

DIFFERENTIAL EFFECTS OF GROWTH FACTORS ON BULBOSPINAL NEURON
SURVIVAL AND NEURITE OUTGROWTH *IN VITRO*

by

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ABSTRACT

During development, the central nervous system (CNS) is capable of not only growth, but regeneration as well. There are two possible reasons why this ability is lost with maturation: 1) the CNS environment no longer supports growth, and 2) the capacity of the neurons for growth diminishes. There is good evidence that treating injured neurons with the appropriate small polypeptide growth factors can reverse their diminished growth capacity and overcome the inhibition of the mature CNS environment.

Neurons in the brainstem with axonal projections to the spinal cord (bulbospinal neurons) are necessary for the initiation and control of many motor behaviours, notably locomotion. After spinal cord injury, these motor behaviours are permanently compromised due to the ineffectiveness of CNS repair. I hypothesized that treating bulbospinal neurons with the appropriate trophic factor(s) will enhance their growth.

To address this hypothesis, I designed a novel assay to examine trophic effects on bulbospinal neurons specifically. The assay is based on retrogradely labeling the bulbospinal neurons as they develop axonal projections to the spinal cord in the chick embryo. Subsequently, the brainstem tissue is dissociated (whole or vestibulospinal) or explanted (vestibulospinal or reticulospinal) into culture, creating survival or neurite outgrowth assays respectively.

Two families of growth factors, neurotrophins and Fibroblast Growth Factors (FGFs) were examined for trophic effects on bulbospinal neurons. The neurotrophins were largely ineffective, but NT-3 significantly increased neurite outgrowth from reticulospinal (not vestibulospinal) explants. Four FGFs (FGF-1, FGF-2, FGF-5 and FGF-9) were also tested. FGF-2 was the most effective, stimulating survival and neurite

outgrowth for all populations examined. FGF-1 only enhanced bulbospinal neurite outgrowth, FGF-9 only survival, and FGF-5 was largely ineffective. At this stage of development, bulbospinal neurons did not express FGF receptors, but non-neuronal cells *in situ* and *in vitro* did. Astrocyte-conditioned medium also increased bulbospinal neuron survival, suggesting that perhaps the FGFs act by stimulating the production of the appropriate growth factor from non-neuronal cells. In conclusion, indirect effects may be an important mechanism for growth factor action on bulbospinal neurons. Furthermore, NT-3, FGF-1 and FGF-2 are good candidates for promoting regeneration from injured bulbospinal neurons *in vivo*.

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LIST OF ABBREVIATIONS

ACM	astrocyte conditioned medium	GGF	glial growth factor
aFGF	acidic fibroblast growth factor	Gi	inhibitory G protein
AIGF	androgen-induced growth factor	HBGAM	heparin binding growth associated molecule
ANOVA	analysis of variance	HBGF	heparin binding growth factor
AP	alkaline phosphatase	HBSS	Hanks balanced salt solution
BDNF	brain derived neurotrophic factor	HMW	high molecular weight
bFGF	basic fibroblast growth factor	HSPG	heparan sulphate proteoglycan
BrdU	bromodeoxyuridine	Ig	immunoglobulin
CAM	cell adhesion molecule	K-FGF	Kaposi fibroblast growth factor
cAMP	cyclic adenosine mono phosphate	KGF	keratinocyte growth factor
CCD	charge-coupled device	LMW	low molecular weight
Ce	cerebellum	LoC	Locus Coeruleus
cek	chicken expressed kinase	n.	nucleus
CFR	cysteine rich FGF receptor	MAG	myelin associated glycoprotein
ChAT	choline acetyltransferase	MAP	microtubule associated protein
Cnd	nucleus centralis dorsalis	MLF	medial longitudinal fasciculus
CNS	central nervous system	mRNA	messenger ribonucleic acid
Cnv	nucleus centralis ventralis	NCAM	neural cell adhesion molecule
CSPG	chondroitin sulphate proteoglycan	NGF	nerve growth factor
dATP	deoxy adenosine triphosphate	NT-3	neurotrophin-3
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	NT-4	neurotrophin-4
DiO	3,3'-dioctadecyloxacarbocyanine perchlorate	PBS	phosphate buffered saline
DMEM	Dulbecco's modified Eagle's medium	PKA	protein kinase A
DNA	deoxyribonucleic acid	PNS	peripheral nervous system
DNase	deoxyribonuclease	Ra	Raphe
DRG	dorsal root ganglion	RGC	retinal ganglion cell
E	embryonic day	Rgc	Reticularis gigantocellularis
ELISA	enzyme-linked immunosorbent assay	RNase	ribonuclease
ER	endoplasmic reticulum	Rpc	Reticularis parvocellularis
FCS	fetal calf serum	RP	Reticularis Pontis
FGF	fibroblast growth factor	RPO	Reticularis Pontis Oralis
FGFR	fibroblast growth factor receptor	RPgc	Reticularis Pontis gigantocellularis
GABA	gamma aminobutyric acid	RPpc	Reticularis Pontis parvocellularis
GAP	growth associated protein	SC	subcoeruleus
GDNF	glial derived neurotrophic factor	SSPE	sodium chloride, sodium phosphate, ethylene diamine tetra acetic acid
GFAP	glial fibrillary acidic protein	TM	transmembrane
		trk	tropomyosin receptor kinase
		TK	tyrosine kinase
		VeD	vestibularis descendens
		VeL	vestibularis lateralis
		VeM	vestibularis medialis

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- CHAPTER 1 - GENERAL INTRODUCTION

1.1 OVERVIEW

One of the enduring enigmas in the field of neuroscience is the absence of central nervous system (CNS) regeneration and functional recovery after injury. Due to the structural and functional complexity of the CNS, the properties, events and changes that conspire to prevent regeneration necessitate an examination at many levels. From studies at behavioural, tissue, cellular and molecular genetic levels, a hierarchy emerges that serves to outline this rapidly expanding field of research.

The first section of the Introduction establishes the control pathways for motor behaviour, and the structure/function of the spinal cord at the cellular level. The second section outlines some cellular and molecular changes that occur after CNS injury, as well as recent advances that give a greater understanding of CNS regeneration failure. Lastly, the third section details how a particular class of molecules, growth factors, may serve as an important component of any CNS repair strategy.

1.2 CNS INJURY RESULTS IN PERMANENT LOSS OF FUNCTION.

1.2.1 Motor control overview

It has long been recognized that damage to the CNS, for example the spinal cord, produces permanent, irreversible functional deficits (Barnard and Carpenter, 1950;

Bjorklund et al, 1971; Ramon y Cajal, 1928). The spinal cord provides the input/output pathway for most sensory/motor functions, connecting the brain with the somatic sensory and motor apparatus via the peripheral nervous system. Spinal cord pathways to the periphery are topographically organized in a segmented fashion, with each spinal segment responsible for communication with a defined subset of the body surface (Landmesser, 1978; Romanes, 1964). For example, the spinal segments in the cervical enlargement control sensorimotor functions of the upper limbs, while segments of the lumbar enlargement control lower limb functions. Thus, following damage to the spinal cord between the cervical and lumbar enlargements, upper limb function is preserved. However, due to the loss of communication with supraspinal areas, lower limb motor and sensory function is compromised (Sholomenko and Steeves, 1987).

1.2.2 Motor control is mediated by the brainstem.

In all vertebrates, much of the motor output from the brain to the spinal cord is relayed through centers in the brainstem (Barnes, 1984). The commands to initiate volitional movements originate in higher cortical centers that connect with specific regions of the brainstem (Kuypers and Lawrence, 1967). These brainstem relay centers are connected in turn to the appropriate spinal level (Holstege and Kuypers, 1982), which controls the output to the appropriate muscles. All connections within the CNS are characterized by their point of origin (where the cell body of the neuron is found) and the point of termination (where the axon finds synaptic targets). Thus, connections from the

cortex to the brainstem (the “bulbous” brain) are termed corticobulbar, and from the brainstem to the spinal cord, bulbospinal.

In mammals, one direct pathway exists between the cortex and the spinal cord, called the corticospinal tract. This tract only mediates finely controlled movements of the distal limbs, for example, the fine finger movements required to grasp and manipulate objects (Lawrence and Kuypers, 1968). This represents a very small subset of the brain’s motor output; the vast majority (e.g. running, jumping, breathing) is controlled via corticobulbar (Kuypers and Lawrence, 1967) and bulbospinal (Steeves et al, 1987; Valenzuela et al, 1990) connections to the appropriate level of the spinal cord.

In this scheme, the cortex sends commands to the brainstem to initiate a movement such as running. The brainstem processes the commands and directs the spinal cord (via the bulbospinal connections to the lumbar cord in this example) to activate the appropriate leg muscles. The lumbar spinal cord then signals the appropriate muscle contractions via motor neurons to produce the motor output recognized as running (Grillner and Wallen, 1985). After a spinal cord injury (at a spinal level between the brainstem and lumbar spinal cord) the bulbospinal connections to the lumbar spinal cord are interrupted, resulting in the loss of this motor behaviour (Sholomenko and Steeves, 1987). **An important conclusion from this is that to restore motor function after spinal cord injury, the connections between the brainstem and spinal cord must be restored.**

1.2.3 Bulbosplinal projections originate from discrete brainstem nuclei.

The cell bodies of neurons that send axons into the spinal cord are found in specific brainstem nuclei, including the reticular formation, vestibular complex, raphe nuclei, red nucleus, and locus coeruleus (Cabot et al, 1982; Kuypers and Martin, 1982; Webster and Steeves, 1988). Spinal interneurons further process the supraspinal neuron commands and then control the motor output to the periphery via the spinal motoneurons. There are a minority of direct monosynaptic connections between brainstem neurons and spinal cord motoneurons (Holstege et al, 1979), but most of the brainstem motor output is further processed by spinal interneurons.

The reticular formation of the pons and medulla, which receives command input from cortical and subcortical regions, mediates much of the motor output of the brainstem (Parent, 1996). This structure, extending the length of the pons and medulla, acts as an initiator for many motor behaviours such as locomotion (Eidelberg, 1981; Grillner and Wallen, 1985; Steeves et al, 1987). Focal electrical stimulation of the appropriate reticular formation regions is sufficient to produce locomotor movements in decerebrate animal preparations (Steeves et al, 1987). Neuroanatomical tract tracing from the spinal cord reveals neurons located in the nucleus (n.) Centralis (dorsal and ventral; Cnd and Cnv respectively), n. Reticularis parvocellularis (Rpc), n. Reticularis Pontis parvocellularis and gigantocellularis (RPpc and RPgc respectively) and n. Reticularis Pontis Oralis (RPO; Martin et al, 1979a; 1979b; Martin et al, 1981; Tohyama et al 1979a; 1979b).

The mammalian vestibular complex can be subdivided into superior, lateral, medial and descending vestibular nuclei. These nuclei receive and process input from the

vestibular apparatus of the inner ear, as well as the cerebellum and reticular formation (Parent, 1996). The lateral, medial and descending vestibular nuclei are the origin of vestibulospinal projections (Martin et al, 1979b; Tohyama et al, 1979a). Lateral vestibulospinal projections control anti-gravity posture mostly by facilitating extensor muscle output (Lund and Pompeiano, 1965, 1968). Vestibular nuclei connections with the reticular formation and cerebellum ensure that postural control is integrated with overall motor behaviour (Carleton and Carpenter, 1983). Medial and descending vestibulospinal neurons projecting to the cervical cord also mediate vestibulo-ocular reflexes to produce the neck/head movements that allow visual stability during body movements (Boyle and Pompeiano, 1980; Parent, 1996).

The raphe nuclei are a group of mostly serotonergic neurons divided between several distinct nuclei in the medulla, pons and midbrain. Only medullary and caudal pontine raphe neurons project axons to the spinal cord (Brodal et al, 1960; Cabot et al, 1982), including neurons in the raphe pallidus and obscurus (mostly medullary) and raphe magnus (located in rostral medulla and caudal pons; Brodal et al, 1960). Functionally, these nuclei modulate not only motor behaviours of somatic (Eccles and Lundberg, 1959; Engberg et al, 1968; Iwamoto et al, 1980) and autonomic motor systems (Cabot et al, 1979; Coote and Macleod, 1974; Henry and Calaresu, 1974), but also the transmission of sensory information as well (Guilbaud et al, 1977; Duggan and Griersmith, 1979).

Like the multifunctional raphe, the locus coeruleus (LoC) of the pons is thought to provide a modulatory influence upon several spinal cord functions. It is implicated in the control of pain pathways, generating analgesia via terminations in the dorsal horn (Segal

and Sandberg, 1977; Sandberg and Segal, 1978). Somatic motor output is also enhanced with activation of LoC neurons, via ventral horn terminations (Strahlendorf et al, 1980).

The red nucleus of the midbrain, the origin of the rubrospinal tract, is involved in modulating the motor output of the brainstem (Arshavsky et al, 1988). It participates in feedback loops from the spinal cord via the cerebellum (Condé, 1988; Daniel et al, 1987; Giuffrida et al, 1988) to aid in error detection/correction of the motor output as it progresses (Arshavsky et al, 1988). This nucleus also plays a role in the coordination of fine motor control for distal extremities (Jarrat and Hyland, 1999).

All bulbospinal neurons project axons within circumscribed tracts in the ventral and lateral spinal cord funiculi. Some of these axons may extend the entire length of the spinal cord, and may branch at numerous spinal levels to produce axon collaterals innervating multiple target fields. This overall organization of bulbospinal pathways is very well conserved between mammals and birds (O'Donovan et al, 1992; Steeves et al, 1987; Webster and Steeves, 1988; Webster et al, 1990).

To summarize this section, the neurons in the brainstem that comprise the motor control system are found in the reticular formation, vestibular nucleus, red nucleus, raphe nucleus and locus coeruleus. These neurons project axons, some of which extend the entire length of the spinal cord, to innervate one or more spinal target areas to initiate and modulate motor output. Damage to the spinal cord results in the interruption of these axons, and the loss of the associated function. **Achieving the goal of motor function restoration after spinal cord injury requires the restitution of appropriate bulbospinal connections to the spinal cord.**

1.3. WHY DOES CNS REGENERATION FAIL?

1.3.1 *The CNS structure favours stability over regeneration ability*

The anatomy of the higher vertebrate CNS hints that repair after injury was never a major design consideration. The CNS is encased in a strong, bony compartment (the skull and spinal column), and protected from sudden shocks by a cushion of cerebrospinal fluid surrounding the brain tissue. This construction suggests that prevention of damage was a higher priority evolutionary strategy than the capability to repair damage. To build the brain and spinal cord during development requires significant time and resources. The payoff is a highly complex structure capable of highly complex motor and sensory functions critical to the survival of the organism. The loss of these functions after injury represents a catastrophic impairment that cannot be repaired quickly. These two points suggest that resources diverted to repairing CNS damage are wasted, as the outcome is almost certainly the death of the organism.

A second consideration follows from the complexity of the CNS structure. It is estimated that the human brain contains 10^{11} neurons, each connecting with an average of 10,000 other neurons (Parent, 1996). During development, conditions exist that allow the growth of these appropriate connections with high fidelity. Once development is complete, it is hypothesized that further growth is actively inhibited to avoid the potential of spurious and inappropriate growth "short-circuiting" the fine structure of the mature CNS. These two considerations provide a conceptual framework for designing experiments that address the failure of regeneration in mature animals:

1. During development, conditions are favourable for CNS growth.
2. After development is complete, CNS growth is actively inhibited..

As CNS maturation occurs, the balance between promotion and inhibition can be altered by two mechanisms: 1) neuron growth potential diminishes, and 2) environmental promotion of growth diminishes or changes to inhibition . Thus during development, the neurons are intrinsically capable of growth, and the environment allows and even supports that growth. From this, the hypothesis emerges that with increasing age, CNS neurons become intrinsically incapable of growth and/or the environment no longer promotes, and actively inhibits growth. Evidence addressing both of these aspects will be considered in the following sections.

1.3.2 Development and regeneration

During development of the CNS, it is obvious that neurons are capable of growing and connecting with their targets, but what happens when the developing CNS is injured? It is now well accepted that injury to the CNS during this growth permissive stage results in anatomical and functionally appropriate regeneration (Hasan et al, 1993; Varga et al, 1996; Wang et al, 1994; 1996; 1998a,b; Xu et al, 1989; 1991). Experiments have characterized time periods in the development of a variety of species during which anatomical and functional recovery occurs after CNS injury.

The Lamprey provides one example of a species with an extended larval development. Injury to the spinal cord during this period results in the regeneration of

bulbospinal motor control pathways (Lurie and Selzer, 1991). Furthermore, this regeneration is functionally appropriate, with good return of swimming behaviour correlated with the re-innervation of spinal targets by supraspinal axons (Davis et al, 1993a,b; McClellan et al, 1988; 1990).

A second model was developed based on easy experimental access to marsupial young at an early stage of development. Marsupial young are born early in development, after which they are protected in the maternal pouch while development proceeds *ex-utero*. This provides good experimental access to a developmentally immature nervous system, and several research groups are using the North American or Brazilian opossum for this reason. Regeneration of cut axons contributes to the repair process seen after injury to the early post-natal marsupial spinal cord (Martin and Xu, 1988; Wang et al, 1996; 1998a,b; Xu and Martin, 1990; 1991). Furthermore, the propensity for repair of spinal cord injury diminishes with the increasing age of the animal.

A third model, developed in our lab, is based upon an avian species: the domestic chicken *Gallus domesticus*. Avian development *in ovo* provides easy experimental access to essentially all stages of development. The development of bulbospinal pathways in the chick occurs during embryonic days (E)3 to E12 (Glover, 1993; Okado and Oppenheim, 1985) of the 21 day developmental process. Bulbospinal neurons are born by about E3 (McConnell and Sechrist, 1980), and then begin extending axons seeking their spinal targets. Functional connections with neurons in the spinal cord are evident by E6/E7 (Shiga et al, 1991; Sholomenko and O'Donovan, 1995), and the pathways complete their development by E11/E12 (Glover, 1993; Okado and Oppenheim, 1985). Retrograde tracing techniques were used to examine regeneration

after spinal cord injury in embryonic chick (Hasan et al, 1993; Shimizu et al, 1990). These experiments defined the early embryonic period during which anatomical regeneration occurred after spinal cord injury. Anatomical restoration of bulbospinal projections was observed if the injury occurred prior to E13 (Hasan et al, 1993). Functional assessments with brainstem stimulation elicited motor output from the spinal cord, indicative that appropriate connections were restored (Hasan et al, 1993). Spinal cord injuries on or after E13 (restrictive period) resulted in diminished repair effectiveness, and after E15 injury repair failed completely as in the adult CNS (Hasan et al, 1993; Shimizu et al, 1990).

From these data, it is clear that the developing CNS is not only capable of growth, but also of regeneration. There are numerous hypotheses regarding the loss of regenerative capacity as the CNS matures, from intrinsic neuronal changes to altered environmental factors. Considering the CNS neurons themselves, intrinsic changes occur that likely contribute to regenerative failure. These changes include measurable deficiencies in gene expression and other phenotypic alterations after mature CNS injury, discussed in the next section.

1.3.3 Intrinsic neuronal determinants of growth

In neuronal development, the expression of specific genes is increased during axonal extension. These changes in gene expression are characteristic of a genetic neuronal growth program. Typically referred to as growth associated proteins (GAPs), the up-regulated gene products include structural elements necessary to build the growing

axon such as tubulin (Miller et al, 1987; Miller et al, 1989; Oblinger and Kost, 1994). Other gene products such as GAP-43 (De La Monte et al, 1989; Jacobson et al, 1986; Reh et al, 1993) and Bcl2 (Merry et al, 1994) are similarly expressed at high levels during axonal growth. The function of GAP-43 remains unknown, but its expression during development is highly correlated with the actively growing neuronal state. Bcl2 expression is not only neuroprotective against naturally occurring cell death, but may be involved in axonal outgrowth (discussed in greater detail below). Thus, a genetic expression profile representing the growth state of the neuron can be determined by examining the state of GAP gene expression.

After adult CNS injury, damaged neurons may engage in an "abortive sprouting" response, during which new growth cones may form close to the injury site. This response may be characterized by increases in GAP gene expression (Bisby and Tetzlaff, 1992; Doster et al, 1991; Koistinaho et al, 1993; Robinson, 1994), as well as decreases in the expression of genes associated with a fully differentiated neuronal phenotype such as neurofilaments (Tetzlaff et al, 1991) and neurotransmitter synthesis enzymes. For example, after septal transection, cholinergic neurons in the mammalian basal forebrain atrophy, decrease synthesis of choline acetyltransferase and other cholinergic markers, and fail to regenerate (Armstrong et al, 1987). Similarly after cervical spinal cord injury, neurons in the red nucleus initially increase the expression of GAPs such as tubulin and GAP43, in characteristic preparation for a growth response (Tetzlaff et al, 1991). Several days after injury, these neurons then decrease the expression of GAPs and become atrophic as well (Tetzlaff et al, 1991).

