic chiasm (Fischer *et al.*, 2001). These data suggest that more than one type of inhibition will need to be overcome to elicit long distance axonal regeneration in the optic nerve. Other inhibitory molecules include those traditionally associated with the lesion site glial scar, such as chondroitin sulphate proteoglycans and tenascin-C

(Sandvig *et al.*, 2004). In addition, there are the more recently implicated inhibitory molecular guidance cues, such as the ephrins and semaphorins (Chapter 3; Pasterkamp *et al.*, 1999) which can be re-expressed after injury or be associated with myelin debris in the distal optic nerve (Benson *et al.*, 2005). If MT-I/II is involved in overcoming myelin-based growth inhibitory proteins at the secretase level of the signalling pathway (Siddiq & Filbin, 2005), other inhibitory molecules present at the lesion site and in the distal optic nerve, presumably act *via* pathways downstream of the secretases (Ellezam *et al.*, 2002).

In this study we observed a regenerative effect at four weeks post surgery after only a bolus dose of MT-I/II. If this regenerative effect is through a direct interaction of MT-I/II with cell signalling molecules in the growth cone (Siddiq & Filbin, 2005), it presumably would have to persist in the growth cone for an extended period of time. However, if MT-I/II is metabolized rapidly, it would suggest a role in transcriptional regulation of other proteins which may not be metabolized so rapidly (Sun *et al.*, 1996; Zhang & Del Bigio, 1998).

Four hours after intravitreal injection of fluorescently tagged MT-I/II, greater than 95% of retinal ganglion cells were fluorescently labelled (C. Bartlett, *Pers. comm.*). By 24 h after delivery most retinal ganglion cells were no longer fluorescently labelled (C. Bartlett, *Pers. comm.*), suggesting that MT-I/II had been transported anterogradely into axons and growth cones or metabolized. Observations of fluorescent labelling in optic nerve axons followed a similar pattern to that seen in the cell soma, with fluorescence observed at 2 and 4 h, but gone by 24 h (C. Bartlett, *Pers. Comm.*). Furthermore, anti-MT-I/II antibody showed the same time course, eliminating the possibility that the fluorescent tagged was cleaved from MT-I/II (C. King, *Pers. Comm.*; G. Plant, *Pers. Comm.*). Taken together (although these data have not yet been subjected to peer review), these data suggest that MT-I/II was transported anterogradely within 2-4 h, but was metabolized by 24 h, and was no longer present in either the cell soma or axons in the optic nerve. Therefore, MT-I/II presumably provides most of its axonal regenerative effect through mechanisms involving gene transcription.

Any effect of MT-I/II on gene transcription must take place within 24 h after delivery, while it is localized within the cell soma. It has previously been shown that MT-I/II can modulate transcriptional activity *in vitro*, because its expression is important for

appropriate gene transcription during development (Kimura *et al.*, 2000). Therefore, MT-I/II may have a role in transcriptional regulation of proteins important for regeneration in the visual system, such as GAP-43, L1, TAG-1 (Jung *et al.*, 1997; Klocker *et al.*, 2001).

In the present study, the low dose of MT-I/II had a superior regenerative effect, with a greater axon density seen in the distal optic nerve than in the high dose group. The result suggests that the low dose was closer to the optimal concentration of MT, when delivered as a bolus dose, and could reflect a bell-shaped dose response curve, similar to that reported for neuroprotective factors *in vivo* (Chen & Weber, 2001; Weishaupt *et al.*, 2004). Furthermore, the high dose of MT may actually be detrimental to cell survival in retina.

Unexpectedly, the zinc sulphate group showed a similar regenerative effect as the high dose MT-I/II group. This suggests that the regeneration shown in this study may be a result of mechanisms different from or additional to MT-I/II's proposed modulation of secretase activity and supports a role for transcriptional regulation. There are at least two possible mechanisms for the regenerative effect of MT-I/II and zinc by transcription regulation. The first is that MT-I/II may be acting as a zinc transporter, providing zinc to transcription factors for proteins important in the regenerative response (Herdegen *et al.*, 1993). A zinc transporter role for MT-I/II has been proposed in light of its usual cytoplasmic location changing to a nuclear location at the G_1/S transition in the cell cycle (Hesketh, 2004). MT-I/II is capable of binding seven zinc molecules, and if all are bound, the high dose MT-I/II contains approximately six times more zinc than the zinc sulphate and low MT-I/II doses. The zinc sulphate group, however, contains almost double the amount of zinc than the low dose of MT-I/II. The superior regeneration in the low dose MT-I/II group over the zinc sulphate group may be explained by a more efficient delivery of zinc to transcription factors at optimal levels for transcription, compared with the high dose MT-I/II and zinc sulphate groups.