This initial injury response, essentially recapitulating developmental events, does not always occur in injured CNS neurons. Retinal ganglion cells subjected to optic nerve crush did not re-express GAPs unless the injury was very close to the origin of the optic nerve (Doster et al, 1991). Returning to the rubrospinal example introduced above, after thoracic spinal cord injury (a greater distance from the rubrospinal cell body compared with the cervical injury response described above) there was no measurable up-regulation of GAPs in rubrospinal neurons (Tetzlaff et al, 1994; Fernandes et al, 1999). Thus, mature neurons in general are less responsive, and display a distance-dependent response to injury. In contrast, thoracic spinal cord injuries during the permissive period for repair in the immature embryonic chick resulted in anatomical and functional regeneration (Hasan et al, 1993).

Similar intrinsic neuronal changes with tissue maturity were evident with *in vitro* preparations. Cultured embryonic Purkinje cells were capable of extensive axonal outgrowth, but post-natally derived neurons were not (Dusart et al., 1997). The entorhino-hippocampal projection (Li et al., 1995), and retinotectal projection (Chen et al., 1995) also displayed age-dependent changes that resulted in axonal growth deficits. These data suggest that intrinsic neuronal changes with maturation contributed to the failure of CNS regeneration.

A potentially interesting role for Bcl2 as an intrinsic determinant of axonal outgrowth ability, distinct from its anti-apoptotic function, was recently described. High levels of Bcl2 expression corresponded with the axonal extension phase during CNS development (Merry et al, 1994). In the developing retino-tectal system of the mouse, Bcl2 was highly expressed at E16 when this projection was actively completing its

development. By E18, Bcl2 expression decreased markedly. In coculture experiments, retinal explants from wild-type E18 mice were unable to innervate E18 tectal explants, but retinal explants from E18 mice overexpressing Bcl2 showed enhanced neurite outgrowth and ability to innervate E18 tectal target tissue. Retinal explants from E16 Bcl2 knockout mice were unable to innervate E16 tectal explants (Chen et al, 1997), Further supporting the role for Bcl2 as an intrinsic determinant of axonal growth. Some cell lines, such as a dopaminergic cell line transfected with a Bcl2 expression vector, also showed robust neurite outgrowth compared to control vector transfected cells (Oh et al, 1996). It will be interesting to follow future developments in this area.

In summary, the developing CNS is capable of anatomical and functional regeneration after injury. This property is diminished or lost as the CNS matures, although an abortive regenerative response recapitulating developmental events may occur after much of the axon is lost to injury (as when damage occurs close to the cell body). The data reviewed in this section provided insight into possible intrinsic neuronal changes with maturation such as developmentally regulated gene expression (Bcl2) that manifested as alterations in gene expression after injury (GAPs). **In conclusion, the success of putative regeneration strategies will require intrinsic neuronal changes in GAP gene expression, recapitulating the developmental growth program.** It should be noted that these intrinsic neuronal changes occur within a changing (maturing) environment, an equally important consideration relating to the failure of CNS regeneration.

1.3.4 The mature CNS environment inhibits axonal growth

Almost one hundred years ago, Ramon y Cajal (1928) already predicted the presence of inhibitory influences in the CNS that prevent regeneration. At the time, mounting clinical evidence suggested that the prognosis for recovery from peripheral nervous system (PNS) damage was considerably better than recovery from central nerve damage. This simple observation prompted Ramon y Cajal (1928) to introduce segments of peripheral nerve into a CNS lesion site. He observed the elaboration of axons into the peripheral nerve graft environment, presumably by injured CNS neurons. This experiment was re-visited using modern techniques to confirm that injured CNS axons sprout into peripheral nerves grafted at the lesion site.

Richardson and co-workers (1980) demonstrated that after optic nerve transection (injury in a CNS environment) and peripheral nerve grafting, injured retinal ganglion cells regenerated axons into the graft. Differences between the PNS and CNS environment are the result of dissimilar non-neuronal cells present therein. Schwann cells provide myelination and control the PNS environment by secreting soluble and basement membrane protein components, and regulating the composition of the extracellular space. Oligodendrocytes and astrocytes provide these same functions for the CNS. The contributions by oligodendrocytes and astrocytes to regeneration failure will be reviewed briefly.

1.3.5 CNS myelin inhibits axonal growth

The failure to recover after central nervous system (CNS) injury has been attributed to numerous characteristics of the cells and environment that make up the brain and spinal cord. One hypothesis suggests inhibitors of neurite growth exist within mature CNS myelin, and are normally present in the myelin sheaths of the mature nervous system to prevent spurious sprouting and preserve the fine structure of the CNS. If that is true, an unfortunate side effect may be the inhibition of the re-growth of axons after CNS injury.

Recently, several proteins were identified that contribute to CNS myelin inhibition of axonal growth, including NI35/250 (novel oligodendrocyte cell membrane bound proteins of 35kDa and 250kDa; Caroni and Schwab, 1988) and myelin associated glycoprotein (MAG; McKerracher et al, 1994; Mukhopadhyay et al, 1994). NI35/250 is a protein or protein complex found in higher vertebrate myelin with powerful growth cone collapse and neurite growth inhibitory properties (Bandtlow et al, 1990; Schwab and Caroni, 1988). Antibodies to NI35/250 (the IN-1 antibody) introduced into the rat CNS at the time of spinal cord injury suppressed the inhibition due to myelin, and allowed regeneration of small numbers of corticospinal axons for distances up to 11mm from the lesion site (Schnell and Schwab, 1990; 1993).

MAG is a membrane-bound myelin protein thought to promote adhesion between oligodendrocytes and axons (Filbin, 1995). Biochemical fractionation of myelin proteins revealed that part of the CNS inhibitory properties separated with the MAG fraction, and that immunodepletion of this fraction with anti-MAG antibodies restored its growth-permissiveness (McKerracher et al, 1994). In a concurrent paper, Mukhopadhyay *et al*

(1994) demonstrated neurite growth inhibition produced by a MAG substrate for cultured cerebellar granule neurons and adult dorsal root ganglion neurons (DRG). These data suggest that myelin contains several neurite growth inhibition components, all of which need to be neutralized for a successful CNS repair strategy.

Another approach for addressing all the inhibitory properties of myelin at once uses a targeted oligodendrocyte/myelin disruption technique. A cocktail containing an antibody directed against oligodendrocyte cell surface antigens combined with serum complement was injected into the spinal cord. The cocktail initiated an immune attack specifically targeted to oligodendrocytes (Sergott et al, 1984; Ozawa et al, 1989), and created a myelin-free zone at the injection site (Dyer et al, 1998; Keirstead et al, 1992; 1995; 1997). When applied to the developing chick spinal cord on E11 prior to the onset of myelination (E13), this cocktail produced a dysmyelination, delaying the onset of myelination until E17 (Keirstead et al, 1992). As previously discussed, prior to E13 in the untreated developing chick, recovery from spinal cord injury occurred (Hasan et al, 1993), but injuries sustained on or after E13 were not repaired. With the antibody cocktail treatment, the permissive period for regeneration was extended until E15, further supporting the hypothesis that myelin serves to inhibit CNS growth or regeneration (Keirstead et al, 1992).

Using this technique in the adult rat CNS also improved the regeneration of bulbospinal connections after spinal cord injury (Dyer et al, 1998). In these experiments, an antibody to galactocerebroside (an oligodendrocyte-specific cell surface antigen) combined with a source of complement infused into the spinal cord with osmotic minipumps, produced a myelin-depleted zone involving several spinal segments around

the infusion site. Up to 40% of severed bulbospinal axons regenerated after spinal hemisection within the myelin-depleted zone, as determined by retrograde tracing from below the lesion site (Dyer et al, 1998). These studies demonstrate that myelin contains inhibitory substances, and interventions targeting myelin directly improved the regenerative capacity of the mammalian CNS. These interventions provide data important for understanding the failure of CNS regeneration, but still fail to restore all axons lost to injury.

1.3.6 Other glial cells contribute to CNS regeneration failure

A second glial cell type in the CNS, the astrocyte, also plays an important role in the response to CNS injury. At the lesion site itself, astrocytes quickly undergo a transformation to a reactive state distinguished by hypertrophy, proliferation and increased expression of the astrocytic marker glial fibrillary acidic protein (GFAP; reviewed in Raivich et al, 1999). This glial scar of reactive astrocytes also secretes basement membrane proteins such as tenascin (Hirsch and Bahr, 1999) and chondroitin sulphate proteoglycans (CSPG; Guo et al, 1993; Levine, 1994; McKeon et al, 1995), two known inhibitory substrates for axonal extension *in vitro* (Faissner and Kruse, 1990; McKeon et al, 1991). However, there is still some debate about whether the astrocytic scar is inhibitory, as increased growth factor production is also evident (Raivich et al, 1999).

To summarize this section, the early developing CNS provides all the ingredients that engender appropriate CNS growth, whether in the course of normal development or regeneration after spinal cord injury. Both intrinsic neuronal changes and extrinsic environmental factors conspire to restrict the regenerative capacity of the CNS as it matures. Intrinsic changes include the post-injury failure to up-regulate appropriate genes thought necessary for growth. **Extrinsic environmental changes during development include the appearance of myelin proteins inhibitory to axonal growth, as well as the maturation of astrocytes capable of producing an inhibitory astrocytic scar at the site of injury.**

1.4. STRATEGIES TO PROMOTE CNS REGENERATION

1.4.1 Growth factor overview

The discussion in the preceding sections introduced 2 hypotheses regarding the failure of CNS regeneration:

1. Intrinsic neuronal changes result in a decreased propensity for regeneration
2. Extrinsic environmental factors (myelin, astrocyte scar) prevent regeneration

Growth factors are a class of secreted, typically small polypeptide molecules expressed at high levels during the development of the CNS. Currently, there are well over 50 known growth factors, grouped as families of structurally related molecules. Maturation of the

CNS is accompanied by decreased expression of most growth factors, perhaps contributing to the decreasing growth promoting environment alluded to earlier. This section introduces the function of growth factors in the development of the CNS, and demonstrates how the application of the appropriate growth factors may overcome the barriers to CNS regeneration described above.

1.4.2 The neurotrophic factor hypothesis

After the birth of a neuron, axonal extension occurs towards putative target regions, relying on pathfinding cues expressed along the growth trajectory. These cues include cell surface proteins on intermediate and final targets, as well as soluble factors released to create molecular gradients guiding the developing axon. The discovery of Nerve Growth Factor (NGF) by Levi-Montalcini (1966) opened a new field of neuroscience based upon the neurotrophic factor hypothesis. At its simplest, the neurotrophic factor hypothesis states:

“The secretion of soluble growth factors in limited amounts from target cells will attract and maintain appropriate innervation.”

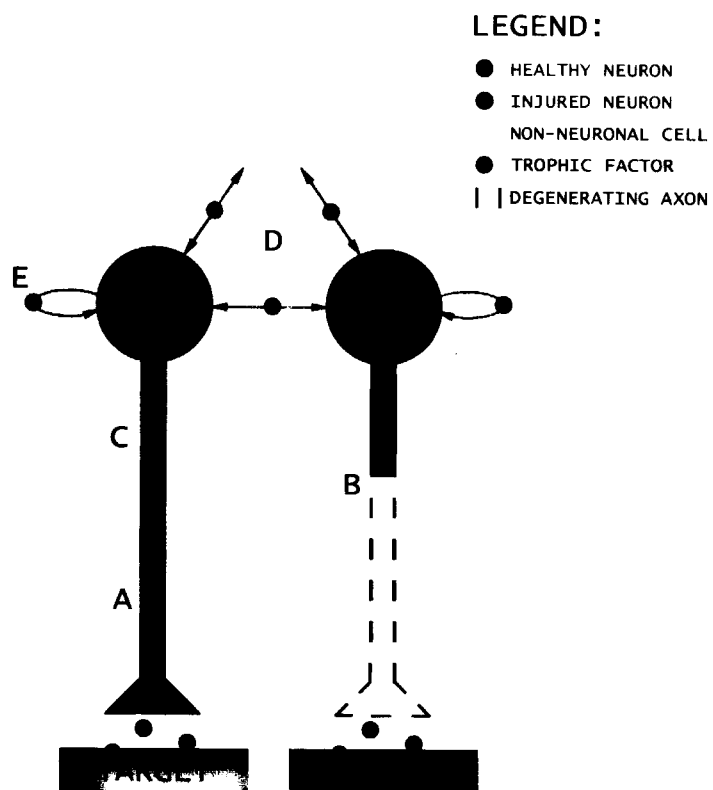
From the properties of NGF discovered by Levi-Montalcini and others, a model of axonal growth can be envisioned whereby axons grow toward the target growth factor source, and compete with other axons for access to the limited supply of growth factor. Successful innervation results from successful competition for access to the limited quantities of available growth factor, and those axons unable to secure sufficient growth

factor resources will degenerate. The continued availability of the target-derived growth factor maintains the health of the innervating axon parent cell body by a retrograde transport mechanism (Figure 1A), best characterized in populations of NGF-dependent neurons such as sympathetic neurons (reviewed in Korsching, 1993). The neurotrophic factor hypothesis has since become much more complex, with the discovery of anterograde transport from cell body to target (Figure 1C), paracrine (Figure 1D) and autocrine (Figure 1E) and even intracrine interactions. These additional pathways of trophic factor signaling provide a rich process for communication between neurons and their surrounding cells.

Growth factors interact with specific cell surface receptors on the developing or mature neurons. These receptors typically transduce the growth factor binding into an intracellular signal by activating one of the many intracellular second messenger pathways. For example, the neurotrophins are a family of four related factors including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4. Each binds to one of three high affinity receptors, tropomyosin receptor kinase (trk)A (NGF), trkB (BDNF and NT-4) and trkC (NT-3; reviewed in Meakin and Shooter, 1992).

The trk receptor structure includes an internal tyrosine kinase domain that auto-phosphorylates upon ligand binding and receptor dimerization, activating the phosphorylation of intracellular second messenger system substrates. These substrates include proteins in the *ras* and phosphatidyl inositol-3 kinase pathways that mediate the neurotrophic effects of the neurotrophins (reviewed in Kaplan, 1998). There is also a low affinity receptor called p75, which binds all four neurotrophins and may be involved with signaling apoptosis (reviewed in Casaccia-Bonnet et al, 1999).

Figure 1: The Neurotrophic Factor Hypothesis. Retrograde transport of a target-derived factor maintains the health of the appropriate innervation for that target (A). After injury (B), the supply of target-derived trophic factor is disrupted, leading to negative consequences for the injured neuron. Recently, evidence for anterograde (C), paracrine (D) and autocrine (E) mechanisms of growth factor action reveal a much richer but poorly understood communication between cells.



One hypothesis suggests that the binding of a neurotrophin to its receptor and the resultant activation of intracellular second messenger cascades, produces both immediate local effects at the growth cone or axon terminal, as well as long term effects via changes in gene expression at the cell body. Immediate local effects result from the tyrosine kinase activation and rapid phosphorylation of proteins participating in intracellular secondary messenger cascades. Long term effects may occur with the internalization of the neurotrophin/receptor complex and retrograde transport back to the cell body. The result of the internalization into a retrograde transport vesicle leaves the activated intracellular kinase domain of the receptor exposed to the cytoplasm as it is transported along the axon into the cell body. The activated kinase domain has the potential to continue signaling during its transport to, and after arrival at, the cell body. Changes in gene expression signaled in this way may produce long-term effects on neuronal function, but the exact nature of the retrograde signal is not known. In subsequent sections, the effects of growth factors that counter-act the barriers to regeneration described previously will be reviewed.

1.4.3 Growth factors administered after injury increase GAP expression

The rubrospinal neuron injury model discussed in section 2.1.2 showed that after cervical spinal cord injury, the up-regulation of GAP gene expression initially occurred, then declined concomitant with a marked neuronal atrophy (Tetzlaff et al, 1991).

Rubrospinal neurons express the trkB receptor (Kobayashi et al, 1997), which binds the neurotrophin family members BDNF and NT-4. Administration of either BDNF or NT-4

in the vicinity of the red nucleus after spinal cord injury resulted in sustained expression of GAPs and reversal of neuronal shrinkage, as well as increased numbers of rubrospinal neurons that grew into a peripheral nerve transplanted into the cervical injury site (Kobayashi et al, 1997). **Thus, administration of the appropriate growth factor reverses regressive neuronal changes after injury that may contribute to regeneration failure in the CNS.**

1.4.4. Growth factor treatment overcomes the CNS inhibitory environment

One inhibitory molecule found in the nervous system is collapsin-1 (Shepherd et al, 1996). As its name suggests, it has a potent growth cone-collapsing activity for numerous neuronal populations (Kuhn et al, 1999; Rabacchi et al, 1999). Treatment of DRG neuronal growth cones with collapsin-1 induced a rapid and reproducible retraction of filopodia (Tuttle and O'Leary, 1998). DRG cultured in the presence of NGF were more resistant to this effect, but if cultured in the presence of BDNF they became more sensitive to collapsin-1. This sensitivity to collapsin-1 in chronic BDNF-treated cultures was also overcome with NGF treatment (Tuttle and O'Leary, 1998). These experiments illustrate the complex interactions possible between growth factors and inhibitory substrates.

The response of neurons to inhibitory myelin proteins such as MAG (McKerracher et al, 1994, Mukhopadhyay et al, 1994) can also be influenced by growth factors. Priming cerebellar neurons in the presence of BDNF or glial-derived neurotrophic factor (GDNF) rendered them resistant to the inhibition effected by

MAG/myelin (Cai et al, 1999). The priming procedure was thought to increase cAMP levels, activating protein kinase A (PKA) to produce the anti-inhibitor state. Myelin inhibitor proteins were thought to block this by activating an inhibitory G protein (Gi), which would inhibit the increase in cAMP levels (Cai et al, 1999).

Other inhibitory components of myelin present barriers to CNS regeneration as introduced in section 2.2.1. One model utilized to demonstrate this was the rat retinal ganglion cell (RGC) injury paradigm. RGCs do not regenerate their axons after optic nerve crush. Administration of BDNF at the time of injury increased re-growth of RGCs by almost 1mm (Weibel et al, 1994). Concomitant infusion of IN-1 myelin inhibitor antibodies with BDNF further increased RGC growth to almost 2mm (Weibel et al, 1994). Placement of peripheral nerve pieces into the vitreous of the eye also stimulated regeneration of axotomized RGC (Berry et al, 1996). This was likely due to the variety of soluble growth factors produced by the Schwann cells within the transplanted peripheral nerve segments.

A similar response was seen with injured corticospinal axons in the dorsal columns of the spinal cord. Dorsal hemisection of the spinal cord severed these axons, and resulted in the loss of distal limb function below the level of the lesion (Schnell et al, 1994). Corticospinal neurons are known to express trkC receptors, which bind the neurotrophin NT-3. Administration of NT-3 after dorsal column injury increased sprouting of corticospinal axons, assessed by anterograde tracing from the cortex (Schnell et al, 1994). This model was used to demonstrate that IN-1 antibodies to myelin-associated neurite growth inhibitors, administered at the time of injury, increased the regeneration of some corticospinal axons as described in section 2.2.1, although there

was no return of lost function. Administration of IN-1 antibodies combined with NT-3 infusion at the injury site results in even greater growth of corticospinal axons (Schnell et al, 1994).

Most recently, BDNF was used to promote the regeneration of rubrospinal neurons after cervical spinal cord injury (Liu et al, 1999). Fibroblasts genetically modified to secrete BDNF were implanted into a cervical lesion site designed to completely interrupt the rubrospinal tract unilaterally. Anterograde tracing demonstrated long re-growth of some rubrospinal axons through the spinal white matter, terminating in appropriate gray matter regions. Furthermore, partial restoration of function was observed that was abolished by a second injury interrupting the regenerated fibers (Liu et al, 1999).

In summary, the selected experiments described above confirmed the effectiveness of growth factor administration for overcoming the inhibitory environment of the CNS. The data from experiments described in the preceding section (intrinsic neuronal changes promoted by growth factors), combined with growth factor effectiveness against myelin inhibition *in vitro* and *in vivo* provide a compelling rationale to examine growth factor responses for all bulbospinal neurons. **It is clear that the appropriate growth factor(s) for bulbospinal neurons will be a necessary part of repair strategies for the restoration of motor function after spinal cord injury.**

1.5 HYPOTHESIS

Based on the evidence presented above, I hypothesize that:

Bulbospinal neurons will respond to the appropriate growth factor with enhanced survival or neurite outgrowth.

The difficulty testing this hypothesis arises from the very nature of bulbospinal neurons. With the exception of Locus Coeruleus (LoC) and Raphe neurons (Ra), there are no phenotypic markers for the majority of bulbospinal neurons distinguishing them from the majority of neurons in the brainstem. LoC and Ra neurons can be distinguished by their transmitter phenotype (noradrenergic and serotonergic respectively) but this distinction does not specifically identify the spinally-projecting neurons resident in each group. Therefore, the first task is to develop the tools to allow the specific study of bulbospinal projection neurons.

1.6 BULBOSPINAL NEURON MODEL DEVELOPMENT

To study the trophic factor responsiveness of bulbospinal neurons, a screen for receptor expression would seem to suffice. However, this limits the study to known receptors, bypasses the observation of indirect effects on bulbospinal neurons, and more importantly, does not specifically examine the physiological effect of trophic factors in the mixed cell environment existing in the brainstem. *In vitro* assays that allow the direct observation of growth factor effectiveness upon bulbospinal neurons were developed based on a novel retrograde tracing protocol.