Alternatively, the concentration of zinc used in our study as a "control" may have been high enough to *per se* induce MT-I/II expression in retinal ganglion cells (Haq *et al.*, 2003; Li *et al.*, 2005; Murata *et al.*, 1999). If this is the case, it is possible that the regenerative effect observed in the zinc group is due to the action of induced MT-I/II.

The zinc sulphate group may induce a MT-I/II concentration which is more similar to that in the high dose group, thus giving similar regenerative effects.

Increased regeneration after peripheral nerve graft surgery

Although there was no significant difference between the number of regenerating axons in the peripheral nerve graft model, the trend towards higher numbers of RT-97 positive axons at 2 mm behind the eye in the experimental group, suggests that MT-I/II may be able to increase the number of retinal ganglion cells extending an axon into the peripheral nerve graft. Further studies with greater power should be undertaken to check if there is a real regenerative effect of MT-I/II in the peripheral nerve graft model. Any regeneration effect of MT-I/II in this model would presumably be *via* similar mechanisms to those proposed after optic nerve transection.

Although retinal ganglion cell axons regenerate into a peripheral nerve graft, the proportion which does so corresponds to only 10% of the surviving population. The remaining viable retinal ganglion cells do not undergo axon regeneration, presumably because they do not express the proteins necessary for axonal growth. Additionally, glial and myelin-associated inhibitory molecules are present in the peripheral nerve itself (Cui *et al.*, 2003; Hunt *et al.*, 2002). Presumably, there is also myelin debris at the optic nerve-peripheral nerve graft interface (Berry *et al.*, 1988; Hall & Berry, 1989). Therefore, MT-I/II may be able to encouraging a further subset of surviving retinal ganglion cells to regenerate axons into the peripheral nerve graft that would not normally do so, by overcoming myelin-based growth inhibitory proteins (Siddiq & Filbin, 2005), or by transcriptional regulation of proteins necessary for axonal growth (Jung *et al.*, 1997; Klocker *et al.*, 2001).

Any trend towards an increased number of axons in the peripheral nerve graft of MT-I/II animals might have been expected to correspond to an increased number of FG labelled cells in flat mounted retinae compared with control animals, but this was not observed in our results. This discrepancy is reconciled by technical consideration of the FG application site 1 cm behind the eye, a location where no difference in axon number is observed between MT-I/II and control nerves grafts. Future experiments applying FG closer to the eye would be able to confirm any trend towards greater numbers of retinal ganglion cells contributing *de novo* axon growth to the regenerated projection in MT-I/II compared with control animals.

Additionally, the trend towards a greater number of axons at 2 mm behind the eye in MT-I/II, but not control nerve grafts may be due to branching of regenerating axons. Branching of regenerating axons has been described in the retina after optic nerve transection (Sawai *et al.*, 1996). Branching also occurs during the regeneration of peripheral nerves, such as the facial nerve, and can lead to severe impairment of coordinated movement due to the simultaneous innervation of antagonistic muscles (Angelov *et al.*, 2005). Regenerating retinal ganglion cells axons have not been reported to branch within the peripheral nerve after grafting, but it has been shown that administration of BDNF and NT-4/5 can increase branch length within the retina (Sawai *et al.*, 1996). Although there is evidence that MT-I/II can promote branching *in vitro* (A. West, *Pers. comm.*), no branching was observed in our nerve grafts when analysed by confocal laser scanning microscopy. The lack of branching observed in the nerve grafts supports the idea that exogenous MT-I/II administration is increasing *de novo* growth of axons into to peripheral nerve graft.

Although branching was not seen in MT-I/II or control nerve grafts, there were signs that axons may loop within the z-series of optical sections at 2 mm behind the eye, a feature not seen in control animals. The putative looping could explain increased axon numbers 2 mm behind the eye. Because thin cryosections were used in this experiment, and anterograde tracing was not performed, it was not possible to confirm this observation. Future experiments to analyse putative looping of regenerating axons in the peripheral nerve graft could use anterograde tracing from retinal ganglion cell somas (Dunlop *et al.*, 2002; Sawai *et al.*, 1996; Watanabe *et al.*, 2003), and axons in the nerve graft analysed to discriminate between *de novo* growth and looping of regenerating axons (Dunlop *et al.*, 2002).

Retinal ganglion cell survival in both surgical models

Neither the high or low doses of MT-I/II delivered to each experimental eye after optic nerve transection resulted in a survival effect on retinal ganglion cells above that seen in vehicle injected control animals. The low dose also had no survival effect on retinal ganglion cells in the peripheral nerve graft model. If MT-I/II exerts its effect by increasing axonal elongation (Chung *et al.*, 2003), overcoming inhibition of myelin-based growth inhibitory proteins (Siddiq & Filbin, 2005), or by transcriptional regulation of proteins associated with axonal growth (Kimura *et al.*, 2000), it may not be expected to increase cell survival. This idea is supported by observations in cortical cultures, where addition of MT-I/II does not increase neuronal survival (A. West, *Pers. comm.*).

In the present study, we used a bolus dose of MT-I/II to investigate survival and regeneration at four weeks post surgery. Neuronal survival has previously been shown to require the continual supply of trophic support, suggesting that continuous delivery may be beneficial (Goldberg & Barres, 2000). Furthermore, MT-I over-expression significantly reduces apoptotic cell death in two models of induced neurodegeneration in the brain (Penkowa *et al.*, 2003; Penkowa *et al.*, 2002; Siddiq & Filbin, 2005). Taken together, these data suggest that the bolus dose used in this study may not have been sufficient to induce neuroprotection four weeks after surgery.

A more intensive treatment regime with MT-I/II, therefore, may increase retinal ganglion cell survival after optic nerve transection and peripheral nerve graft surgery. A possible regime may take advantage of viral constructs containing MT-I/II. These viral constructs can be delivered to retinal ganglion cells by a single injection, after which the infected cells will continually express a low level of MT-I/II. Viral constructs containing various trophic factors have been shown to increase neuronal survival after different types of insults, including excitotoxitiy (Bemelmans *et al.*, 2005; Schuettauf *et al.*, 2004), in a model for Huntington disease (Kells *et al.*, 2004), glaucoma (Martin, 2004) and optic nerve transection (Kugler *et al.*, 1999; Schuettauf *et al.*, 2004). Presumably, MT-I/II viral expression will also augment neuroregeneration in both optic nerve transection and peripheral nerve graft paradigms (A. Harvey, *Pers. comm.*).

The potential of using chimeric peripheral nerve grafts, constructed to express MT-I/II should also be investigated. Chimeric peripheral nerve grafts expressing CNTF have

recently been demonstrated to increase both survival and regeneration (Hu *et al.*, 2005). Axonal growth cones may be able to use the MT-I/II provided by the chimeric graft to overcome inhibition by myelin-based growth inhibitory proteins (Siddiq & Filbin, 2005).

Further studies of exogenously administered MT-I/II both *in vitro* and *in vivo* are now required to fully understand its mode of action. Potential mechanisms are by direct interaction with intracellular signalling pathways or by gene transcription of proteins required for axonal growth. MT-I/II may even have the potential to act as a multifunctional molecule depending upon the dose, frequency and location with which it is administered. MT-I/II has already been implicated in a number of cellular functions including apoptosis, proliferation, differentiation (Davis & Cousins, 2000) and, in the present study, in axonal regeneration.

Chapter 5:

General discussion

The consequence of changes in molecular guidance cue expression after injury

Most studies of the central nervous system after injury have focussed on damaged neurons and their immediate connections, both afferent and efferent. My results in Chapter 2 highlight that changes in the Eph-ephrin molecular guidance system are regulated through polysynaptic connections in the visual system. The involvement of polysynaptic connections implies that if we hope to manipulate guidance molecules to increase functional recovery after injury, it will be important to take into consideration more than just the structure of interest, but in addition, all the afferent and efferent pathways.