The one feature all bulbospinal neurons share, by definition, is the elaboration of an axon into the spinal cord. This feature provides the means for specifically identifying this functionally important class of neuron, distinguishing them from all other cells in the brainstem with projections to different parts of the neuraxis. It was first necessary to find the appropriate retrograde tracing dye, which when applied *in vivo*, would persist after culturing. In this way the focus of attention remains with bulbospinal neurons growing amongst the cells normally found in the developing brainstem.

The developing chick embryo was chosen for these experiments for a number of reasons. First, it provides easy access to a developing CNS for the retrograde labeling of bulbospinal neurons. This easy access to the developing CNS made the chick embryo one of the favoured developmental animal models, hence a great deal is already known about its anatomy and physiology. Lastly, it is a biped as are humans, and the organization of the bulbospinal motor control system is well conserved between birds and mammals (O'Donovan et al, 1992; Steeves et al, 1987; Webster and Steeves, 1988; Webster et al, 1990).

Besides the rubrospinal neuron studies already described, little is known regarding growth factor responsiveness or trophic factor receptor expression for bulbospinal neurons, a functionally important class of neuron. The next chapter characterizes the tools developed to assay the bulbospinal neuronal response to growth factors. Subsequent chapters examine bulbospinal neuron trophic factor responsiveness and possible mechanisms of growth factor action.

- CHAPTER 2 - THE BULBOSPINAL NEURON MODEL

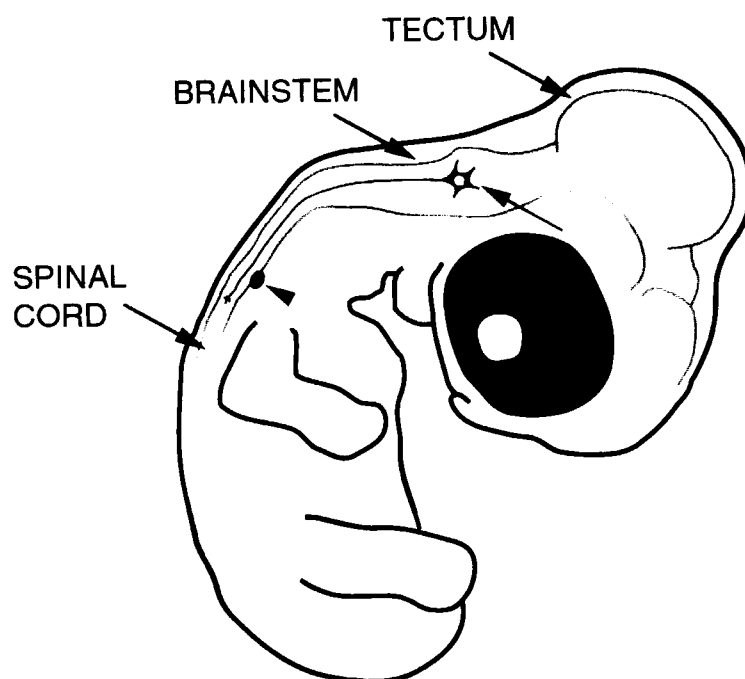
2.1 INTRODUCTION

The heterogeneity of neuronal phenotypes in the brainstem, including local and long projection neurons, presents a challenge for the study of trophic effects on bulbospinal neurons specifically. No phenotypic marker is currently known that identifies and distinguishes all bulbospinal neurons from the greater majority of brainstem neurons, although all bulbospinal neurons share the characteristic of maintaining an axonal projection into the spinal cord.

Two novel assays for the examination of bulbospinal neuronal survival and neurite outgrowth mediated by trophic factors were designed. The assays took advantage of the one developmental event in common between all bulbospinal neurons: the projection of an axon into the spinal cord. To identify bulbospinal neurons engaged in this process, a retrograde tracing dye was implanted into the pathway of the growing axons, the cervical spinal cord.

During the course of normal development, axons from bulbospinal neurons in specific nuclei in the brainstem seek their target neurons by growing caudally into the spinal cord. The axons extend past the cervical dye implantation site and encounter the lipophilic dye. The dye diffuses into and labels the lipid cell membranes of the growth cones. Diffusion of the dye through the lipid cell membrane, combined with normal vesicular traffic, aids the spread of the dye throughout the membrane compartments of the neuron.

Figure 2: A cartoon illustration of a stage 28 chick embryo. The relationship between the DiI crystal implantation site (red arrowhead) and the developing spinal cord (CNS is outlined in gray) is shown. As bulbo-spinal neurons (red arrow, not to scale) develop axonal projections extending into and through the dye-saturated region surrounding the implantation site, the dye dissolves into the lipid cell membrane. Vesicle traffic between the growth cone and the cell body retrogradely transports the dye, labeling the cell body in the brainstem.



This technique generated survival assays based on DiI-labeled neurons derived from the entire pons and medulla (including reticulospinal, vestibulospinal, raphespinal and coerulespinal neurons). Alternatively, careful microdissection isolated specific nuclei from several animals. Survival assays thus contained labeled neurons from a single bulbospinal projection (e.g. vestibulospinal neurons). This technique was used for experiments described in Chapter 3 and Chapter 4.

The second assay measured trophic effects on neurite outgrowth with explant cultures from specific micro-dissections of brainstem nuclei. Several hours after plating, the serum-containing plating medium was replaced with serum free experimental media (with or without trophic factors). Neurite outgrowth was measured and compared between explants exposed to serum free control and trophic factor supplemented culture medium.

In this chapter, the model of bulbospinal neuron survival is characterized, and the use of Fetal Calf Serum (FCS, a universally known source of undefined trophic support) as a positive control for neuron survival is evaluated. For the neurite outgrowth assay, the composition of the growth substrate is an important determinant of growth. For these experiments, laminin is used because it is a permissive substrate for a wide variety of CNS neurons (Powell and Kleinman, 1997). The concentration of laminin in the solution used to coat the culture dishes determined the amount of protein adhering to the culture dish. Therefore, the concentration of laminin that provided maximal neurite outgrowth in medium containing 10% FCS was determined, and used for the rest of the neurite outgrowth experiments.

2.2 METHODS

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Fertilized White Leghorn chicken eggs (*Gallus domesticus*) were obtained from a local supplier and set in a forced-air incubator at 37.5°C in a humidified atmosphere. On E4.5 (approximately stage 28 as defined by Hamburger and Hamilton, 1951), a small, round piece of shell was removed from the top of the egg in the area of the air sac and set aside. This produced a window allowing access to the embryo through holes made in the air sac shell membrane and underlying extra-embryonic membranes. After the operation described below, the round piece of shell was replaced on the egg, and the shell sealed with paraffin wax. The egg was then returned to the incubator to allow development to proceed.

2.2.1 Retrograde Tracing

Retrograde tracing from the spinal cord of developing chick embryos *in ovo* was used to specifically label bulbo-spinal neurons prior to the culturing procedure. Several different retrograde tracing dyes were evaluated, including carbocyanine dyes (DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate and DiO, 3,3'-dioctadecyloxacarbocyanine perchlorate, Molecular Probes Inc., Eugene, OR), fluorescein, rhodamine or biotinylated dextran amines (Molecular Probes), cholera toxin B fragment (Boehringer Mannheim, Laval, Que.) and True Blue dye. The water-soluble dyes (all but DiI and DiO) were mixed with sterile saline solution at concentrations ranging from 5 to 25%, and injected in 0.1-0.5µl volumes directly into the cervical spinal

cord of E4.5 chick embryos using a glass electrode with an inside diameter of 40-80 μ m. Up to two dozen animals were treated with each retrograde tracing dye in this manner, in place of the carbocyanine dye implantation procedure outlined below and used for all other animals.

Crystals of the carbocyanine dye DiI or DiO were prepared by first making a saturated solution of the dye in 95% ethanol, containing suspended crystals of undissolved dye. After much of the ethanol evaporated, this suspension was then made into a fine colloidal paste using a glass mortar and pestle. As more of the solvent evaporated, an optimal consistency was maintained with the addition of small quantities of 95% ethanol. The paste was used to coat the tip of a #00 insect pin and the pins set aside to air dry.

A crystal of the carbocyanine dye was implanted in contact with the ventral surface of the mid-cervical spinal cord by pushing a DiI-coated insect pin into the space between the developing vertebrae and the cord. Fine forceps were then used to hold the crystal in place while the pin was withdrawn. The egg was returned to the incubator for approximately three days to allow for retrograde labeling of developing bulbospinal neurons. The DiI crystal implantation procedure was well tolerated with animal survival rates in excess of 90% three days following the implantation.

2.2.2 Histology

To examine the efficacy of the labeling procedure, some animals not used for cultures were prepared for *in situ* fluorescence microscopy by immersion fixation in 4%

paraformaldehyde overnight, followed by 3 rinses in PBS. The brainstems and dye implantation sites in the neck were then dissected and cryoprotected in 18% sucrose-PBS for an additional 24 hours, and cut at a thickness of 40 μ m in the coronal plane using a Zeiss Microm cryostat. Sections were mounted on Superfrost Plus slides (Fisher Scientific) and examined using the epifluorescence microscope. The number of retrogradely labeled neurons on every fifth section (200 μ m separation) from the spinomedullary junction to the isthmus region was then counted and corrected using the Abercrombie (1946) correction factor to estimate the total number of neurons which were retrogradely labeled by DiI in each brainstem.

2.2.3 *Dissociated Brainstem Cultures*

To produce the bulbo-spinal neuron survival assay, dissociated cell cultures were prepared from the hindbrains of retrogradely labeled E8 chick embryos. The embryos were removed from the egg on E8, decapitated, and the hindbrain was dissected free in ice-cold Ca^{++} and Mg^{++} free Hank's balanced salt solution (HBSS, Canadian Life Technologies, Burlington ON). Care was taken to remove any visible meninges and associated vasculature prior to culturing. Three hindbrains were pooled in ice-cold HBSS to produce enough tissue to perform the assay.

Dissociated brainstem neurons were cultured essentially as described by Goslin and Banker (1991). Briefly, each hindbrain was manually sliced into ~1 mm cubes using a sterile razor blade. The resulting tissue fragments from several animals were pooled, and incubated in HBSS containing 0.125% trypsin (Canadian Life Technologies) and

0.01% DNase (Pharmacia, Uppsala, Sweden) for 20 minutes at 37°C. The digest was stopped with the addition of ice cold 10% fetal calf serum (FCS, Canadian Life Technologies), the tissue was rinsed several times with HBSS containing 10% FCS, then triturated using HBSS containing 0.0033% DNase and 10% FCS through a 1ml pipette tip to produce a single cell suspension. The suspension was collected in a 15ml centrifuge tube and a 1ml underlay of pure FCS was added. The suspension was then spun at 150xg through the FCS underlay to remove cellular debris. The cell pellet was resuspended in culture medium and the number of viable cells determined based on the exclusion of 0.06% nigrosin dye. The suspension was then diluted to allow a plating density of 7.5×10^4 cells/cm², and 0.4ml placed in each well of the chamber slides (Nunc Inc., Naperville IL).

Prior to culturing, the chamber slides were incubated overnight with 100µg/ml polylysine, rinsed three times with sterile distilled water, then coated using 75µg/ml laminin (Canadian Life Technologies) for at least 2 hours. Cells were plated in Dulbecco's Modified Eagles Medium (DMEM, Canadian Life Technologies) supplemented with 10% FCS, and all cultures were grown in a humidified 37°C incubator containing a 5%CO₂/95% air atmosphere. The cells were allowed 2 hours to settle to the bottom of the chambers and attach to the polylysine/laminin substrate, and recover from the dissociation procedure. The medium was then exchanged for the experimental medium, DMEM supplemented with 10% FCS, or the serum-free control medium. The cultures were replaced in the incubator and allowed 48 hours to grow. A single experiment consisted of 4 replicate wells for each control or treatment, and was repeated at least twice in independent trials.

To assess whether the process of mitosis could contribute to an increase in the number of labeled neurons observed at the conclusion of the experiment, some cultures were also treated with bromo-deoxyuridine (BrdU, Boehringer Mannheim) at the time of plating. These cultures were then fixed as described below and stained using fluorescein-conjugated anti-BrdU antibodies according to the manufacturer's suggested protocol (Boehringer Mannheim).

After 48 hours, the cultures were fixed with freshly hydrolyzed 4% paraformaldehyde (BDH Laboratory Supplies, Poole, England) for at least 15 minutes, rinsed with phosphate buffered saline (PBS), and the culture chambers removed from the glass slide. Coverslips were applied using PBS/glycerol mounting medium containing Hoechst 33258 (2 μ g/ml), a DNA binding dye. Cultures were scored for the number of viable, DiI-labeled, phase-bright neurons that appeared healthy on the basis of morphology (neurites extending at least 3 cell body diameters) and Hoechst 33258 nuclear staining. All of the labeled neurons in approximately one fifth of the area of each well (4 parallel excursions with the 40x objective across each well) were counted and these numbers were averaged across the four replicate wells. Statistical comparisons were made between the serum free control (scaled to 100%) and experimental wells within each experiment using one-way analysis of variance (ANOVA). If required, multiple comparisons between treatments were performed using the Newman-Keuls test.

2.2.4 Immunocytochemistry

Some of the initial cultures, not used for exposure to experimental media, were characterized for the presence of neural cell types using immunocytochemistry. After fixation of the dissociated cultures as outlined above, cells were treated with PBS containing 0.03% Triton X-100 (PBS-T, BDH Laboratory Supplies) and 10% normal goat serum for 20 minutes to permeabilize the cells and block non-specific binding sites. One of three primary antibodies was then applied in PBS-T plus 10% normal goat serum for 2 hours at room temperature. Following three PBS-T rinses, the secondary antibody was applied for 1 hour followed by three PBS-T rinses, then coverslips applied as above. Primary antibodies included anti-Glial Fibrillary Acidic Protein (GFAP, antibody # G-A-5, 1:500 dilution), -vimentin (MAB1633, 1:500 dilution, Chemicon, Temecula, CA) and -MAP1b (MAB366, 1:500 dilution, Chemicon), the secondary antibody was fluorescein-conjugated goat anti-mouse IgG (1:500 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA). For the purpose of GFAP antibody immunofluorescence staining, some cultures were maintained for up to 1 week prior to fixation as described above. These cultures received fresh replacement medium at the midpoint of the experiment (the fourth day *in vitro*).

2.2.5 Vestibulospinal Explant Neurite Outgrowth Assay

Animals with labeled bulbospinal neurons were prepared and hindbrains isolated as described above. Labeled brainstems were placed on the stage of a McIlwain Tissue Chopper (Brinkmann, Westbury NY) and chopped into 300 μ m coronal slices. Several candidate slices from each hindbrain were selected from the region thought to contain the vestibulospinal neurons. Using the epifluorescence microscope, the slices were rapidly screened for the presence of vestibulospinal neurons at low power (5x objective). Slices containing vestibulospinal neurons were set aside for micro-dissection. Using a dissecting microscope, each slice was cut diagonally just medial to the vestibular neurons, separating the vestibulospinal neurons from reticular and raphe neurons. These explants were collected and plated individually in 24-well culture plates (Fisher Scientific, Nepean, Ontario, Canada) containing 1ml of DMEM + 10% FCS pre-coated for 2 hours with 25, 50, 75 or 100 μ g/ml laminin. A single experiment consisted of at least 4 replicate wells per treatment, and was repeated at least twice. The cultures were grown for approximately another 32 hours, for a total of 41 hours, then fixed with 10% formalin in 0.1M PBS containing 10% sucrose. Images of the DiI-labeled neurites were captured on a Nikon inverted microscope equipped with a Princeton Micromax cooled charge-coupled device (CCD), imported into Photoshop 4.0/5.0 (Adobe Systems Inc., San Jose, CA), where tracings were made of the labeled neurites. The neurite length analysis was performed on a G3 Power Macintosh computer using the public domain NIH Image program v1.60 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). An image taken of a

micrometer scale was used to calibrate the quantification, performed using the built-in object measurement function of NIH Image. The numerical neurite length data was exported to Microsoft Excel (Microsoft Corp. Redmond, WA) for further analysis. Each experiment included serum-free control treated explants, and neurite lengths were compared only within individual experimental trials, not across separate experiments. This was done to decrease the potential variability as a result of uncontrollable properties such as batch-to-batch variations in media formulations. Neurite lengths for each explant exposed to a particular treatment were measured, pooled and analyzed for mean neurite length. Statistical comparisons were made between the multiple treatments within one experiment using ANOVA. Neurite lengths for each treatment were also separated into 200 μ m length bins, and plotted using a "longer than x" graphical representation. This provides a plot with length bins on the X-axis and the percentage of neurites longer than "x" on the Y-axis. Thus, the first data point (neurite length of 0 μ m) is assigned a Y-axis value of 100%, as all measured neurites are longer than 0 μ m. All photomicrographs presented in this thesis were processed using Adobe Photoshop 4.0/5.0 for the preparation of montages and addition of symbols and labels.

2.3 RESULTS

2.3.1 *Evaluation of Retrograde tracers*

Several different retrograde tracing dyes were evaluated, including carbocyanine dyes (DiI and DiO), fluorescein, rhodamine or biotinylated dextran amines (Molecular Probes), cholera toxin B fragment (Boehringer Mannheim, Laval, Que.) and True Blue dye. The primary criteria for this assay included robust and extensive labeling of the greatest number of bulbospinal projection neurons, and retention of the label after culturing and fixation. Of the tracers used here, the lipophilic dyes DiI and DiO were the only retrograde tracers to label bulbospinal neurons in sufficient quality and quantity to perform the bioassay. Furthermore, the labeling persisted even after dissociation and plating, and re-distributed throughout the cell as new membrane was added in the course of neurite extension.

In animals prepared for histological evaluation, the labeling with DiI proved the best both in number of neurons (~8,000/animal, Table 1) and quality of the labeling. The bright fluorescence and resistance to photobleaching also made DiI the preferred label for these experiments. DiO was almost equally effective but the labeling appeared less robust and although not quantified, appeared to label fewer neurons. The higher autofluorescence of brain tissue observed with fluorescein dye filter sets likely contributed to the weaker appearance of the DiO labeling, and the perception of decreased neuron numbers. The DiO labeling was also more sensitive to photobleaching; therefore, most of the experiments were done with DiI.

An examination of the DiI implantation site from several animals revealed the DiI crystal in close contact with the ventral and ventrolateral surfaces of the spinal cord (Figure 3). Little damage to the spinal cord from the implantation procedure was evident. Tissue sections taken from 1mm rostral to the implantation site revealed extensive labeling of ventral and ventrolateral tracts, where the majority of the bulbospinal projections are growing. Some labeling was also seen in propriospinal neurons, and the dorsal columns. Dorsal column labeling presumably arose from ascending sensory projections that also have the opportunity to grow through the implantation site and take up the dye, as well as dorsal root ganglion neurons in close proximity to the implantation trajectory of the DiI encrusted pin.

In vivo, neurons exhibited labeling which varied from a punctate staining of vesicles located in the cell body and proximal neurites, to extensive labeling of the cell membrane and other internal membrane compartments. The distribution of labeled cells observed in the brainstem matched previously published data. Spinally projecting neurons were observed throughout the reticular formation, as well as the Raphe nuclei and vestibular complex (Figure 4A,B). The hindbrain pictured in Figure 4 was cut into serial 300 μ m thick slices, and the slices arranged from caudal to rostral (Figure 5). Starting with the medulla, labeled neurons were observed in the dorsal and ventral portions of the Central nucleus (Cnd and Cnv respectively, Figure 5A-B) and the Raphe nuclei (Figure 5C-F). Moving rostrally, labeled neurons were also seen in the parvo- and gigantocellular nuclei of the reticular formation (Rpc and Rgc respectively, Figure 5D-H).

Figure 3: The DiI implantation site. A cartoon illustration (A) indicating the location of the DiI crystal (arrowhead) and the origin of the tissue sections shown in B and C. A section through the DiI implantation site (B) reveals the DiI crystal (DiI, outlined in white) in between the developing vertebra (V) and the spinal cord (SC, outlined in gray). Approximately 1mm rostral to the implantation site (C), the ventral and lateral funiculi (trajectory of bulbospinal projections, arrowheads) display the DiI label. Propriospinal neurons are also labeled with DiI (arrow). Scale bar (for B, C) 200 μ m.