As well as the bilateral increase in ephrin-A2 expression that I have shown after laser lesion (Chapter 2), bilateral cellular proliferation has been observed after injury (Panagis *et al.*, 2005; Rice *et al.*, 2003). In the visual system after unilateral optic nerve crush, glial cells proliferate bilaterally in the *superior colliculus* (Bodeutsch *et al.*, 1999; Panagis *et al.*, 2005; Setkowicz *et al.*, 2004). In addition, following traumatic brain injury in rats, endogenous neural stem/progenitor cells proliferate bilaterally in the subgranular zone and the subependymal zone (Rice *et al.*, 2003). The demonstration of bilateral changes in these injury models, both molecular and cellular, presumably are intrinsic attempts by the brain to heal itself after injury (Rice *et al.*, 2003) and may allow some functional compensation. Future research could address whether the bilateral change of guidance molecule expression observed may be involved in rewiring the newly-generated cells.

Unlike injury to the optic nerves that can be categorized definitively as unilateral or bilateral, injuries to the brain and spinal cord are harder to categorize. After stroke, for example, depending on its severity and location, cell death may be confined to one side of the brain or spread bilaterally. In stroke research much work has focused on increasing neuronal survival in the peri-infarct region, by seeking to understand the molecular mechanisms that are activated during programmed cell death (Rickhag *et al.*, 2006). Less work has focussed on bilateral changes, although a study in gerbils demonstrated that bilateral neuronal proliferation can occur after stroke (Du Bois *et al.*, 1985). Bilateral molecular and cellular changes, therefore, could provide alternate pathways with high plasticity immediately after trauma, which could be utilized by physical therapist to regain some lost function.

The reported difference that unilateral or bilateral optic nerve transection has on ephrin-A2 expression in the *superior colliculus* is of potential clinical importance (Knoll *et al.*, 2001; Rodger *et al.*, 2001). Unilateral optic nerve transection results in bilateral ephrin-A2 up-regulation (Rodger *et al.*, 2001), while bilateral transection results in no change of the ephrin-A2 graded expression in the *superior colliculus* (Knoll *et al.*, 2001). If it can be confirmed that the different outcomes are due to the lesion type and not to methodological differences (Chapter 2; Knoll *et al.*, 2001; Rodger *et al.*, 2001), then the studies raise serious issues about the way central nervous system injury should be treated. An injury affecting only one side of the brain must be considered in a more holistic way, taking into account the bilateral changes induced by the initial insult. After a bilateral injury, on the other hand, manipulation of guidance molecules may be required to direct regenerating axons appropriately. Taking into account the symmetrical organization of the nervous system could prove more effective, potentially tailoring treatments to the type of injury sustained.

The bilateral up-regulation of ephrin-A2 in the *superior colliculus* observed after unilateral optic nerve transection (Rodger *et al.*, 2001) must also be contrasted with unilateral up-regulation after the direct, unilateral physical injury associated with insertion of the peripheral nerve graft (Chapter 3). Different mechanisms presumably underlie ephrin-A2 up-regulation after denervation of a target, compared to direct physical injury. I have shown that denervation of the *superior colliculus* after optic nerve transection causes collicular cells to relay information to ipsilateral structures regarding the loss of input, *via* existing polysynaptic pathways (Chapter 2). After direct, unilateral physical injury to the *superior colliculus*, however, there is presumably cell death associated with the trauma (King *et al.*, 1997; Vickers *et al.*, 2000), which is more rapid than that induced by denervation (Smith & Bedi, 1997). Thus, reorganization may not occur in the *superior colliculus* (Lund & Lund, 1973, 1971) and therefore there will be no accompanying signalling to ipsilateral structures.

Direct physical injury to the neocortex by needle stab injury results in neurofilament changes which mimic the early stages of Alzheimer's disease neuronal pathology (King *et al.*, 1997). Needle stab injuries are similar to the injury caused through insertion of a peripheral nerve graft, which uses a pulled glass micropipette to insert the distal tip of the nerve into the *superior colliculus*. Taken together, these data suggest a possible role for ectopically up-regulated ephrin-A2, associated with neurofibrillary tangles in the

stab wound injury model, for the early stages of Alzheimer's neuronal pathology (King *et al.*, 2001; King *et al.*, 1997). Ephrin-As are expressed in visual cortex during development and their possible up-regulation in Alzheimer's disease neuronal pathology may be an ultimately doomed attempt to increase plasticity of connections in response to continued insult to the brain. Such a hypothesis of chronic insult to the brain resulting in Alzheimer's disease has been previously proposed, but molecular guidance cue expression is yet to be investigated (Vickers *et al.*, 2000).

Multiple requirements for appropriate topography

Understanding how EphA5 and ephrin-A2 respond to surgical interventions is important if we hope to re-establish topographic connections between the eye and the *superior colliculus via* peripheral nerve grafting. A further requirement to improve the topographic order of the regenerated projection after peripheral nerve grafting, however, is to increase the number of retinal ganglion cell axons contributing to this projection. Although it has not yet been possible to test the requirement of size of the regenerated projection in mammals, in goldfish, retinotectal topography is not restored if the population of regenerating axons is reduced to less than 10-15% of its original number (Udin & Gaze, 1983). Recently it has been demonstrated that with the combined treatment of CNTF and a non-degradable cAMP analogue, the number of regenerating axons can be increased to 10-15% (Cui *et al.*, 2003). If these 10-15% of axons that can regenerate along a peripheral nerve graft can also be encouraged to enter the *superior colliculus*, it will be possible to start testing animals for visual function that requires topography.

Multiple signalling pathways are required for optimal regeneration

I have shown that MT-I/II increases regeneration through the lesion site and that there is a trend of increased axon number in the peripheral nerve graft (Chapter 4). The protective and/or regenerative effect of some peptide trophic factors, including BDNF (Mansour-Robaey *et al.*, 1994; Zhang *et al.*, 2005) and CNTF (Cho *et al.*, 1999; Zhang *et al.*, 2005), can be potentiated by elevation of cAMP (Cui *et al.*, 2003; Meyer-Franke *et al.*, 1995; Shen *et al.*, 1999). Elevating cAMP levels increases recruitment of TrkB, the high affinity BDNF receptor to the plasma membrane (Meyer-Franke *et al.*, 1998), and increases CNTF receptor alpha mRNA in the retinal ganglion cells (Park *et al.*, 2004). Furthermore, the action of CNTF and cAMP has been shown to act *via* multiple

signalling pathways (Park *et al.*, 2004). Future studies could investigate possible molecules that may potentiate the regenerative effect of MT-I/II observed in this study.

Our current knowledge of MT biology suggests that cell surface receptors may not be required to exert its regenerative effect, due to direct up take by retinal ganglion cells (C. Bartlett, *Pers. comm.*), and to date, MT-I/II receptors have not been identified. If MT-I/II is entering the cell, its regenerative effect may due to the action of different pathways to those used in CNTF-cAMP potentiation, which use cell surface receptors (Park *et al.*, 2004).

The utilization of different intracellular pathways during axonal regeneration is highlighted by the suggestion that GAP-43 axon growth is distinct from growth stimulated by elevated cAMP levels (Andersen *et al.*, 2000). In axons that can spontaneously regenerate, GAP-43 is up-regulated as demonstrated by high GAP-43 levels in neurons that regenerate axons into a peripheral nerve graft (Campbell *et al.*, 1991; Ng *et al.*, 1995). Additionally, inosine stimulates axon growth both *in vitro* and *in vivo* after unilateral transection of the corticospinal tract, and is associated with GAP-43 up-regulation (Benowitz *et al.*, 2002). Regeneration of axons into a peripheral nerve graft is also significantly increased by administration of inosine (Wu *et al.*, 2003). Up-regulation of GAP-43 alone, however, is not sufficient for retinal ganglion cells to regenerate axons through the lesion site (Doster *et al.*, 1991). Recently, GAP-43 positive axons were shown to regenerate in the injured spinal cord only if combined with chondroitinase ABC treatment and neural stem/progenitor cell transplantation (Ikegami *et al.*, 2005). Elevation of cAMP, however, can promote axons to regenerate through the lesion scar (Bhatt *et al.*, 2004; Cai *et al.*, 2001; Ellezam *et al.*, 2002). In cells with elevated cAMP levels, GAP-43 decreased in a dose dependent manner (Andersen *et al.*, 2000). These data suggest that combinatorial drug administration that targets multiple regenerative pathways is likely to achieve optimal regeneration of retinal ganglion cell and other central nervous system axons.