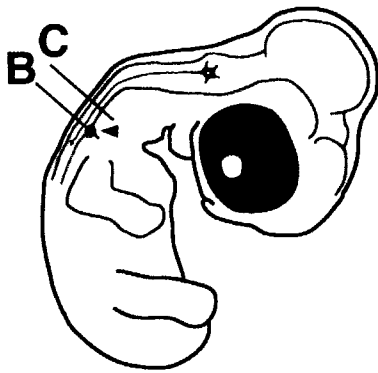
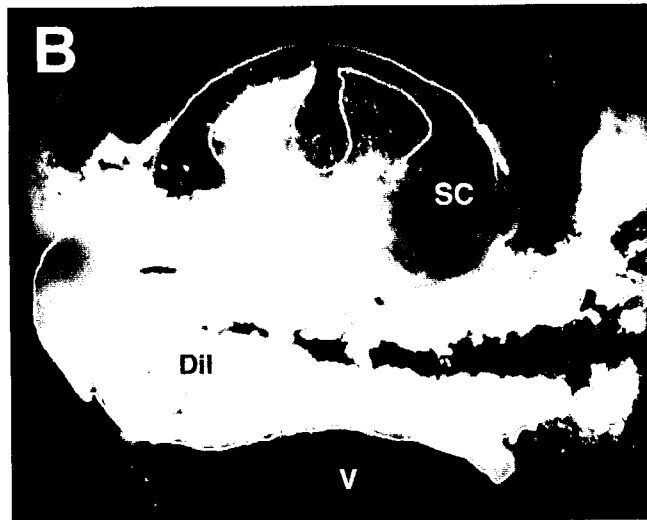
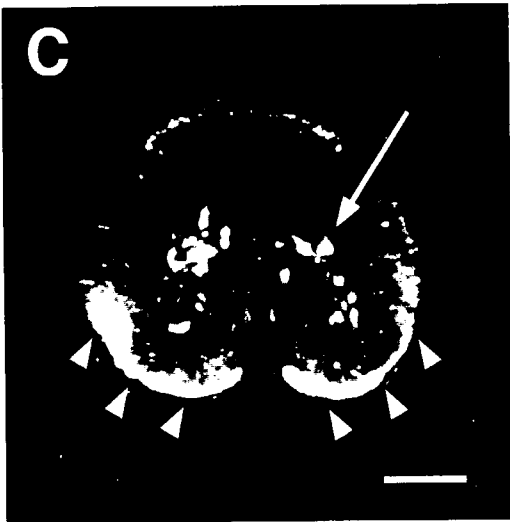
A**B****C**

Figure 4: DiI-labeled bulbospinal neurons in a freshly prepared wholemount of E8 chick hindbrain. The ventral surface (A) revealed bulbospinal neurons distributed throughout the Reticular formation (R) and medullary Raphe nuclei (Ra). The dorsal surface showed the Vestibular Complex, note that the Cerebellum (Ce) was reflected to expose the floor of the fourth ventricle. The vestibulo-cochlear nerve (nVII/VIII) is indicated in these photomontages. Scale bar 1mm.

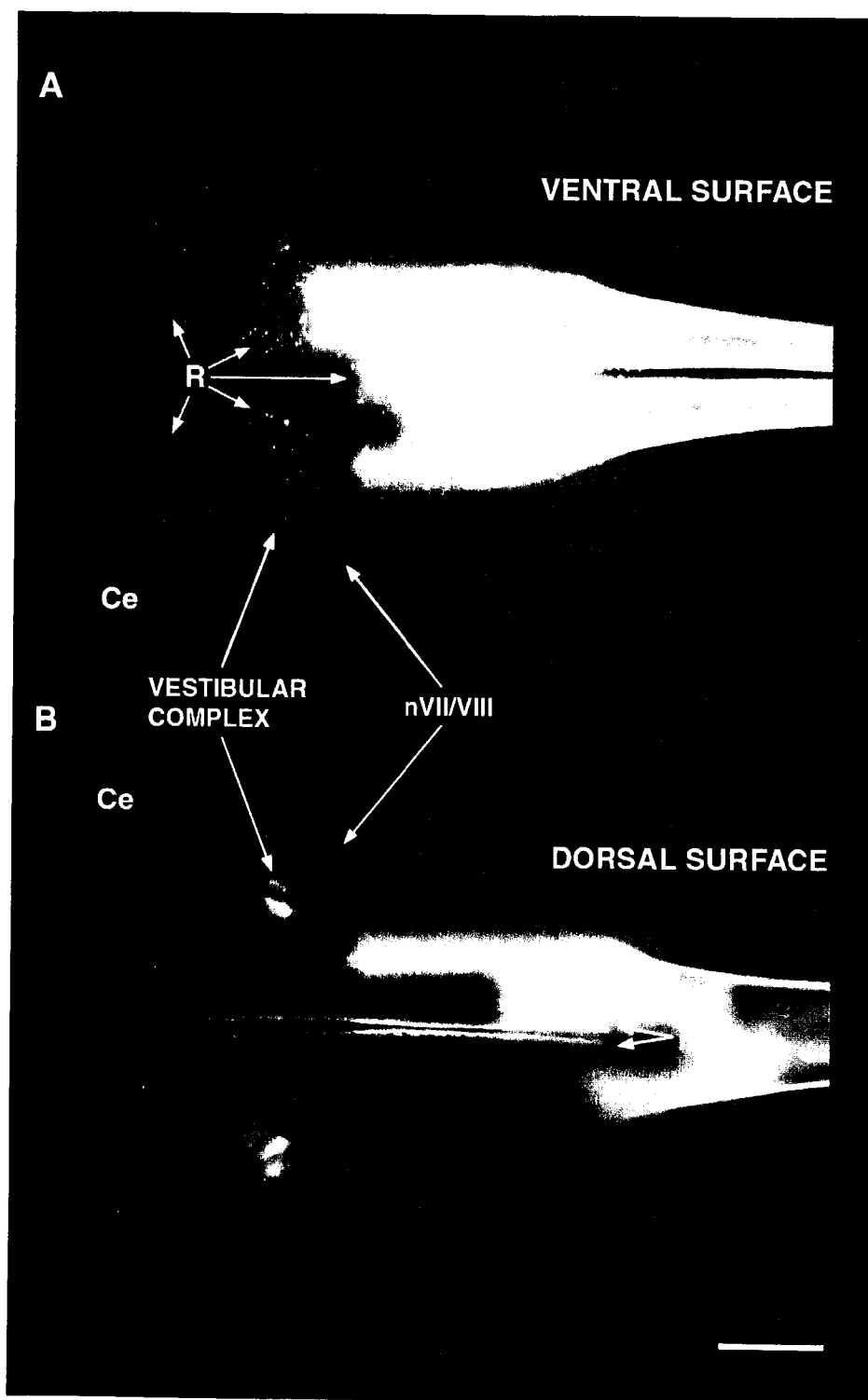
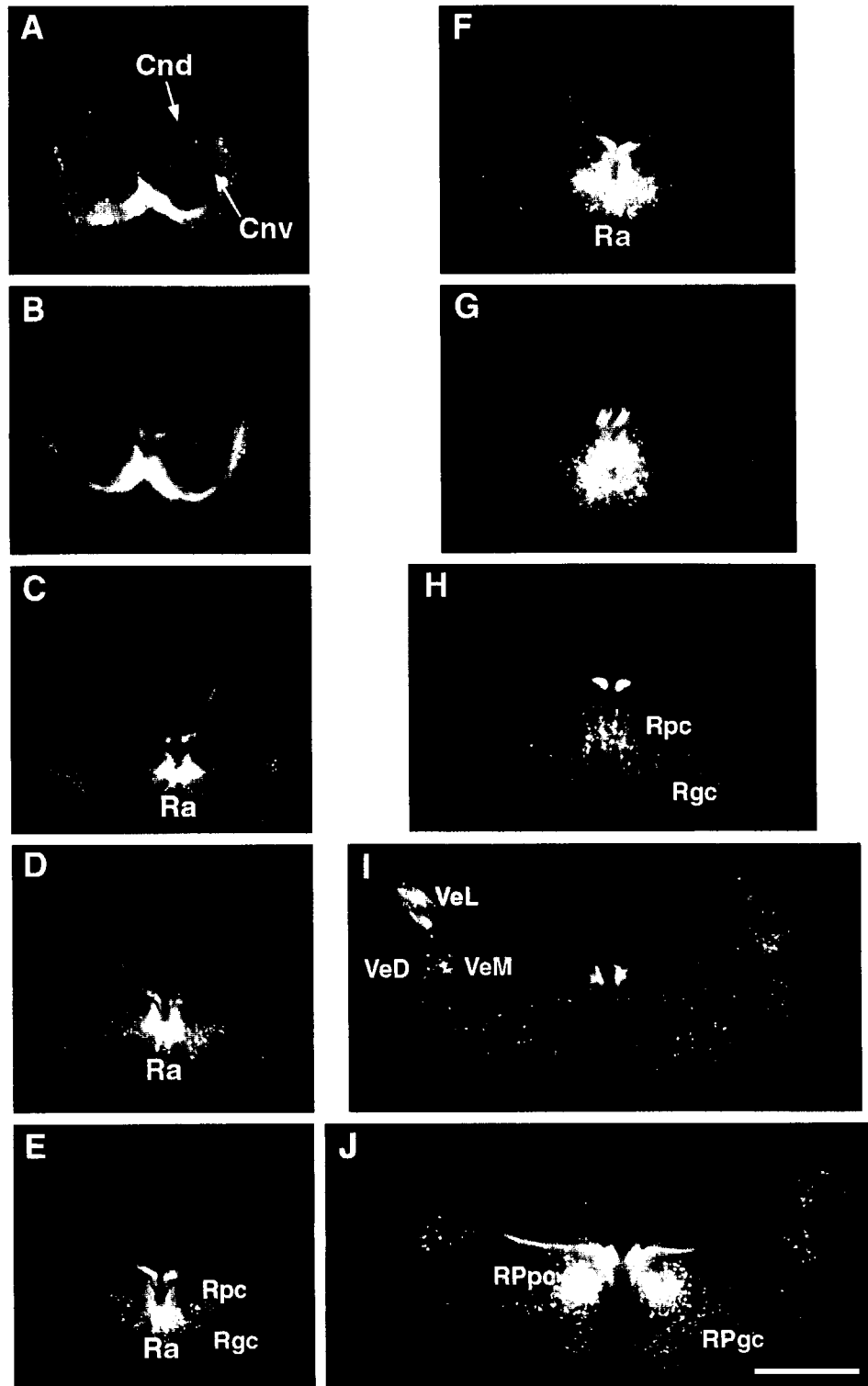
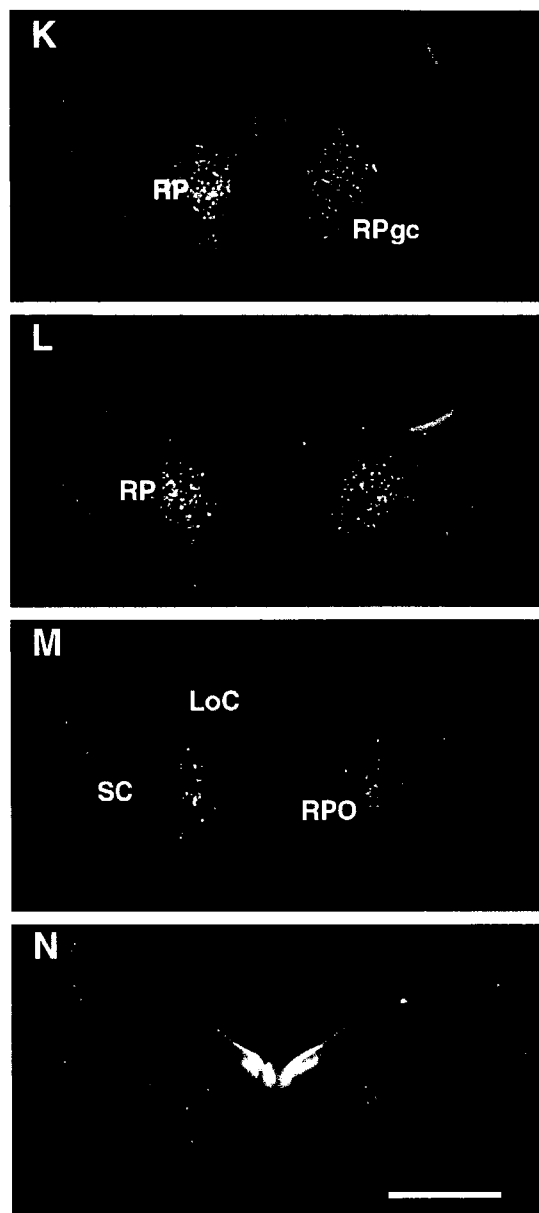


Figure 5: The distribution of DiI-labeled bulbospinal neurons in the E8 chick brainstem. A series of slices from a fresh-cut hindbrain (300 μ m thickness), arranged caudal to rostral, demonstrated labeled neurons within Cnd and Cnv (A, B); Raphe nuclei (Ra, C-F), note also the Medial Longitudinal Fasciculus (MLF, F); Rpc and Rgc, (D-H); the vestibular complex (I, J) including VeD, VeM and VeL; RPpc and RPgc (J-K); RP (K-L); RPO (M,N); LoC and SC (M). Scale bar 500 μ m





Neurons in the most caudal extent of the vestibular complex (Figure 5I), defined as including neurons in the Vestibularis Descendens (VeD), Vestibularis medialis (VeM) and Vestubularis lateralis (VeL), were visible. The vestibular nuclei straddle the ponto-medullary junction; hence labeled vestibulospinal neurons are similarly observed in the caudal pons (Figure 5J). Raphe projections within the pons are not labeled, and are known to project to more rostral targets in the neuraxis. The pontine reticular formation contributes spinal projections from several nuclei, including the Reticularis Pontis parvocellularis (RPpc, Figure 5J-K), Reticularis Pontis gigantocellularis (RPgc, Figure 5J-K), Reticularis Pontis (RP, Figure 5K-L) and Reticularis Pontis Oralis (RPO, Figure 5M-N). Also at the rostral extent of the pons, neurons were labeled in the Locus Coeruleus (LoC, Figure 5M) and Subcoeruleus (SC, Figure 5M). Labeling was also seen in some midbrain neurons in the Red nucleus (data not shown). Note that the Red nucleus was not well labeled, likely due to the late time course of rubrospinal tract development (Shiga et al, 1991). Therefore, the midbrain was not included in any of the culture experiments. Cultures were only prepared from the hindbrain, which includes the medulla and pons (cerebellar anlagen removed).

An estimation of the number of neurons that could be labeled using this technique was calculated from neuronal counts obtained with a serial-sectioning paradigm. Between the spinomedullary junction caudally and the isthmus, every fifth 40 μ m section was assessed for the number of visible DiI-labeled neurons. The counts were corrected using the Abercrombie (1946) correction factor, yielding an average of ~8000 labeled neurons per animal. A maximum of approximately 11,000 neurons was seen in one of the animals (Table 1).

The majority of neurons were located in the reticular formation, although the vestibular complex likely represented the greatest concentration of labeled neurons because it occupies a very small, circumscribed area within the brainstem. In contrast, labeled spinally projecting neurons in the reticular formation were more sparsely distributed, extending in bilateral, nearly continuous columns from the spinomedullary junction to the rostral pons.

Brainstem Region						
Animal	Raphe Nuclei	Medullary Reticular	Vestibular Complex	Pontine Reticular	Locus Coeruleus	Total
1	1,550	4,183	1,003	3,856	573	11,165
2	1,207	3,097	663	2,500	333	7,857
3	797	1,837	720	1,623	127	5,104
4	1,233	2,740	1,290	2,113	147	7,523
Average						7,912

Table 1: Neuronal counts of DiI labeled neurons in 4 randomly selected E8 chick embryos. An average of 7,912 neurons per animal were labeled by the DiI implantation procedure.

2.3.2 Dissociated cell culture assay characterization.

Enzymatic and mechanical disruption of the brainstem tissue and subsequent plating of the single cell suspension produced dissociated cell cultures for the survival assay. The dissociated neurons, a small proportion of which were labeled with DiI (and thus originally projected into the spinal cord), re-grew in a mixed cell environment under serum free conditions with or without the experimental trophic source. The environment

of the re-growing dye-labeled neurons included neighbouring cells of glial, neuronal or non-neural identity (e.g. fibroblasts and blood vessel epithelial cells) as well as the laminin substrate upon which the axons re-grew. Forty-eight hours after plating, the cultures were fixed and scored for the number of DiI-labeled surviving neurons.

After dissociation and plating, the culture wells contained cells at a density of approximately 7.5×10^4 cells/cm². Over the ensuing 48 hours, the cells re-established their phenotypic characteristics, including cell type-specific morphology. The neurons typically began extending neurites within the first 9-12 hours, and the fastest growing DiI labeled neuronal axons achieved lengths in excess of 2mm. This represents an average axonal growth rate of 55µm/hour.

Concomitant with the reappearance of characteristic cellular morphology was the re-expression of cell type-specific markers. As all differentiated neurons express Microtubule-Associated Protein (MAP) 1b (Sato-Yoshitake et al, 1989), its expression was used as a phenotypic marker to identify neurons in some of the experiments where an accounting of the total neuronal population was required (Figure 6A). MAP1b immunoreactivity was only observed in cells with neuronal morphology (as identified with phase contrast microscopy), within the cell body and neurites.

The immunocytochemical characterization of the dissociated cultures was also performed with antibodies raised against Vimentin and GFAP. Vimentin is an intermediate filament protein expressed by a variety of non-neuronal cells, including pre-differentiated glia, fibroblasts and endothelial cells (Tapscott et al, 1981). Vimentin antibody staining revealed that the majority of non-neuronal cells in the cultures expressed this antigen (Figure 6B). GFAP is an astrocyte-specific intermediate filament

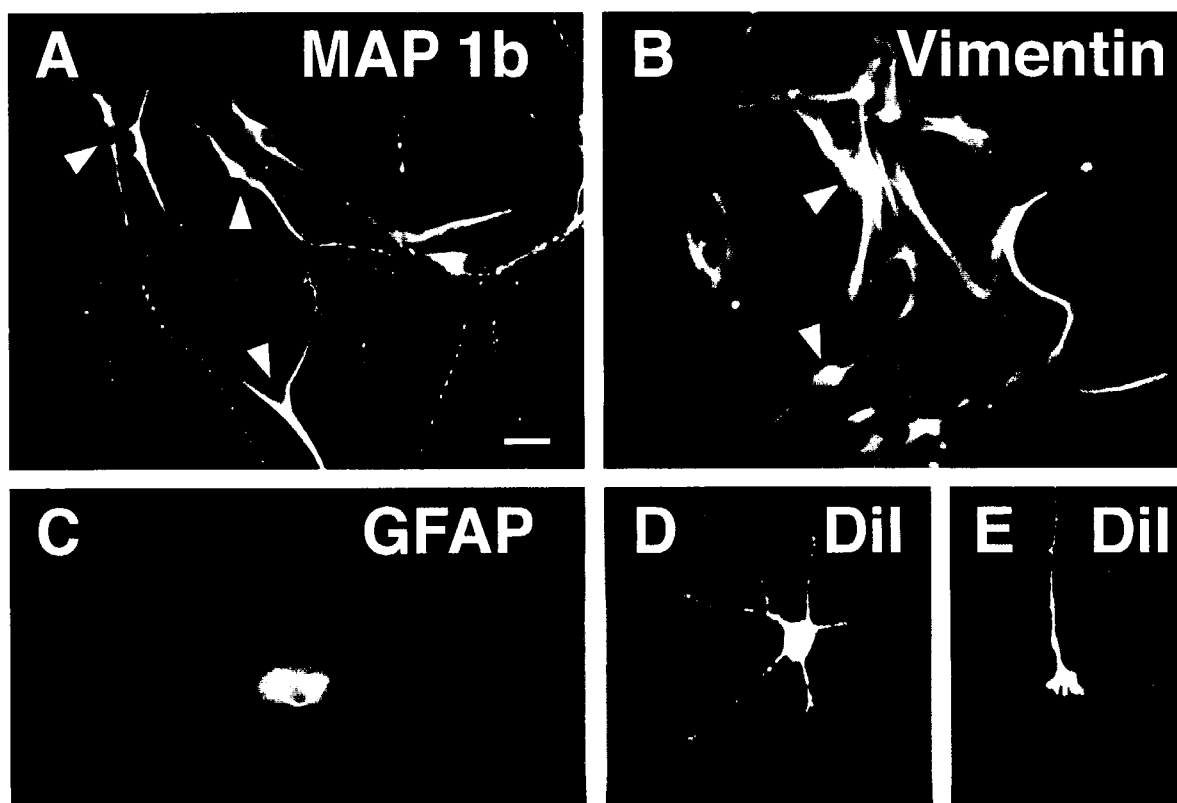
protein, routinely used as a marker for differentiated astrocytes (Cohen et al, 1979). Rarely were any GFAP positive cells observed after 48 hours *in vitro* (Figure 6C). Longer duration cultures were necessary to observe significant numbers of GFAP-positive cells, which began to appear on the third day *in vitro* and increased in number as time progressed (data not shown).

To assess the effect of trophic factor treatments, survival in the experimental wells (containing trophic factors) was compared with survival in serum free control medium-treated wells. The DiI label provided the initial screen for healthy surviving bulbospinal neurons. The presence of the dye allowed the investigation to focus only on bulbospinal neurons, and ignore the majority of the cells in the wells. The quality of the DiI labeling observed *in vitro* was similar to that observed *in situ*. The neuronal labeling varied from a punctate staining of the cell body and proximal neurites, to a robust labeling of membrane compartments throughout the neuron (Figure 6D), including the axon and the growth cone situated up to 2mm away from the cell body (Figure 6E).

Typically there were several hundred DiI-labeled neurons in each well, dependent upon the treatment conditions. Each well was scanned using four parallel excursions of the objective at a standard interval across the well for all the experiments. Each DiI-labeled neuron (Figure 7A,D) was characterized as surviving if it met the following criteria:

1. non-apoptotic nucleus (Hoechst 33258 stain)
2. a phase-bright morphology
3. neurites extending at least 3 cell body diameters

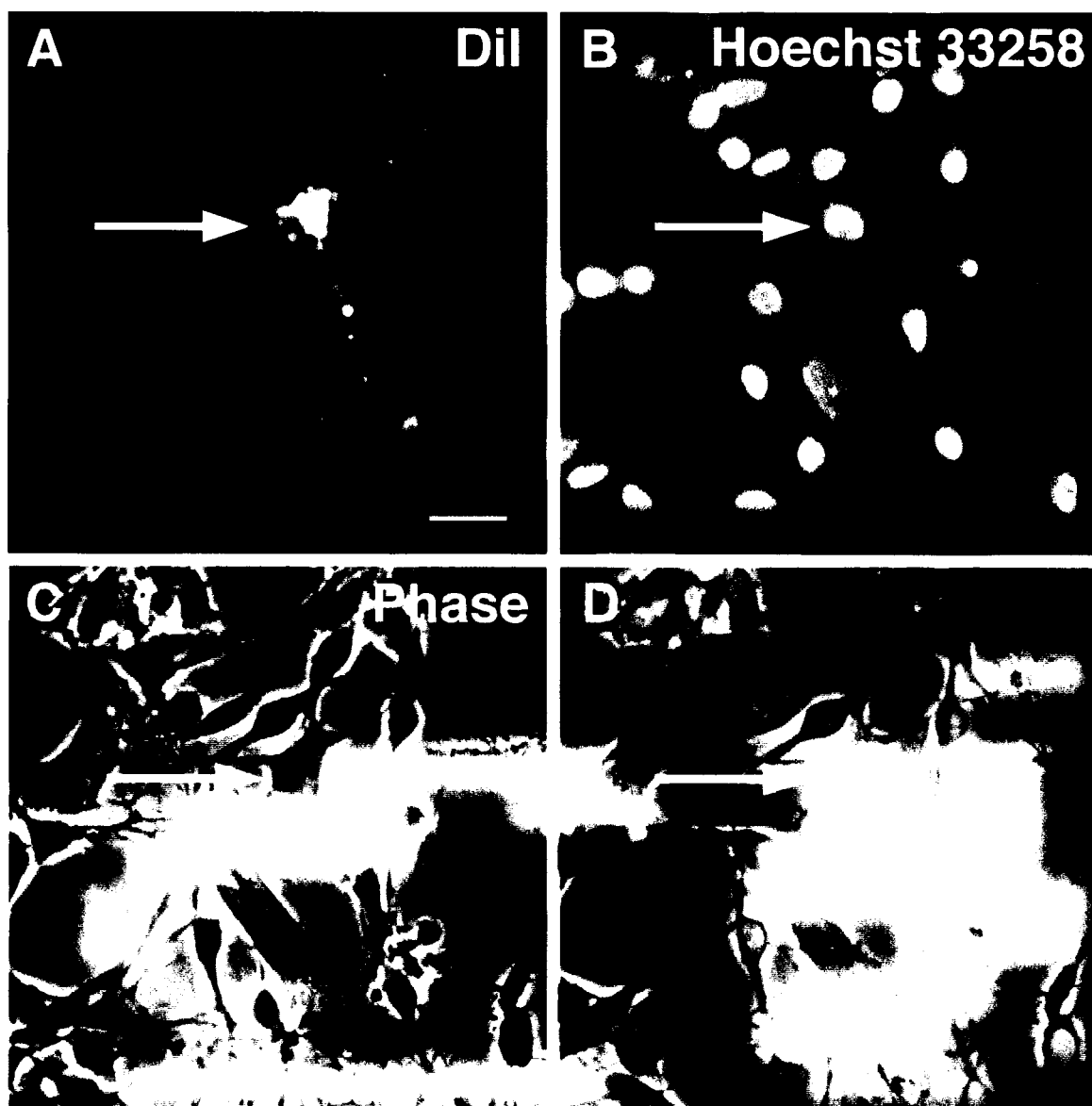
Figure 6: Characterization of the dissociated cell culture assay. MAP1b immunoreactivity (A) distinguished neurons from the non-neuronal cells present. Vimentin immunoreactivity (B) suggested that the majority of non-neuronal cells were predifferentiated glial cells, fibroblasts or endothelial cells. Rarely was GFAP immunoreactivity (C), specific for differentiated astrocytes, observed after 48hours *in vitro*. Some neurons were heavily labeled with DiI (D), including the extent of the axon (up to 2mm in length) and growth cone (E). Scale bar, 20 μ m.



If a neuron is not initially prepared to survive, then it is unlikely to extend neurites of any significant length, and will instead begin the process of apoptosis. Even if initially primed for growth, neurons which may at first exceed the 3 cell body diameter neurite length criterion and then begin to die will also undergo apoptosis with characteristic changes in nuclear morphology (Clarke and Oppenheim, 1995). For this reason, the nuclear stain Hoechst 33258 was incorporated into the mounting medium, to provide an assessment of the nuclear morphology of any DiI-labeled neurons under consideration (Figure 7B,D). One hallmark of the apoptotic process is the condensation and clumping of nuclear material on the periphery of the nucleus (Clarke and Oppenheim, 1995), made visible because Hoechst 33258 stains DNA directly. If any condensation or clumping of nuclear material was apparent then the cell was not included in the survival counts.

After culturing, neuronal cell bodies will typically maintain a more spherical shape in spite of the growth of the neurites. Using phase contrast optics (Figure 7C,D), this appears as a darker rounded shape surrounded by a high contrast phase-bright ring. In contrast, non-neuronal cells establish a very flat, low contrast phase-dull appearance and can be distinguished from neurons on this feature. A second feature of healthy surviving neurons is the growth of neurites that become the dendrites and axon(s). A lack of neurites would indicate that there is insufficient trophic support to engender the continued differentiation of that neuron (or the cell's identity was non-neuronal). In the low plating density conditions used here, it was possible to follow specific neurites from their source cell bodies often for distances greater than 3 cell body diameters. Furthermore, the presence of the DiI label eased the task of identifying the trajectory of a particular neurite, and whether it satisfied the second criterion above.

Figure 7: Appearance of DiI-identified bulbospinal neurons in dissociated cell culture. Bulbospinal neurons typically exhibited a punctate DiI labeling pattern (*A, D*). The criteria for identifying healthy, surviving DiI labeled neurons in the survival assay included normal Hoechst 33258 nuclear staining (*B, D*). Further criteria included a phase bright neuronal morphology with neurites extending at least 3 cell body diameters (*C, D*). Scale bar, 20 μ m.



It was possible that dye transfer between labeled and unlabeled cells artificially inflated the number of labeled neurons counted in these experiments. This issue was addressed by combining tissue isolated from one animal subjected to DiI retrograde tracing, and one animal subjected to DiO retrograde labeling. If the lipophilic dyes could leak out or otherwise transfer from cell to cell, then cells stained with both label should appear in the resulting dissociated culture. The combined tissue was then dissociated and plated following the normal culturing procedure. Neurons were observed containing either DiI or DiO, but none stained with both dyes were found (data not shown).

Some cultures were grown in the presence of BrdU to determine whether mitosis of neurons artificially inflated the observed number of neurons labeled with DiI. Cells with a non-neuronal morphology stained with the BrdU antibody were readily apparent, but BrdU-stained neurons were not observed; nor were any DiI-labeled neurons double stained with the BrdU antibody (data not shown).

2.3.3 Neuronal survival in serum-treated cultures

The survival-promoting effects of FCS were quantified using the dissociated hindbrain cell culture assay. Dissociated cell cultures prepared from the hindbrain were grown in DMEM containing 10% FCS, and survival of bulbospinal neurons compared with cultures grown in serum free DMEM. The survival of bulbospinal neurons in medium containing 10% FCS increased $54 \pm 11\%$ ($p < 0.005$) above serum free control cultures (Figure 8).

2.3.4 Characterization of the laminin substrate effect on neurite outgrowth.

Increasing concentrations of laminin were used to coat the bottom of 24well culture plates prior to plating vestibulospinal explants in serum-containing medium. The neurite outgrowth observed under these conditions was typically long and unbranched, with little fasciculation (Figure 9A). The neurites were traced and the tracings used to measure neurite length (Figure 9B). The estimated maximal rate of neurite outgrowth was similar to the dissociated neuronal cultures, approximately 60 μ m/hour. Neurite outgrowth was limited after coating with a 25mg/ml laminin coating solution (mean length 561 \pm 54 μ m), and increased with 50 (mean length 695 \pm 43 μ m) and 75mg/ml (mean length 977 \pm 40 μ m). With a coating concentration of 100mg/ml (mean length 1045 \pm 56 μ m), no change over 75mg/ml was observed (Figure 10). The initial *in vivo* DiI labeling of these vestibulospinal neurons was accomplished via a spinally projecting axon. Thus, these neurites likely represent regrowing projections because the original axon was severed during the explant procedure.

It is interesting to note that the re-growing neurites typically curve in a clockwise direction. This effect was noted previously for retinal ganglion cells from *Xenopus* (Grant and Tseng, 1986), fish (Heacock and Agranoff, 1977), chick and mouse (Halfter et al, 1987), but remains largely unexplained. Presumptive non-neuronal cells were observed to migrate away from the explants with increasing time in culture, however the neurites typically grew at a faster rate than the migrating cells. Thus the growth cones of the neurites were growing in contact with the laminin substrate with no direct involvement of non-neuronal cell surfaces.

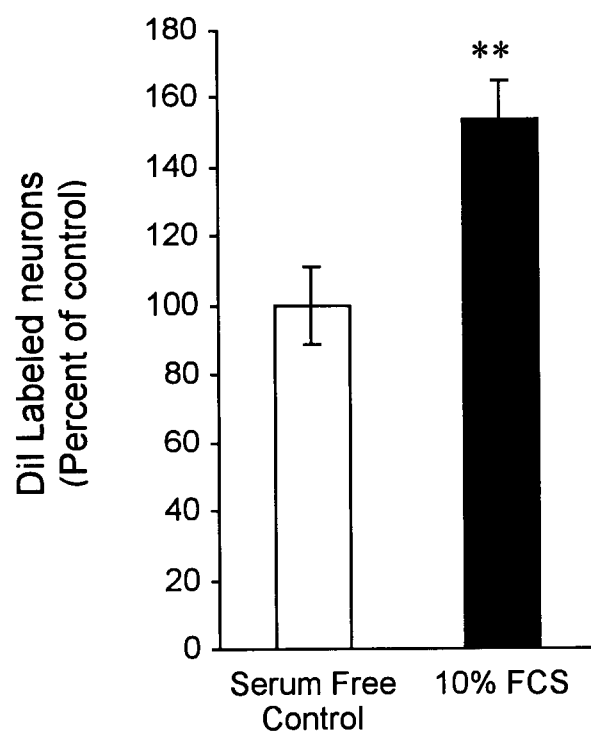
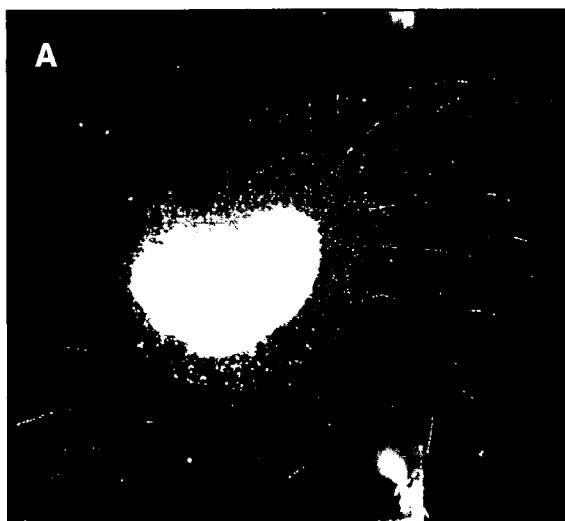


Figure 8: Bulbospinal neuron survival in serum-containing medium. Bulbospinal neuron survival was increased $54 \pm 11\%$ with 10% FCS treatment compared to serum free DMEM treated cultures (** $p < 0.005$).

Figure 9: Neurite outgrowth from a Vestibular Complex explant. A microphotomontage shows DiI-labeled neurites extending from a vestibular complex explant grown in serum free control medium (A). The corresponding tracing used to measure neurite length (B). Scale bar, 1mm (B).



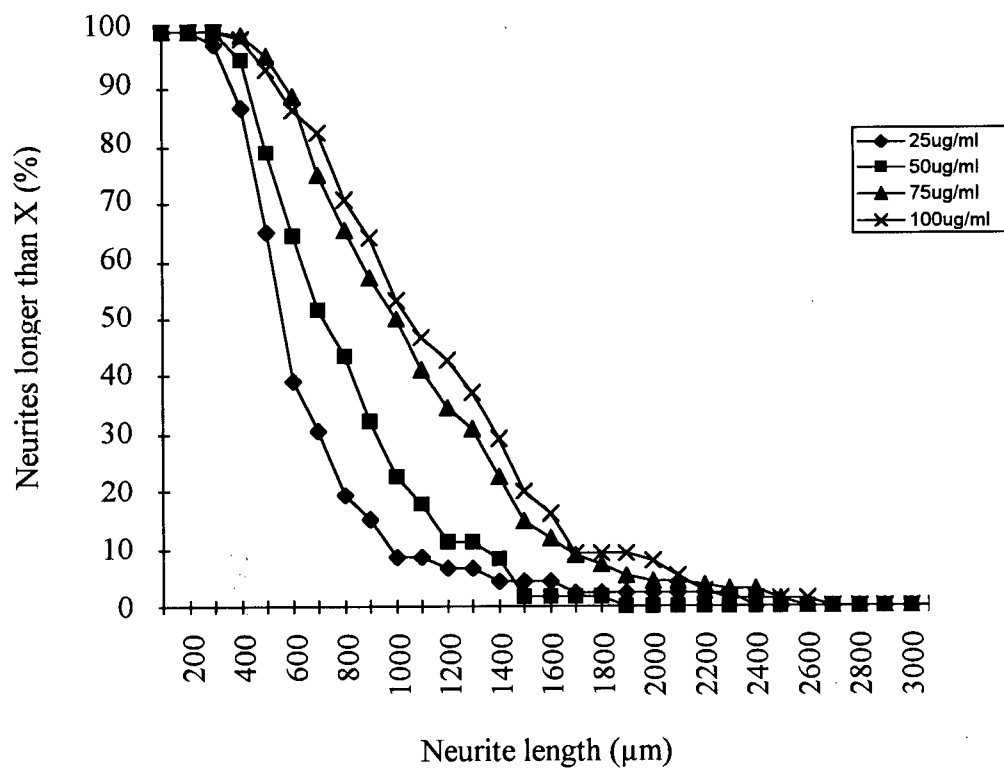


Figure 10: Effect of laminin coating concentration on neurite outgrowth. Increasing concentrations of Laminin in the solution used to coat the culture wells resulted in increased neurite length. A maximal effect was observed at a concentration of 75 $\mu\text{g/ml}$.

2.4 DISCUSSION

2.4.1 *Summary*

I developed two novel assays based on retrograde labeling of chick embryo bulbo-spinal projection neurons prior to their culture. Dissociated cell cultures prepared from brainstems labeled in this fashion allowed the study of survival effects mediated by trophic factors on a population of neurons otherwise difficult to study. Explant cultures prepared from specific bulbo-spinal nuclei produced a neurite outgrowth assay for testing growth factor-mediated neurite outgrowth.

2.4.2 *Retrograde Tract Tracing*

The success of the assay is dependent upon the quality of the pre-culture retrograde labeling, which will vary with the retrograde tracing dyes currently available. The retrograde tracing dyes previously used in our lab with avian species include True Blue, the dextran amines, horseradish peroxidase, fluorogold, and the carbocyanine dyes. To produce the optimal retrograde labeling for these experiments, each dye was tested with a standard injection technique followed by a histological evaluation of the retrograde staining. All the dyes successfully label brainstem spinal neurons from injections or implants in the cervical spinal cord of E5 chick embryos, but with varied success. Only DiI and DiO were effective at robustly labeling sufficient numbers of neurons for observation in culture post-labeling. Also, only the carbocyanine dyes (DiI and to a

lesser extent DiO) labeled neurons sufficiently well to maintain readily visible staining levels in the cells after the dissociation, plating and subsequent differentiation *in vitro*.

The DiI label procedure was well tolerated by the animals, with a survival rate in excess of 90% after 3 days (E5-E8). For experiments using longer survival times, most animals surviving the initial 48 hours post-labeling survived until E17, after which few animals survived and even fewer hatched. It was noted that after E17, the few remaining animals had difficulty maintaining water balance in the egg, and re-absorbing extra-embryonic fluid prior to hatching. This was likely a result of the disruption of extra-embryonic membranes necessary for the dye implantation procedure.

The distribution of neurons in the brainstem labeled after this procedure corresponded well with previously published data (Cabot et al, 1982). Spinally projecting neurons were found throughout the reticular formation, a nearly continuous column of cells that began in the caudal medulla and extended rostrally the length of the hindbrain. Labeled neurons were also observed within the medullary Raphe nucleus, but not within pontine Raphe nuclei. The pontine Raphe are known to project to rostral CNS targets, and therefore would not have the opportunity to pick up the retrograde label from the spinal cord (Brodal et al, 1960). The vestibular complex also contained labeled neurons in the Vestibularis Descendens (VeD), Vestibularis medialis (VeM) and Vestibularis lateralis (VeL). These three subdivisions of the vestibular complex are known to extend axons into the spinal cord via the lateral lemniscus spinalis or the medial longitudinal fasciculus (MLF; Glover, 1993). In the pons, labeled neurons were appropriately distributed within the reticular formation, but with fewer numbers in the Locus Coeruleus / subcoeruleus. Thus, the labeled neurons observed in the dissociated

cultures derived from a number of sources within the brainstem, but all shared the characteristic of a spinally projecting axon.

2.4.3 *Immunocytochemical Characterization in vitro.*

The immunocytochemical characterization revealed numerous non-neuronal cells in the assay, as revealed by vimentin immunoreactivity. The vimentin immunoreactivity represents a number of non-neuronal phenotypes including predifferentiated glioblasts, fibroblasts, and epithelial cells (Tapscott et al, 1981). It was estimated that as many as one half to two thirds of the cells were non-neuronal. The majority of these vimentin stained cells likely represented immature glia (Dahl, 1981; Dahl et al, 1981), as longer term cultures contained a large percentage of GFAP-positive astrocytes (data not shown). The presence of a significant number of non-neuronal cells may confound the interpretation of the results of trophic experiments, by providing an indirect route for the growth factor action. Thus, the applied trophic factor may stimulate non-neuronal cells to secrete an appropriate trophic factor for the survival or neurite outgrowth of the labeled neurons. Consideration of this mechanism will be given in greater detail in Chapter 5.

Immunocytochemical staining for the glial intermediate filament GFAP was performed to identify astrocytes in the cultures. Cells stained by the GFAP antibody were rarely observed after 48 hours *in vitro*. *In vivo*, astrocytes begin differentiating and expressing GFAP on E10-11 (Bignami and Dahl, 1975), and similarly GFAP staining does not appear consistently until approximately 3 days post-plating (dissociation and plating initiated on E8, data not shown).

The total hindbrain neuronal population can also be studied, using an antibody to a neuron-specific protein, MAP1b (Sato-Yoshitake et al, 1989). Comparisons between the neuron-specific MAP1b staining, and cellular morphology revealed by Phase Contrast microscopy, were used to train for the rapid and positive identification of neurons simply by assessing the morphology under Phase Contrast optics. The use of Phase Contrast optics to routinely identify neurons (rather than MAP1b antibody staining) was necessary because the MAP1b staining procedure included exposure of the cells to detergents in order to facilitate intracellular access of the antibody. DiI is very soluble in detergents, so exposure to detergents effectively removed the DiI staining. The training in identification of neurons using phase optics proved especially important, as there was a small but significant population of non-neuronal cells labeled with DiI. These non-neuronal cells likely became labeled via the phagocytosis of DiI labeled neuronal debris that resulted from the dissociation procedure. Thus, the cultures were scanned for DiI-labeled cells and the neuronal identity confirmed with Phase Contrast optics. The health of the DiI labeled neurons was then assessed with Hoechst 33258 staining, which revealed nuclear morphology, so that apoptotic cells were excluded from the surviving neuron counts.

2.4.4 Dissociated Cell Survival Assay

For this preliminary characterization of the technique, cultures were grown in DMEM supplemented with 10%FCS, a universally acknowledged promoter of cell survival with undefined trophic components (Goslin and Banker, 1991). The survival

under this treatment condition was compared with serum free DMEM (negative control) using Student's t-test to examine the significance of the difference in mean survival (expressed as a percentage of the control well). I found that 10% FCS treatment increased survival to 154% of serum free control levels. This is a well-known effect of FCS, which reportedly contains many undefined promoters of neuronal survival (Goslin and Banker, 1991). Furthermore, these data validate the use of 10% FCS as a positive control for future experiments with defined trophic medium (see Chapter 3, 4, 5)

2.4.5 Neurite Outgrowth Assay

The initial characterization of the neurite outgrowth assay aimed to determine an optimal concentration of laminin for coating the culture wells. The objective was to provide a permissive substrate, avoiding the study of neurotrophic effects upon overcoming barriers to growth presented by the plastic culture dish surface. Several concentrations of laminin were tested in the 2hr coating protocol, and 75mg/ml was found to provide maximal outgrowth. This concentration of laminin will be used in future experiments examining the effect of growth factors on bulbospinal neurite outgrowth (see Chapter 3, 4).