Delivery strategies must optimize both regeneration and topography

In this study, MT-I/II was delivered by bolus intravitreal injection. However, we must be aware that any delivery strategy used for neuroprotective and neuroregenerative factors may disrupt the expression and interpretation of guidance molecules as a result of physical injury (Chapter 3). Damage to retinal ganglion cells can modify EphA5 expression in non-damaged areas of the experimental retina and even the uninjured ipsilateral eye (Chapters 2 & 3; Rodger *et al.*, 2001). Although the laser lesions used in chapter 2 are more damaging than peripherally located injections used to administer MT, it must be confirmed that even relatively minor insults do not affect the expression of EphA5 and other guidance molecules.

Regardless of whether or not intravitreal injections *per se* trigger a change in EphA5 expression across the retina, alternative, non-invasive delivery methods are preferred due to the sensitive nature of the eye. The small size of MT-I/II (6-7 kDa) may facilitate delivery to retinal ganglion cells by topical application to the cornea. Topical application of antibody fragments, 28 kDa in size, to rabbit corneas over a 4-12 h period, results in the presence of the antibody in the vitreal chamber at potentially physiologically useful concentrations between 50 and 150 ng/ml (Williams *et al.*, 2005). A further alternative is intraperitoneal injection of survival and regeneration factors (Zhang *et al.*, 2005). Intraperitoneal injection of the nitric oxide synthase scavenger nitro-L-arginine increases retinal ganglion cell survival after optic nerve transection at one and two weeks post surgery (Zhang *et al.*, 2005). For both topical application and intraperitoneal injection strategies, it must be confirmed that the more diffuse delivery of MT-I/II does not result in unwanted changes in the eye or other parts of the body.

The MT-I and MT-II gene promoters are highly inducible by controlled electric stimuli (Rubenstrunk *et al.*, 2003). Electrical stimulation across either the cornea or the optic nerve after optic nerve transection for 1 and 2 h respectively has been shown to increase survival of retinal ganglion cells 7 d post injury (Morimoto *et al.*, 2002; Morimoto *et al.*, 2005). In the transcorneal electrical stimulation study, most of the survival was due to increased IGF-1 expression in retinal ganglion cells, however, the mechanism of IGF-1 activation is not yet known (Morimoto *et al.*, 2005). Additionally, a voltage gradient applied across lesioned adult spinal cord for one month induces regeneration of

transacted dorsal column axons (Borgens *et al.*, 1990; Borgens *et al.*, 1986; Borgens & Bohnert, 1997).

Furthermore, functional electrical stimulation systems implanted in patients with motor-complete thoracic level spinal cord injury have recently been shown to be beneficial for upright mobility and bladder and bowel function (Johnston *et al.*, 2005). Taken together, these data suggest that some of the benefits seen by electrical stimulation may be due to MT-I/II induction. Future studies of these experimental and clinical paradigms will be required, to investigate whether *in vivo* electrical stimulation after neuronal injury can induce MT-I/II. Future treatments, therefore, could apply electrical current across the retina, spinal cord or brain after injury, with the specific aim of increasing MT-I/II to take advantage of its protective (Penkowa *et al.*, 2002; Penkowa & Hidalgo, 2003) and regenerative effects (Chapter 4).