2.4.6 Conclusions

Although the classical approach to neuron culture involves the use of less mature tissue to decrease the proportion of non-neuronal cells, this is not possible due to the requirement for retrograde labeling from the spinal cord prior to the culturing. Thus, the bulbospinal neurons described here are (for the most part) already involved with final target cell selection in the spinal cord at the time of culture. Due to the more mature nature of the source tissue, the cultures contain a higher proportion of non-neuronal cells. This may impact significantly upon the results seen with trophic factor treatment due to the possibility of indirect effects mediated by the non-neuronal cells. Perhaps this environment will provide an even better test for neurotrophic effects, more similar to the intact brain environment where CNS repair strategies will be deployed.

- CHAPTER 3 - NEUROTROPHIN EFFECTS ON BULBOSPINAL NEURONS *IN VITRO*

3.1 INTRODUCTION

The previous chapter described a novel technique for studying the effect of trophic factors on bulbospinal neurons from the whole hindbrain. To specifically identify these neurons within mixed cell cultures from the hindbrain, this technique takes advantage of one attribute these neurons share: the extension of an axon into the spinal cord.

The axonal extension event during development provides a pathway to specifically label the cell bodies of spinally projecting hindbrain neurons, using a retrograde tracing dye applied to the developing spinal cord. As the axons are extending past the dye implantation site, the lipophilic dye (DiI) dissolves into the cell membranes and labels the cell body in the brainstem in a retrograde fashion. By preparing dissociated or explant cultures from labeled brainstems, survival or neurite outgrowth assays (respectively) containing labeled bulbospinal neurons can be prepared by focusing specifically upon bulbospinal neurons marked by the presence of DiI; other cells and neurons can be ignored.

Using these assays, I showed that the application of a trophic source (FCS) to dissociated cell cultures increased the survival of bulbospinal neurons. At the same time, this validated the use of FCS as a positive control for future experiments with defined trophic factors. Furthermore, the optimal concentration of laminin was determined to allow neurite outgrowth from explants derived from specific nuclei in the brainstem.

Thus I have developed the tools to study the effects of trophic factors upon an anatomically and functionally distinct class of neurons in the brainstem.

There exists a large repertoire of growth factors in the developing and adult vertebrate CNS. An evolving hypothesis is that different neuronal populations respond in distinct ways to various growth factors, depending upon determinants both intrinsic to the neuron as well as extrinsic (environmental) factors. With the techniques described here, this hypothesis can now be explored directly for a class of neuron important for motor control.

There are currently well over 50 known growth factors, which often exist as members of related families of molecules. In this and subsequent chapters, I will examine the effects of neurally expressed members from two families of trophic factors: the Fibroblast Growth Factors (FGFs) and neurotrophins. This chapter will examine the effects of neurotrophins, including Nerve Growth Factor (NGF), Brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), on survival and neurite outgrowth of bulbospinal neurons. The neurotrophins bind to a family of receptor tyrosine kinases that includes tropomyosin receptor kinase (trk)A, trkB and trkC (for a review, see Ip and Yancopoulos, 1996). The trkA receptor binds NGF with high affinity, trkB binds BDNF and NT-4, and the trkC receptor binds NT-3. There also exists a lower affinity receptor, p75, that binds all neurotrophins with similar affinity.

All members of the neurotrophins and their receptors are expressed in discrete patterns within the brainstem. Studies of trk receptor expression in the brainstem of mammalian species revealed that all three trk receptors appeared in regions containing

bulbospinal neurons (Gibbs and Pfaff, 1994; Holtzman et al, 1995; Lamballe et al, 1994; Ringstedt et al, 1993; Snyder et al, 1997). For example, *trkA* is expressed in presumptive neurons of the n. Raphe Obscurus, *Rpc* and *Rgc*, and scattered within the VeL (Gibbs and Pfaff, 1994).

Receptor expression in the appropriate region of the brainstem does not automatically mean that bulbospinal neurons express the receptors. A recent paper combining retrograde tracing and *in situ* hybridization for *trk* receptor expression in rat bulbospinal neurons revealed that very few actually expressed *trkA*, although *trkA* expression was observed in regions of the brainstem containing bulbospinal neurons (King et al, 1999). *trkB* and *trkC* expression was more widespread, with 90% and 84% (respectively) of bulbospinal neurons expressing these receptors (King et al, 1999). Rubrospinal neurons are known to express *trkB* and respond to exogenously applied BDNF after spinal cord injury (Kobayashi et al, 1997). However, the rubrospinal projection is not well labeled following the retrograde tracing paradigm outlined here, and therefore was excluded from these experiments.

The hypothesis to be tested is that:

Exposure to the appropriate neurotrophin *in vitro* will enhance the survival and/or neurite outgrowth of bulbospinal neurons.

Whole brainstem dissociated cell cultures (neuronal survival), as well as the vestibulospinal dissociated (neuronal survival) and explant cultures (neurite outgrowth), combined with a pontine reticular explant culture (neurite outgrowth) were used for these experiments.

3.2 METHODS

Bulbospinal neurons were labeled with DiI *in ovo* as described in Chapter 2. The dissociation, plating and culture procedures were as described in Chapter 2. Preparation of explant cultures was completed as described in Chapter 2. Fixation and quantification of neuron survival and neurite outgrowth was performed as described in Chapter 2, any deviations or additions to the procedure are described below. The neurotrophins were recombinant human, generously provided by Amgen Inc (Thousand Oaks, CA). or purchased from R&D Systems Inc. (Minneapolis, MN)

3.2.1 Dissociated bulbospinal neuron survival assay

Cultures prepared from the whole hindbrain were maintained for 48 hours in the presence of NGF, BDNF, NT-3 or NT-4, at a concentration of 5, 25 or 75ng/ml. The mean number of bulbospinal neurons that survived in the presence of the neurotrophins was expressed as a percent of the mean neuronal survival in wells containing serum free control medium. Statistical significance was tested using Student's t-test (when only two groups were compared) or analysis of variance (ANOVA) in multi-treatment experiments.

3.2.2 Dissociated vestibulospinal neuron survival assay

The vestibular complex was microdissected from the hindbrain of 8-10 DiI labeled brainstems using a razor blade. The vestibular complex, situated dorsolaterally in

the hindbrain, was easily isolated from the more medial and ventrally located reticular formation and other bulbospinal populations. The presence of DiI labeling also guided the microdissection, as fresh dissected hindbrains were rapidly screened at low power using the epifluorescence microscope to visualize the exact location of the vestibulospinal neurons (Figure 11A). A coronal cut was made at the rostral and caudal borders of the vestibular nucleus and the resulting slice laid flat. Tissue containing the vestibular neurons was isolated from the reticular formation using a diagonal cut just medial to the location of the labeled vestibulospinal neurons (Figure 11A). Harvesting and pooling of the tissue from 8-10 animals was then followed by the dissociated cell culture procedure outlined in Chapter 2 for the whole hindbrain dissociated cell survival assay. Survival in serum-free DMEM was compared with NGF, BDNF, NT-3 and NT-4 treated cultures (1ng/ml, 10ng/ml and 100ng/ml) by counting the number of DiI-labeled surviving bulbospinal neurons as described above and in Chapter 2.

3.2.3 *Vesibulospinal Neurite Outgrowth Assay*

DiI-labeled hindbrains were prepared, harvested and sliced as described in Chapter 2. Candidate slices were selected from the pool of hindbrain slices and rapidly screened under the epifluorescence microscope for the presence of labeled vestibulospinal neurons. The vestibular complex was cut off the slice as shown in Figure 11A, then plated as described in Chapter 2. Vestibulospinal explants were exposed to control serum free medium, or serum free medium containing one of NGF, BDNF, NT-3

or NT-4 at concentrations of 5ng/ml, 25ng/ml, 50ng/ml or 75ng/ml. Quantification of neurite outgrowth was as described in Chapter 2.

3.2.4 Reticulospinal Neurite Outgrowth Assay

Animals with DiI labeled reticulospinal neurons were prepared and the hindbrains harvested and sliced as described in Chapter 2. Several candidate slices from each hindbrain were selected from the region thought to contain the RPpc/RPgc reticulospinal neurons. Using the epifluorescence microscope, the slices were rapidly screened for the presence of reticulospinal neurons at low power (5x objective). Slices that contained reticulospinal neurons were set aside for micro-dissection. Using a dissecting microscope, each slice was cut just lateral to each group of the reticulospinal neurons, and a third cut made at the midline (Figure 11B). These cuts separated the reticulospinal neurons from surrounding bulbospinal neurons. Note that the Raphe neurons in this region (and more rostral regions) of the brainstem do not project to the spinal cord, and were unlabeled. These explants were collected and plated as described for the vestibular complex explants, in wells pre-coated for 2 hours with 75 μ g/ml laminin. A single experiment consisted of at least 4 replicate wells per treatment, and was repeated at least twice. Experimental media consisted of serum free control DMEM, or DMEM supplemented with 5ng/ml, 25ng/ml, 50ng/ml or 75ng/ml of each neurotrophin. The cultures were grown for approximately another 72hr, then fixed with 10% formalin in 0.1M PBS plus 10% sucrose, which preserved growth cone structure better than 4%

freshly hydrolyzed paraformaldehyde. Quantification of neurite outgrowth was as described for the vestibulospinal explants in Chapter 2.

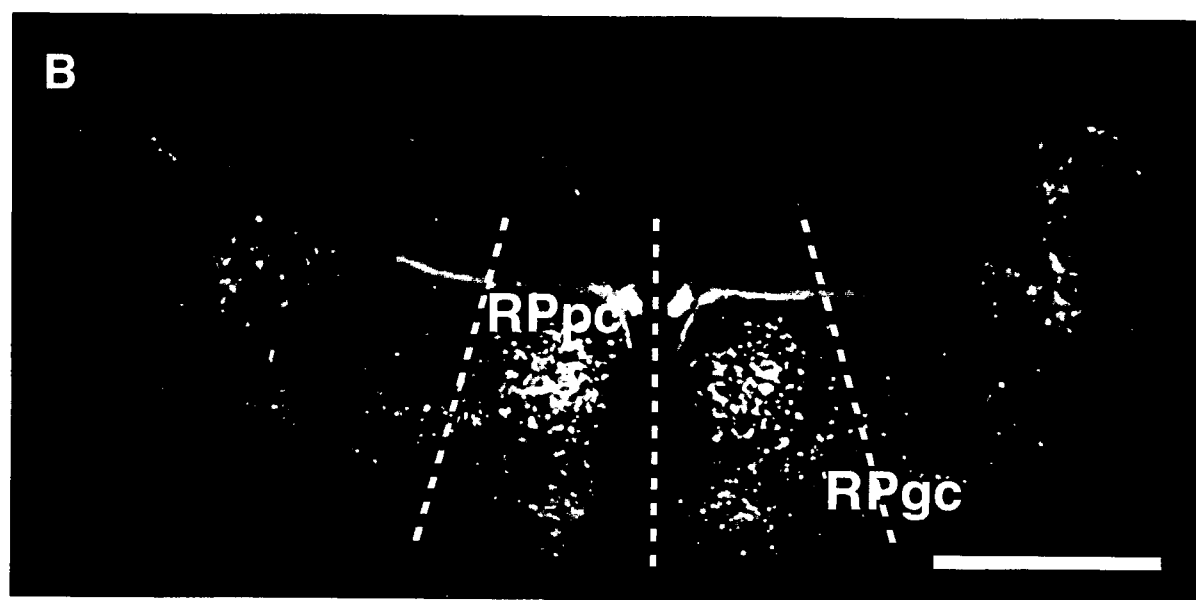
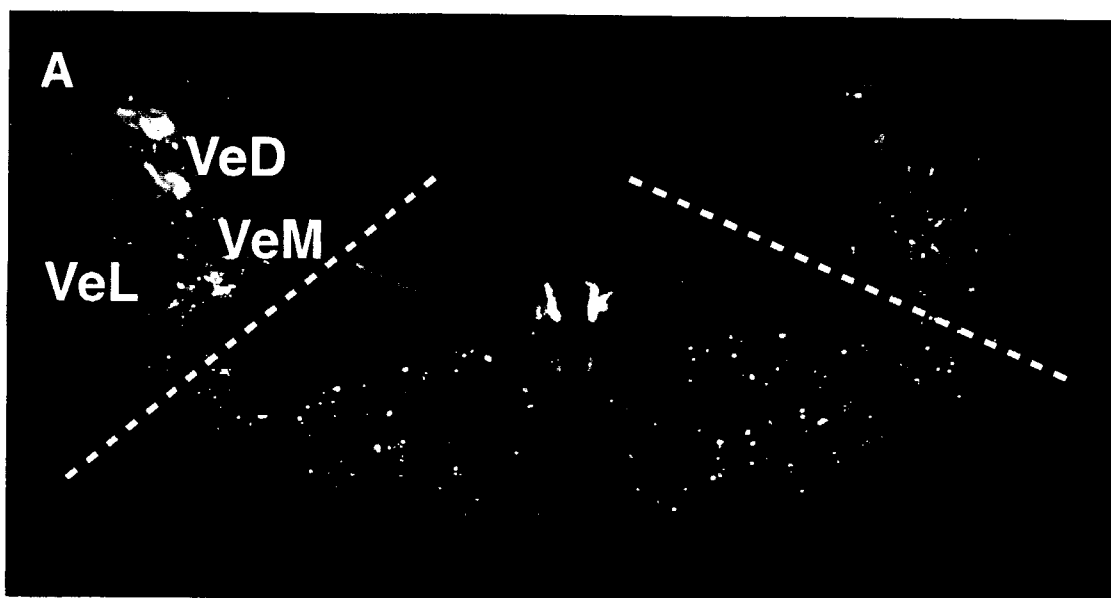
3.2.5 DRG explant cultures

As a positive control experiment, neurotrophin efficacy was determined using a dorsal root ganglion (DRG) neurite outgrowth assay. E9 chick embryos were removed from their shell and immediately decapitated. Spinal columns were dissected out in PBS, then placed in HBSS. Sacral vertebrae were removed and the lumbar spinal cord exposed by removing the ventral surface of the lumbar and several lower thoracic vertebrae. The lower thoracic spinal cord was cut, and the distal end of the cord lifted slowly to remove the lumbar spinal cord from the vertebral column with DRG attached. DRG were harvested from the L1-L6 spinal levels and placed individually in wells of 48 well culture plates pre-coated for 2 hours with 75µg/ml laminin. The laminin coating solution was rinsed out with 0.5ml HBSS and replaced with the culture medium prior to plating the explants. The test media consisted of Neurobasal (Canadian Life Technologies) containing N2 supplements (Canadian Life Technologies) and 500U penicillin/streptomycin, with either no growth factor (serum free control medium) or NGF, BDNF NT-3 or NT-4 at a concentration of 25ng/ml or 100ng/ml.

The DRG were grown for 24hours at 37°C in a humidified 95% air 5% CO₂ atmosphere, with at least 4 DRG in each medium test group. Fixation was performed using 4% paraformaldehyde in PBS overnight in case immunostaining was necessary to better observe the neurites. After 3 PBS rinses, phase contrast photomicrographs were

obtained to qualitatively compare the neurite outgrowth observed from explants in the serum free control wells with the neurotrophin-treated explants.

Figure 11: Micro-dissection of specific bulbospinal populations. A freshly dissected 300 μ m thick slice with DiI labeled vestibular complex neurons (A) was cut along the dashed lines. This produced tissue explants dissociated for the vestibular complex survival assay or simply plated for the neurite outgrowth assay. VeD, n. Vestibulars Descendens; VeL, n. Vestibularis Lateralis; VeM, n. Vestibularis Medialis. A similarly labeled freshly dissected 300 μ m slice with reticular formation neurons (B) was cut where indicated (dashed lines). This produced tissue explants for the pontine reticular neurite outgrowth assay. Scale bar 500 μ m (B)



3.3 RESULTS

3.3.1 *Bulbospinal Neuron Survival*

Using dissociated cultures derived from the pons and medulla, effects upon the survival of DiI-labeled bulbospinal neurons were quantified after exposure to the neurotrophins in concentrations ranging from 10ng/ml to 100ng/ml. NGF did not detectably change bulbospinal neuron survival at any of the concentrations tested, nor did BDNF, NT-3, or NT-4 (Figure 12) after 2 days *in vitro* (DIV). However, all the neurotrophins promoted neurite outgrowth from DRG test cultures, confirming the bioactivity of the human neurotrophins on chick neural tissue (data not shown). Furthermore, concurrent experiments with fibroblast growth factors (FGFs) revealed that the bulbospinal neuronal death occurring during the first 2 DIV was present and could be reversed (see Chapter 4). This suggests that the laminin substrate and culture medium conditions used for the assay did not already maximally stimulate bulbospinal neuron survival.

3.3.2 *Vestibulospinal Neuron Survival*

Effects upon the survival of vestibulospinal neurons were quantified using dissociated cultures derived from the dorsolateral region of the ponto-medullary junction, containing only labeled vestibulospinal neurons amongst the unlabeled cells in that

region. NGF did not promote vestibulospinal neuron survival at any of the concentrations tested after 2 DIV, nor did BDNF, NT-3, or NT-4 (Figure 13).

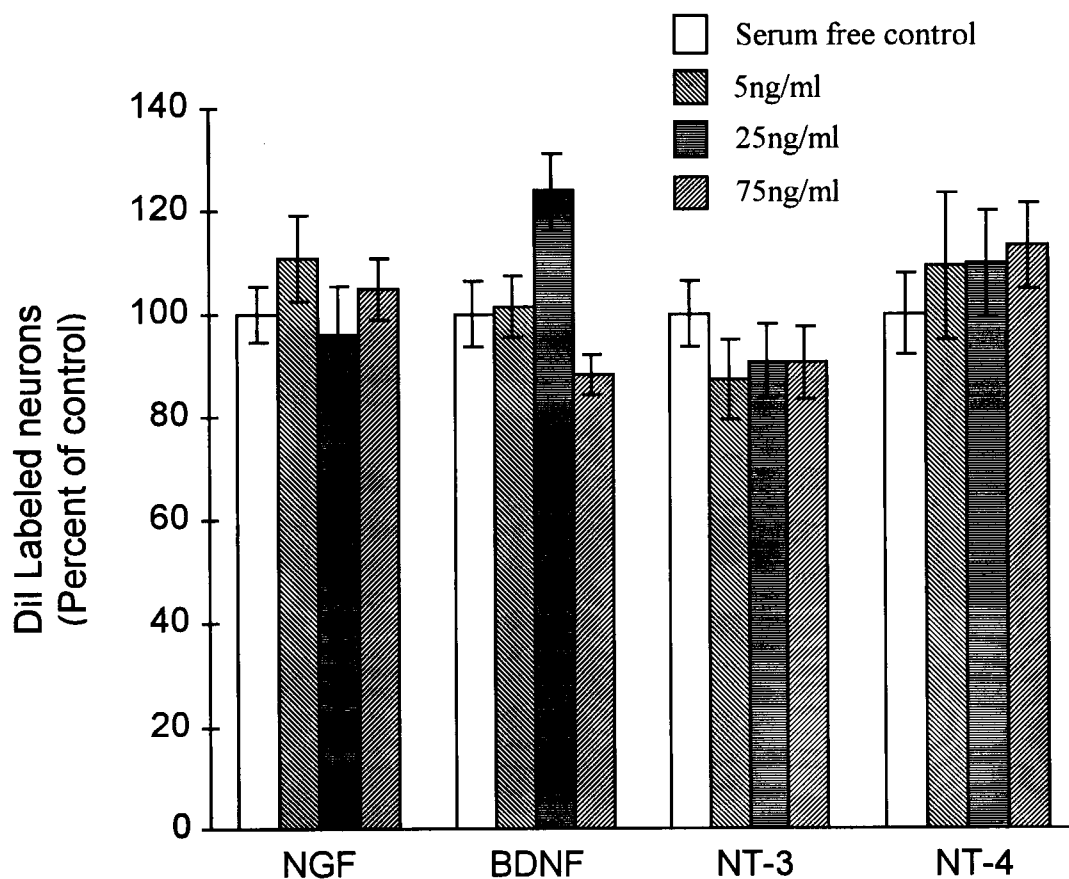


Figure 12: The neurotrophins did not promote bulbospinal neuron survival. Hindbrain dissociates were plated and grown in the presence of NGF, BDNF, NT-3 or NT-4. At the concentrations tested (5ng/ml, 25ng/ml, 75ng/ml), bulbospinal neuron survival was unaltered after 2 DIV.