Visually driven behaviour in peripheral nerve grafted animals

One of the most important considerations in stimulating damaged central nervous system axons to regenerate is whether they restore connections that have the capacity to support normal function. The visual system is ideal for carrying out such behavioural testing due to the abundance of visual tests for specific aspects of visual function. Behavioural testing of peripheral nerve grafted animals demonstrated responses to conditioning stimuli to a similar level as monocular control rats, even though they have a ten fold lower number of axons comprising their projection (Sasaki *et al.*, 1999). Furthermore the recovery of the pupillary light reflex by insertion of the peripheral nerve graft into the pretectum shows most of the characteristics of the normal response (Whiteley *et al.*, 1998). After peripheral nerve grafting, animals can also discriminate between horizontal and vertical strips in a Y-maze. Discrimination of orthogonal stripes requires minimal topography and relay connections to the cortex (Thanos *et al.*, 1997), presumably reflecting the small tendency towards topography along the rostro-caudal axis reported in electrophysiological experiments (Sauvé *et al.*, 2001). Taken together, these data reinforce the idea that the brain is capable of great plasticity. If an anatomical link is provided between the retina and *superior colliculus* after injury, by increasing survival and regeneration, the brain can sculpt what projection it has available to obtain maximal function (Qi *et al.*, 2004).

However, we must be aware that if the regenerated projection is wired inappropriately or simply too restricted in size, it could be detrimental and confusing. A recently identified model for studying abnormal visual connections is the lizard *Ctenophorus ornatus* (Beazley *et al.*, 2003). Unlike the peripheral nerve graft model, the lizard has a large regenerated projection, and this projection can be used to study the benefits of training on the reformation of retinotopic order (Beazley *et al.*, 1997). There is evidence that lizards which have received a unilateral optic nerve transection close their experimental eye during regeneration of retinal ganglion cell axons (P. Chen, *Pers comm.*). Presumably, this behaviour is due to the disordered nature of the regenerated connections formed in the brain, resulting in a confusing input which the lizards can avoid by closing their experimental eye. If the lizards, however, are forced to use their experimental eye for feeding, by covering the un-operated eye, improved functional outcomes and anatomical order of the regenerated projection are seen (Beazley *et al.*, 2003). These data suggest that retinotopic order can be improved once the connections to the brain have formed by training. Future work should address the role of training in animals receiving a peripheral nerve graft to improve the topographic order of the regenerated projection.

Outlook for future research and the treatment of central nervous system injury

Current research suggests that there is no panacea for injury to the brain and spinal cord. Functional recovery will depend on many factors. Patients must be treated as rapidly as possible, as there is certainly a window after injury to save cells (Xu *et al.*, 2005). Various molecular interventions would potentially increase cell survival by providing trophic factors to reduce cell death and allow cells to return to a non-compromised and regenerative-ready state (Goldberg *et al.*, 2002). Additional factors including electrical stimulation may be used to increase regeneration of axons to their targets. Alternatively, surgical intervention could direct regenerating fibres to their main targets using chimeric nerve bridges expressing additional trophic factors (Hu *et al.*, 2005), perhaps MT-I/II. Once regenerating axons arrive at their target, inhibitory molecules would have to be removed by chondroitinase ABC (Fouad *et al.*, 2005; Tropea *et al.*, 2003) combined with Eph-receptor fusion proteins to remove ectopic expression of ephrin-A2 (Rodger *et al.*, 2004). Additionally, MT-I/II may be used to overcome myelin-based growth inhibitory (Siddiq & Filbin, 2005). Regenerating axons that robustly re-innervate their targets could then use the topographically appropriate expression of guidance molecules to find their correct locations (Chapter 2 and 3). At

this point, additional factors, such as BDNF could be applied to the target to increase arborization (Sawai *et al.*, 1996).

Once connections are re-established, the path to functional recovery is far from over. This is when patients would take control of their rehabilitation by intensive work with physiotherapists to ensure the reconstructed pathways receive the correct electrical stimulation, to firstly be maintained (Beazley *et al.*, 2003; Whiteley *et al.*, 1998), and secondly to relearn the functions they carried out before injury (Liu *et al.*, 2002). Learnt functions would presumably take longer, but they would happen, as evidenced by work in mice on a rotating tube. Wild type mice can learn to walk on the rotating tube very quickly, but mice that have received a pedunculotomy take longer to attain a similar performance level (Dixon *et al.*, 2005). This rehabilitation might be ongoing for the rest of the patient's life, in a similar way that everyone is encouraged to exercise regularly and eat healthily.

We remain someway off having a complete treatment for injuries to the central nervous system, due to the uniqueness of each injury. However, as we develop models to overcome the lack of regeneration in adult mammals and further understand how regenerating fibres and molecular guidance cues respond to such surgery, we are bringing the hope of pathway reconstruction closer to those with injuries of the central nervous system.

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