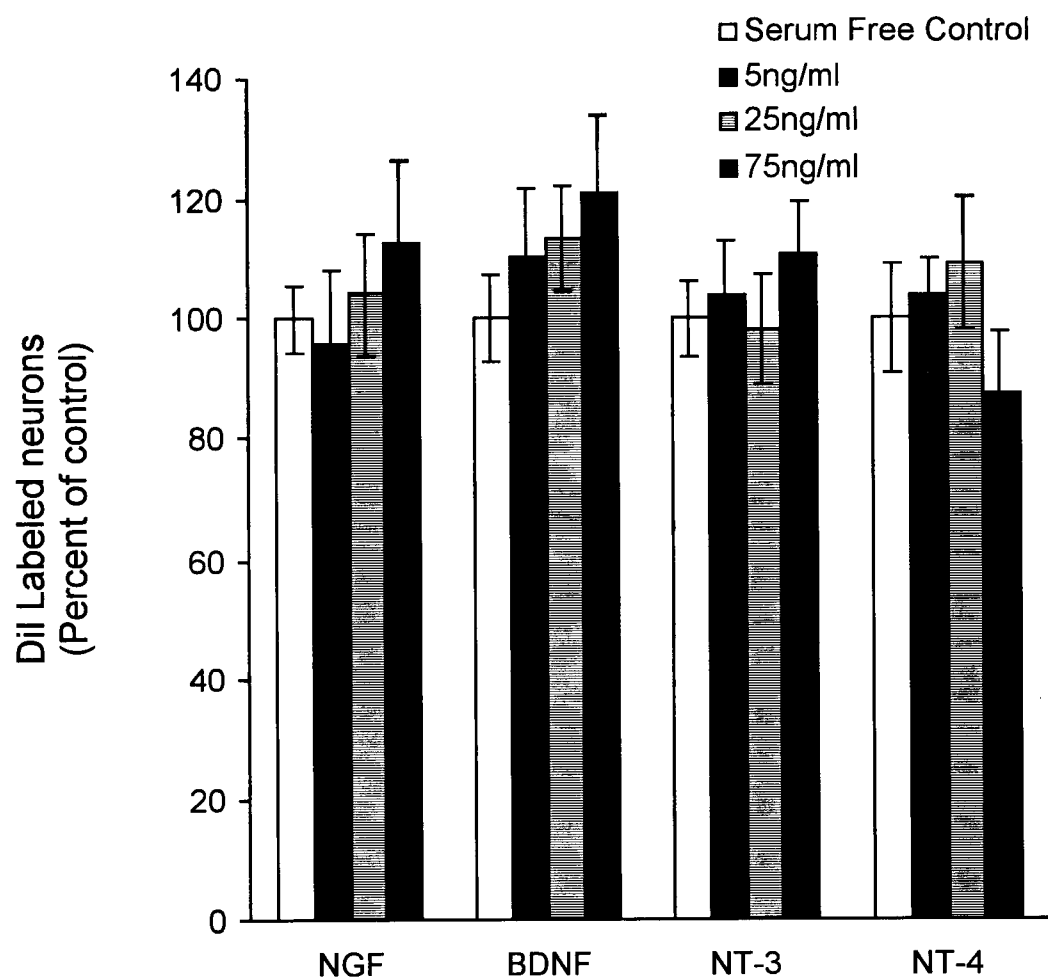


Figure 13: Neurotrophins did not stimulate vestibulospinal neuron survival. Vestibulospinal neurons were plated and grown in the presence of NGF, BDNF, NT-3 or NT-4. After 2 DIV, vestibulospinal neuron survival was not increased at any of the concentrations tested.

3.3.3 Vestibulospinal Neurite Outgrowth

Neurotrophin effects upon neurite outgrowth from vestibulospinal neurons were quantified using explant cultures derived from the dorsolateral region of 300 μ m coronal slices from the ponto-medullary junction in the hindbrain. In a typical experiment, neurite outgrowth from NGF and NT-3 treated explants was compared with serum free control treated explants. No significant difference in neurite length was observed between the NGF or NT-3 treated explants and control explants (Table 2, 50ng/ml for both). In a separate set of experiments, neurite outgrowth from BDNF and NT-4 treated explants was compared to serum free control medium treated explants (Table 3, 50ng/ml for both). Neither BDNF nor NT-4 promoted vestibulospinal neurite outgrowth compared to serum free control treated explants. Additional concentrations (5, 25 and 75ng/ml) were tested for all the neurotrophins with similar results in this assay (data not shown).

Treatment	Mean neurite length \pm Std. error (μ m)	Number of neurites
Serum Free Control	815 \pm 31	127
NGF (50ng/ml)	814 \pm 28	158
NT-3 (50ng/ml)	832 \pm 23	172

Table 2: Neither NGF nor NT-3 promoted vestibulospinal neurite outgrowth. Four explants were used in each treatment group and the neurite length measurements pooled. No significant differences in neurite length were observed between serum free control and NGF or NT-3 treated explants (based on ANOVA).

Treatment	Mean neurite length ± Std. error (μm)	Number of neurites
Serum Free Control	884 ± 28	131
BDNF (50ng/ml)	943 ± 25	180
NT-4 (50ng/ml)	961 ± 34	178

Table 3: BDNF and NT-4 were ineffective at promoting vestibulospinal neurite outgrowth. Neurite length did not significantly change with BDNF or NT-4 treatment (based on ANOVA). In this experiment, the neurite lengths measured from 4 explants per treatment were pooled.

3.3.3 Pontine Reticular Formation Neurite Outgrowth

Effects upon neurite outgrowth of RPpc/gc neurons were quantified using explant cultures derived from the caudal pole of the reticular formation in the pons. NGF did not promote reticulospinal neurite outgrowth at a concentration of 50ng/ml (Table 4), nor did BDNF or NT-4 (Table 5). Additional concentrations of NGF, BDNF and NT-4 were tested with similar results (data not shown). However, in a separate experiment NT-3 did stimulate increased neurite outgrowth from pontine reticular neurons. In a typical experiment, the mean length of NT-3 (50ng/ml) treated neurites was 37% greater than that seen in control serum free medium-treated cultures (NT-3 treated mean neurite length=1049 ± 32μm, control mean neurite length=768 ± 19μm, Figure 14)

Treatment	Mean neurite length ± Std. error (μm)	Number of neurites
Serum Free Control	1326 ± 52	54
NGF (50ng/ml)	1394 ± 36	116

Table 4: NGF did not promote reticulospinal neurite outgrowth. In a typical experiment, pooled neurite length measurements from 3 control and 4 NGF treated reticulospinal explants showed no significant difference in neurite length.

Treatment	Mean neurite length ± Std. error (μm)	Number of neurites
Serum Free Control	1350 ± 69	159
BDNF (50ng/ml)	1240 ± 91	142
NT-4 (50ng/ml)	1390 ± 49	134

Table 5: BDNF and NT-4 did not promote reticulospinal neurite outgrowth. Pooled neurite length measurements from 4 explants per treatment group revealed that no significant changes in neurite length were observed with BDNF or NT-4 treatment.

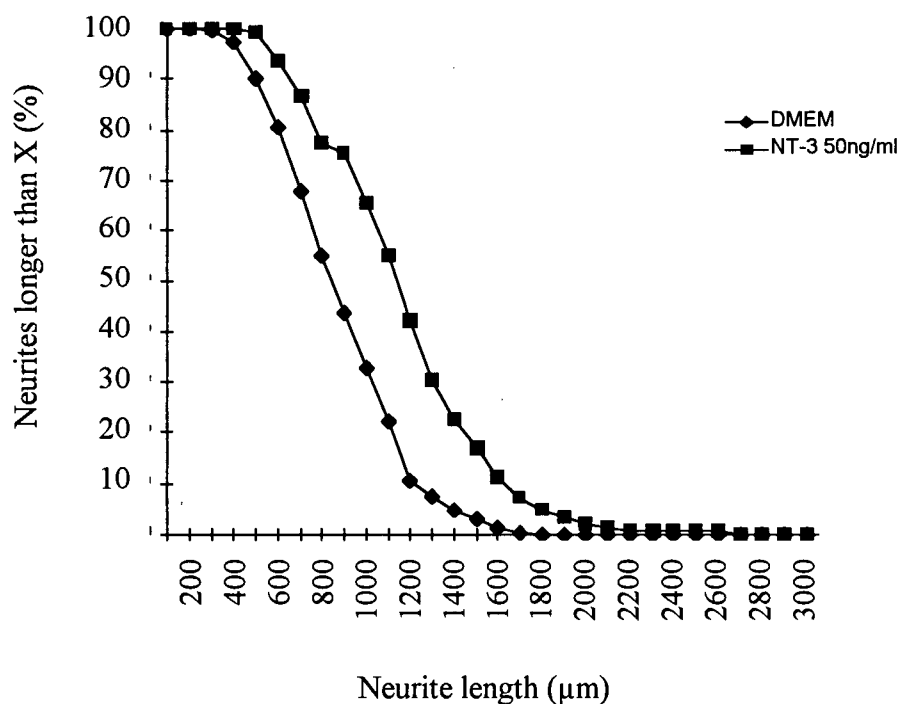


Figure 14: NT-3 increased reticulospinal neurite length. Reticulospinal explant cultures treated with 50ng/ml NT-3 (mean neurite length = $1049 \pm 32\mu\text{m}$) demonstrated a shift in the distribution of neurite lengths towards longer neurites compared with serum free control medium (DMEM) treated explants (mean neurite length = $768 \pm 19\mu\text{m}$).

3.4 DISCUSSION

3.4.1 *Summary*

In this chapter, I applied growth factors from the neurotrophin family of growth factors to address the hypothesis that appropriate trophic support will increase survival and or neurite outgrowth for bulbospinal neurons. The neurotrophins were largely ineffective at promoting survival of bulbospinal neurons derived from the pons and medulla under the conditions tested herein, including a more specific assay containing only vestibulospinal neurons. Neurite outgrowth on a laminin substrate was promoted by NT-3, however only for RPpc/gc reticular neurons. NGF, BDNF and NT-4 were ineffective at stimulating neurite outgrowth for pontine reticular or vestibular nucleus explants.

The presence of extensive sequence homologies between mammalian and chicken neurotrophin polypeptides suggested that the human growth factors would be effective in an assay utilizing chick cells. For example, the amino acid sequences of human (Rosenthal et al, 1991; Yamamoto and Gurney, 1990), rat (Maisonpierre et al, 1991) and pig (Leibrock et al, 1989) BDNF are identical, and chick BDNF differs by only 7 amino acids (Isackson et al, 1991). Furthermore, mammalian BDNF is an effective neurotrophic agent for several chick neuron populations (Lindsay et al, 1985; Hofer and Barde, 1988). The effectiveness of the human neurotrophins was confirmed by the increased neurite outgrowth from chick DRG (data not shown). Thus the lack of neurotrophin responsiveness of bulbospinal neurons in the survival assays was not a result of species differences between chick and human neurotrophin polypeptides. It is

more likely that the responsiveness of the neuronal populations is defined by trk receptor expression. All trk receptors are known to be expressed by specific populations of DRG, but it is not known whether embryonic bulbospinal neurons express detectable levels of trk receptor.

3.4.2 Neurotrophin effects on bulbospinal neuron survival

Studies of the expression of trk receptors in the brainstem of mammalian species revealed that all three high affinity trk receptors were present in almost all regions containing bulbospinal neurons (Gibbs and Pfaff, 1994; Holtzman et al, 1995; Lamballe et al, 1994; Ringstedt et al, 1993; Snyder et al, 1997). For example, trkA is expressed in presumptive neurons of the n. raphe obscurus, gigantocellular and paragigantocellular medullary reticular formation, and scattered within the lateral vestibular nucleus (Gibbs and Pfaff, 1994). However, a recent paper describing the distribution of trk receptor expression combined with retrograde tracing from the spinal cord revealed that the trkA-expressing neurons did not project to the spinal cord (King et al, 1999). Thus, the ineffectiveness of NGF could be explained by the lack of trkA receptor expression in bulbospinal neurons. However, the possibility exists that the distribution of trkA receptors in the avian brainstem is different from the mammalian brainstem, and overlaps with the bulbospinal neuron population. Similarly, the developmental expression of trkA receptors may be different than the pattern observed in adult animals. To answer this question, *in situ* hybridization experiments combined with retrograde tracing should be repeated in the developing and adult chick.

BDNF, NT-3 and NT-4 were also ineffective in promoting bulbospinal neuron survival. The distribution of *trkB* and *trkC* mRNA in the adult mammalian hindbrain overlaps with 90% and 84% (respectively) of the bulbospinal population (King et al, 1999), thus most bulbospinal neurons should be responsive to these three neurotrophins. There are at least three possible explanations to reconcile this inconsistency. First, perhaps bulbospinal neurons are responsive to BDNF, NT-3 and NT-4, but these neurotrophins affect some property other than neuronal survival. Second, it is possible that the early developing bulbospinal neurons used in the present experiments had not yet begun to express neurotrophin receptors. Further experiments with *in situ* hybridization for *trk* receptors at early stages of development are clearly required. Thirdly, subtle differences may exist between avian and mammalian *trk* receptor distributions.

One example of a species difference described in the literature involves the neurons of the Locus Coeruleus (LoC). It is known that neurons in the mammalian LoC respond with increased survival after treatment with BDNF, NT3 and NT-4 but not NGF (Friedman et al, 1993; Sklair-Tavron and Nestler, 1995). In contrast, the avian LoC does respond to NGF with increased survival (von Bartheld et al, 1995). However, caution must be exercised in the interpretation of these experiments as the LoC contains a variety of neuronal populations with terminal fields throughout the neuraxis (Parent, 1996). As well, differences in the methodologies between the two studies could generate conflicting conclusions based on false negative findings. NGF effects on neuronal survival were not observed in the bulbospinal survival assay reported here, however the LoC was not well labeled by the DiI implantation procedure. It is likely that the small number of labeled LoC neurons present in the cultures were not sufficient to produce a measurable change

in bulbospinal neuron survival. The survival assay prepared from whole hindbrain may be most useful for discovering agents with a broad spectrum of activity across the majority of bulbospinal neuron populations. For survival of fetal chick neurons, the data presented here suggested that the neurotrophins do not fall into this category. However, it is possible that there are small populations of neurons within the brainstem that do respond to the neurotrophins.

The populations of bulbospinal neurons found in the pons and medulla include raphespinal, vestibulospinal, coerulespinal and reticulospinal neurons, each with functionally and anatomically distinct characteristics. Thus, functional expression of trk receptors in any of these regions does not guarantee that a survival effect will be observed in dissociated cultures containing all populations of bulbospinal neurons, such as the whole hindbrain dissociated cell survival assay used here. This is due to larger populations of non-responsive neurons masking the survival response from smaller trk-expressing populations.

The criterion used in the present study, survival, may not be the most appropriate test of function for neurotrophins. One drawback of the survival assay is that effects on neurite outgrowth cannot be measured easily. It is possible to measure the axons in the most intensely labeled bulbospinal neurons, however the number of such neurons in a typical experiment is low. Furthermore, the identity (raphespinal, vestibulospinal, reticulospinal, coerulespinal) remains obscure unless the tissue was harvested from a discrete region of the brainstem (for example, the vestibular complex).

A second drawback of the survival assay is the difficulty establishing a baseline for the percentage of neurons that die during the first 2 DIV. Establishing this baseline

would allow an estimation of the numbers of neurons rescued by the growth factor treatment. As mentioned in Chapter 2, there is a small population of non-neuronal DiI-labeled cells present in all the cultures, likely a result of the phagocytosis of DiI-labeled debris. With increasing time, the number of DiI-labeled non-neuronal cells could be expected to increase, via cell division and the continued phagocytosis of debris. Thus, an accurate count of the number of DiI-labeled neurons is not possible until after their differentiation has progressed sufficiently to allow unambiguous identification, which precludes counting during the first 18 hours or more of the culture period.

These data support the conclusion that under the conditions tested in these assays, exogenously applied neurotrophins are not broad-spectrum survival factors for bulbospinal neurons. The next experiments test the hypothesis that the appropriate neurotrophin will increase neuronal survival or neurite outgrowth from a specific population of bulbospinal neurons, the vestibulospinal neurons.

3.4.3 Neurotrophin effects on vestibulospinal neuron survival, neurite outgrowth

The neurotrophins did not detectably alter vestibulospinal neuron survival or neurite outgrowth. However, the recently published description of trk receptor expression in adult mammalian bulbospinal neurons revealed that few vestibulospinal neurons express trkA, and the lowest expression of trkB and trkC occurred in the vestibular complex (King et al, 1999). If a similar expression pattern can be demonstrated for the developing chick embryo, this suggests that there are insufficient numbers of cells expressing enough neurotrophin receptor to produce a measurable

change in survival or neurite outgrowth. This supposition awaits confirmation from *in situ* hybridization experiments for *trk* expression combined with retrograde tracing in the developing and adult chicken. Alternatively, if the *trk* receptors are found in some vestibulospinal neurons, perhaps they are producing effects other than changes in survival or neurite outgrowth. It seems unlikely that the survival and neurite outgrowth for vestibulospinal neurons were already maximally stimulated by the culture conditions used here, as concurrent experiments with fibroblast growth factors revealed significant increases in both parameters (see Chapter 4). These data support the conclusion that neurotrophins are not effective for promoting vestibulospinal neuron survival or neurite outgrowth. The next experiments examined neurite outgrowth from reticulospinal explants with neurotrophin treatment.

3.4.4 Neurotrophin effects on reticulospinal neurite outgrowth

Only NT-3 (50ng/ml) detectably altered neurite outgrowth from RPpc/gc containing explant cultures, increasing the mean neurite length 37% ($p < 10^{-12}$) above serum free control treated explants in a typical experiment. BDNF, NT-3 and NT-4 did not produce a measurable change in reticulospinal neurite outgrowth. Bulbospinal neurons in the mammalian reticular formation do express *trkC* receptors (King et al, 1999), providing a potential mechanism through which NT-3 exerts its effects. Confirmation of *trkC* expression in developing chick reticulospinal neurons is required from future experiments.

Unfortunately, it was not technically possible to produce a survival assay for these reticulospinal neurons. The low density of the labeled neurons in this region of the brainstem did not provide sufficient numbers after dissociation and plating to perform the assay. The study by King and colleagues (1999) described *trkB* expression in mammalian reticulospinal neurons, but no effect of BDNF or NT4 (the cognate ligands for *trkB*) on neurite outgrowth were detectable. If the *trkB* expression pattern can be confirmed for developing chick reticulospinal neurons, it is likely that BDNF or NT4 are affecting another aspect of neuronal function.

3.4.5 General comments

The context within which the neurotrophins are presented to the cells deserves consideration. In the dissociated cell survival assays, there are numerous non-neuronal cells present. These non-neuronal cells were characterized as vimentin-positive based on immunostaining with a highly specific antibody (see Chapter 2). As previously discussed (see Discussion, Chapter 2), these cells represent differentiating glial cells as well as fibroblasts and endothelium-derived cells (Tapscott et al, 1981). One hypothesis that remains to be tested is whether the non-neuronal cells present in the assay are already secreting an appropriate level of the appropriate neurotrophin. Thus the exogenously applied neurotrophin cannot measurably increase survival due to sufficient endogenous expression and release by non-neuronal cells present in the cultures.

One particular type of non-neuronal cell, the astrocyte, is known to secrete all the neurotrophins when fully differentiated (Houlgatte et al, 1989; Oderfeld-Nowak et al,

1992; Rudge et al, 1992; Schwartz and Nishiyama, 1994; Yamakuni et al, 1987; Zafra et al, 1992). The onset of GFAP expression in astrocytes is thought to correlate with the progression to a differentiated phenotype (Dahl, 1981; Dahl et al, 1981). Recall from Chapter 2 that there are very few GFAP-positive cells within the dissociated survival assay, thus in the 48 hour assay period, the astrocytes are still differentiating. Longer-term cultures revealed a massive increase in the number of GFAP positive astrocytes by 4 days *in vitro*, which matches the developmental appearance of GFAP-positive cells seen *in vivo* on E12 (4 days after the tissue is harvested for culturing). It is unknown if differentiating (ie. non-GFAP positive) pro-astrocytes can produce and secrete neurotrophins, it would be interesting to examine the expression of mRNA and protein for neurotrophins in the developing chick brainstem.

Several studies have indicated that there was little cell death after a CNS injury for long projection neurons unless the injury was very close to the cell body of the neuron. For instance, corticospinal neurons suffered severe atrophy or death after an internal capsule lesion, which was reversed with BDNF or NT-3 treatment (Giehl and Tetzlaff, 1996). After a thoracic spinal cord injury in the developing chick embryo, little cell death was found in brainstem regions containing spinally-projecting neurons (Xu and Martin, 1990; C.B. McBride and J.D. Steeves, unpublished observations). However, identifying growth factors that produce a survival response in the dissociated hindbrain survival assay may recognize those factors that also reverse the atrophic changes that occur after adult CNS injury. The data from the neurite outgrowth assay may have greater relevance for preparing a spinal cord injury repair strategy from the viewpoint of promoting the re-extension of the axon towards its spinal targets.

3.4.6 Conclusions

In summary, for most bulbospinal neuron populations, the data presented here did not support the hypothesis that exposure to the appropriate neurotrophin *in vitro* will enhance the survival and/or neurite outgrowth of E8 chick bulbospinal neurons under the conditions tested in these assays. However, NT-3 enhanced reticulospinal neurite outgrowth, suggesting it may be a good candidate for stimulating regeneration of these neurons after spinal cord injury.

- CHAPTER 4 - FGF EFFECTS ON BULBOSPINAL NEURONS *IN VITRO*

4.1 INTRODUCTION

In the previous chapter I analyzed bulbospinal neuron responsiveness to the neurotrophins. These experiments revealed that the neurotrophins did not promote the survival of bulbospinal neurons derived from either the whole hindbrain or the vestibular nucleus. The neurotrophins also did not promote vestibulospinal neurite outgrowth, but NT-3 supported reticulospinal neurite outgrowth. In this chapter I examine the effects of the fibroblast growth factor (FGF) family upon bulbospinal neuron survival and neurite outgrowth. The widespread expression of FGFs and their receptors during development of the brainstem and spinal cord, combined with the known survival and neurite outgrowth promotion activities, provide the rationale for studying their effects upon bulbospinal neurons.

4.1.1 The Fibroblast Growth Factors

The FGFs are a family of at least 19 structurally similar heparin binding growth factors, many of which are neurally expressed (reviewed in Galzie et al, 1997). The first FGFs discovered, acidic and basic FGF (now FGF-1 and FGF-2 respectively) were identified as two structurally related mitogens with differing isoelectric points (Gospodarowicz 1974; Gospodarowicz et al, 1986; Rudland et al, 1974). Once the sequences of FGF-1 and FGF-2 were known, the application of molecular techniques

identified numerous other related members of this large growth factor family.

Sequencing studies also determined that several members of the FGF family were already known by several names in the literature, due to independent discoveries of the same molecule in different contexts. This lead to some confusion, so a standard nomenclature was adopted using a simple numbering system to identify the family members from FGF-1 to FGF-19. Table 6 lists the currently accepted nomenclature matched with the deprecated names for the FGFs.

Accepted Nomenclature	Deprecated Nomenclature
FGF-1	aFGF, HBGF-1, HBGM
FGF-2	bFGF, HBGF-2
FGF-3	<i>int-2</i>
FGF-4	<i>hst-1</i> , KGF
FGF-5	known only by accepted nomenclature
FGF-6	<i>hst-2</i>
FGF-7	K-FGF
FGF-8	AGF
FGF-9	glial activating factor or GGF
FGF-10 to FGF-19	known only by accepted nomenclature

Table 6: FGF nomenclature. The accepted and deprecated nomenclature are listed.

The FGFs and their receptors are found in essentially all tissue types, with diverse but specific distributions and functions for each. The expression and function of these molecules are controlled at many levels, from transcriptional events through translation, secretion and receptor binding.

The effects of the FGFs in the nervous system are many and varied. They influence many aspects of neural cell function including survival and neurite outgrowth from a diverse range of neuronal cell types (Sweetnam et al, 1991). They are widely expressed in the developing and mature nervous system, within the spinal cord (Kalcheim and Neufeld, 1990) and in brainstem regions containing spinally projecting neurons (Gomez-Pinilla et al, 1992). Both neurons and non-neuronal cells such as astrocytes synthesize various members of the FGF family, including FGF-1, FGF-2, FGF-5, FGF-9 (Eckenstein et al, 1991; Emoto et al, 1989; Gomez-Pinilla and Cotman, 1993; Gomez-Pinilla et al, 1992; Gospodarowicz et al, 1984; Grothe and Janet, 1995; Haub and Goldfarb, 1991; Kuzis et al 1995; Pettman et al, 1985; Tagashira et al, 1995; Woodward et al, 1992).

Despite widespread FGF expression and functions, discrete survival effects on specific populations of CNS neurons have been observed. FGF-2 increased survival of cultured dentate gyrus neurons (Lowenstein and Arsenault, 1996), ventral mesencephalic dopaminergic neurons (Mayer et al, 1993), basal forebrain cholinergic neurons both *in vitro* (Perkins and Cain, 1995) and *in vivo* after fimbria/fornix transection (Cummings et al, 1992). Increases in neurite outgrowth occurred with FGF-2 treatment of mesencephalic dopaminergic neurons (Ferrari et al, 1991) and spinal cord neurons (Peulve et al, 1994) *in vitro*. Thus FGFs produce trophic effects thought to be important for the neuronal response to injury, namely survival and neurite outgrowth.

Like the neurotrophins, high affinity tyrosine kinase receptors (FGFR) transduce FGF binding to produce cellular effects (reviewed in Green et al, 1996). There are 4 known mammalian receptors (FGFR1-FGFR4), three of which are expressed in the CNS:

FGFR1, FGFR2 and FGFR3. The fourth (FGFR4) was originally thought not to be neurally expressed, however a recent investigation detected its expression in the cerebellum (Ozawa et al, 1996). Avian species express three known tyrosine kinase FGFR, cek1 cek2 and cek3 (Pasquale and Singer, 1989; Pasquale, 1990), which take their name from the method of isolation as chicken expressed kinases. These proteins were identified from a chick mRNA expression library screened with antibodies raised against conserved sequences found in tyrosine kinase domains. cek1, cek2 and cek3 gene products are expressed in the brain, and are homologous to mammalian FGFR1, FGFR3 and FGFR2 respectively (Avivi et al, 1991; Partanen et al, 1991; Pasquale, 1990).

Two additional types of receptors for FGFs exist, the first is called the cysteine-rich FGF receptor (CFR). It was originally isolated from chick embryo extracts by its FGF binding activity (Burrus and Olwin, 1989). This is an enigmatic molecule, with a short (13 amino acid) intracellular domain, coupled by a transmembrane domain to a repeating, cysteine rich extracellular domain (Burrus and Olwin, 1989). Little is known about the function of this molecule, other than its high affinity FGF binding activity.

The second type of FGF receptor manifests as a low affinity binding to heparan sulphate proteoglycans (HSPG; Moscatelli, 1987). By virtue of the heparan moieties on these proteins, an interaction with FGFs (which contain a heparin binding site) allows or participates in the high affinity binding to the tyrosine kinase FGFR (Kan et al, 1993; Pantoliano et al, 1994). This interaction produces dimeric complexes of FGFR bound to the FGF, resulting in autophosphorylation of the tyrosine kinase receptors and activation of intracellular secondary messenger pathways (reviewed in Green et al, 1996).

The hypothesis to be tested is that:

The appropriate FGF will increase bulbo-spinal neuron survival and/or neurite outgrowth.

This hypothesis will be tested on a laminin substrate using the bulbo-spinal dissociated cell survival assay and the explant neurite outgrowth assay as in Chapter 3.

4.2 MATERIALS AND METHODS

Bulbospinal neurons were labeled with DiI *in ovo* as described in Chapter 2. The dissociation, plating and culture procedures, as well as the explantation procedure, were as described in Chapter 2, with further details provided in Chapter 3. Fixation and quantification of neuron survival and neurite outgrowth was performed as described in Chapter 2 and Chapter 3, any deviations or additions to the procedure are described below.

4.2.1 Dissociated Bulbospinal Neuron Survival Assay

Dissociated cell cultures were prepared from the hindbrain of DiI-labeled animals as described in Chapter 2. Cultures were maintained for 48 hours in the presence of FGF-1 (supplemented with 10units/ml heparin) or FGF-2, at concentrations between 0.1 and 50 ng/ml. The fixation and analysis of survival was performed as described in Chapter 2.

4.2.2 Dissociated Vestibulospinal Survival Assay

Dissociated vestibulospinal nucleus cultures were prepared from 8-10 DiI-labeled brainstems as described in Chapter 2 and Chapter 3. Survival in serum-free DMEM was compared with FGF-1 (supplemented with 10units/ml heparin), FGF-2, FGF-5 and FGF-

9 treated cultures (1ng/ml, 10ng/ml and 50ng/ml) by counting the number of DiI-labeled surviving vestibulospinal neurons as described in Chapter 3.

4.2.3 Vestibulospinal Explant Neurite Outgrowth Assay

Hindbrains were isolated and prepared for vestibulospinal explant cultures as described (see Chapter 2, 3). Experimental media consisted of DMEM containing either recombinant human FGF-1 (supplemented with 10units/ml heparin) FGF-2, FGF-5, FGF-9 (R&D Systems Inc.) at 1, 10 and 50ng/ml. A single experiment consisted of at least 4 replicate wells per treatment, and each medium condition was examined at least twice. Image capture and neurite length analysis were performed as described in Chapter 2.

4.2.4 Reticulospinal Neurite Outgrowth Assay

DiI labeled Reticulospinal explants were prepared as described in Chapter 3. Explants were exposed to serum free DMEM, or serum free DMEM supplemented with 1, 10 and 50ng/ml of recombinant human FGF-1 (supplemented with 10units/ml heparin) FGF-2, FGF-5, FGF-9 (R&D Systems Inc.). A single experiment consisted of at least 4 replicate wells per treatment, and each medium condition was examined at least twice. Quantification of neurite outgrowth was as described in Chapter 2 for the vestibulospinal explant assay.

4.3 RESULTS

4.3.1 Effects of Fibroblast Growth Factors on Bulbospinal Neuron Survival

Fibroblast Growth Factors are known to support the survival of a wide variety of neuronal phenotypes. FGF-1 and FGF-2 effects on bulbospinal neuron survival were tested in dissociated cell cultures derived from the pons and medulla, which contained DiI-labeled reticular, vestibular, raphe and locus coeruleus neurons. FGF-1 did not promote bulbospinal neuron survival at a concentration of 50ng/ml, even in the presence of heparin. In the same experiment, FGF-2 enhanced bulbospinal neuron survival $82 \pm 8\%$ above control levels, and FCS (positive control) increased survival $54 \pm 11\%$ (Figure 15). Due to the possibility that discrete effects of growth factors on specific populations of bulbospinal neurons could be masked by larger populations of unresponsive neurons, further experiments were only performed with specific bulbospinal populations as described below.

4.3.2 Vestibulospinal Neuron Survival in Dissociated Culture

The survival of DiI-labeled vestibular complex neurons was tested in the presence of several FGF family members known to be expressed in neural tissue, namely FGF-1, FGF-2, FGF-5 and FGF-9 (Eckenstein et al, 1991; Emoto et al, 1989; Gomez-Pinilla and Cotman, 1993; Gomez-Pinilla et al, 1992; Gospodarowicz et al, 1984; Grothe and Janet, 1995; Haub and Goldfarb, 1991; Kuzis et al 1995; Pettman et al, 1985; Tagashira et al, 1995; Woodward et al, 1992). FGF-1 and FGF-5 (up to 50ng/ml) were ineffective at

promoting neuronal survival, whereas FGF-2 and FGF-9 (10ng/ml) significantly enhanced vestibulospinal neuron survival by $95 \pm 16\%$ and $70 \pm 18\%$ respectively (Figure 16).

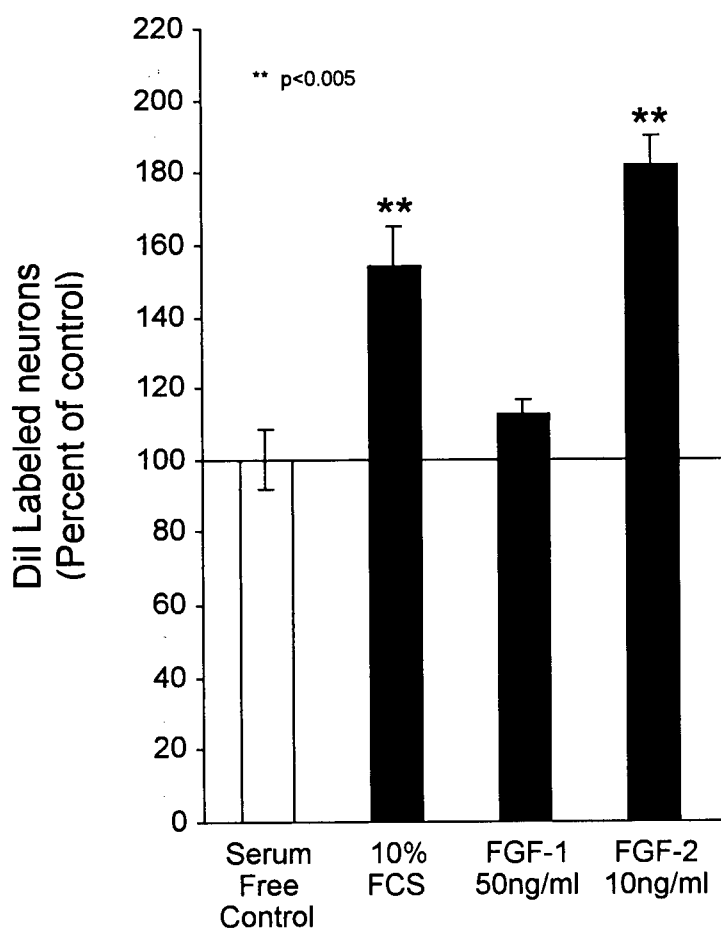


Figure 15: FGF-2 but not FGF-1 promotes bulbospinal neuron survival. The number of surviving DiI-labeled bulbospinal neurons was counted after FGF-1, FGF-2 or FCS (positive control) treatment. Compared to the number surviving in serum free control medium, FGF-2 and FCS (but not FGF-1) promoted bulbospinal neuron survival.

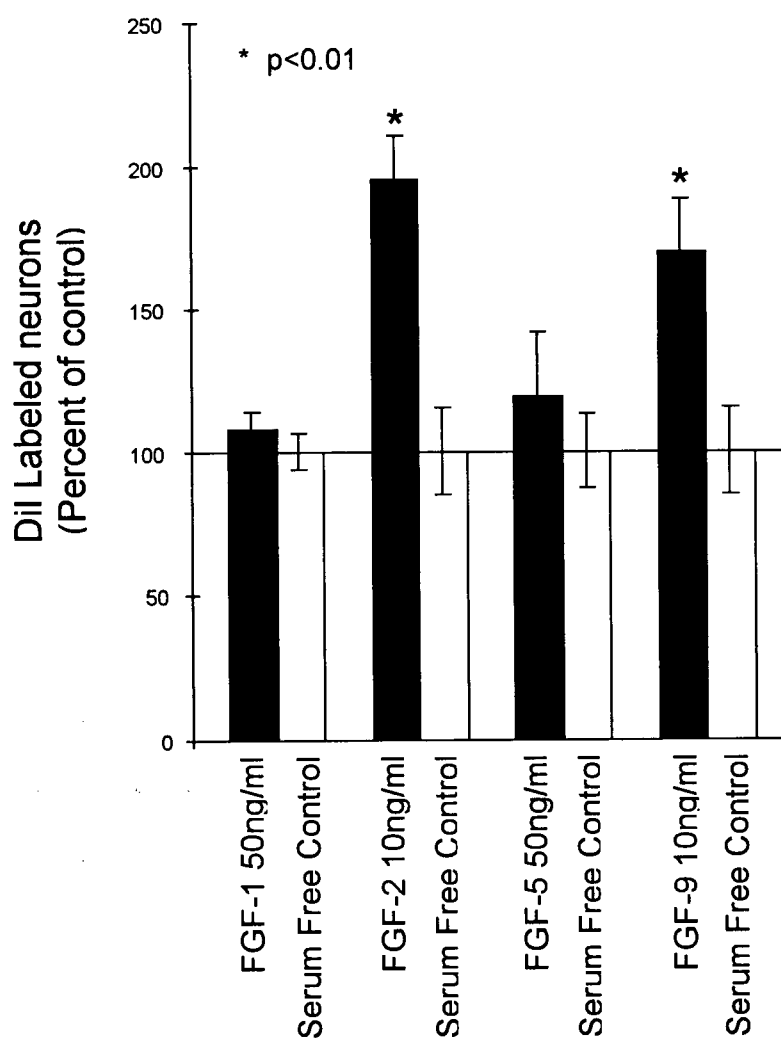


Figure 16: FGF-2 and FGF-9 promoted vestibulospinal neuron survival. In separate experiments, the effects of FGF-1, FGF-2, FGF-5 and FGF-9 on cell survival were measured. Only FGF-2 and FGF-9 enhanced vestibulospinal neuron survival ($p < 0.01$).

4.3.3 Vestibulospinal Neurite Outgrowth from Explant Cultures.

Images of the explants were captured and the neurites traced for quantification of neurite length. The longest neurites grew at an average rate exceeding $60\mu\text{m/hr}$. In a typical experiment, FGF-1 at a concentration of 10ng/ml increased ($p<0.001$) the mean neurite length to $1240 \pm 32\mu\text{m}$ ($n=187$ neurites) compared to serum free control medium ($933 \pm 42\mu\text{m}$, $n=125$), as did FGF-2 treatment (10ng/ml , $p<0.001$, mean neurite length of $1319 \pm 29\mu\text{m}$, $n=208$). Conversely, FGF-5 had only weakly positive (50 ng/ml , $p<0.05$) effects on vestibulospinal neurites, increasing mean neurite length to $1071 \pm 46\mu\text{m}$ ($n=80$) in the same experiment. This result proved inconsistent as subsequent experiments did not show increases in neurite outgrowth with FGF-5 treatment. FGF-9 did not promote vestibulospinal neurite outgrowth at any of the tested concentrations.

Since there was a wide range of neurite lengths in a typical experiment (several hundred microns to several millimeters), consideration of only the mean neurite length might have obscured effects on discrete populations of neurons. Thus, the data has been presented graphically, sorting the total population of neurites by length, and plotting the percentage of the population found within incremental $200\mu\text{m}$ length bins. These plots revealed that FGF-1 and FGF-2 stimulated a shift within the whole vestibulospinal population towards production of longer neurites, while FGF-5 may have a small effect on a slower-growing population (Figure 17). FGF-9 did not alter the distribution remarkably, nor was the mean significantly different than the serum free control (data not shown).

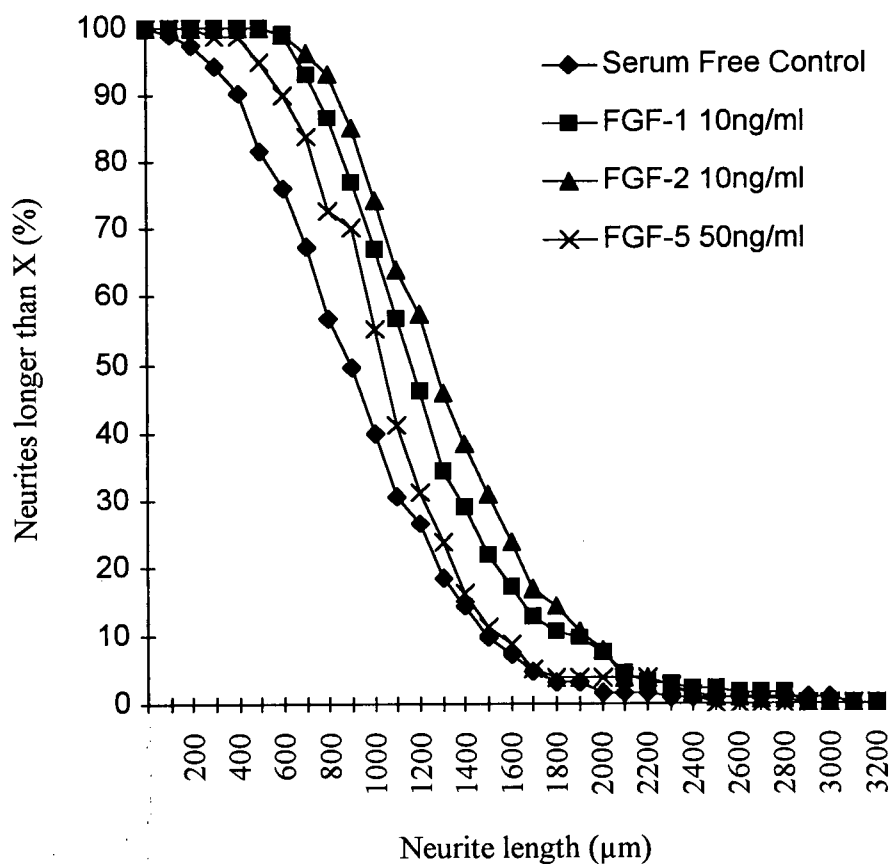


Figure 17: FGF effects on vestibulospinal neurite outgrowth. FGF-1 and FGF-2 stimulated a shift towards longer neurites for the whole population. FGF-5 may only affect a sub-population of slower-growing neurites, as evidenced by the right-ward shift of the top half of the neurite distribution.

4.3.4 Reticulospinal Neurite Outgrowth from Explant Cultures.

Reticulospinal explant cultures were prepared that contained RPgc and RPpc neurons. Treatment with FGF-1 and FGF-2 resulted in 30% and 36% longer neurites respectively ($p < 0.01$). Neurites grown in serum free control medium achieved a mean length of $746 \pm 33 \mu\text{m}$, FGF-1 (10ng/ml) treated neurites averaged $967 \pm 53 \mu\text{m}$, and FGF-2 treated neurites averaged $1015 \pm 86 \mu\text{m}$. The distribution of neurites after FGF-1 treatment showed a shift in the longest population of neurites, but the shorter neurites were similar in length to serum free control treated neurites (Figure 18). FGF-2 treatment resulted in a uniform shift of the entire distribution towards longer neurite lengths (Figure 18). FGF-5 and FGF-9 treatment did not result in a significant change in neurite length, nor was the distribution of neurites altered remarkably (data not shown)

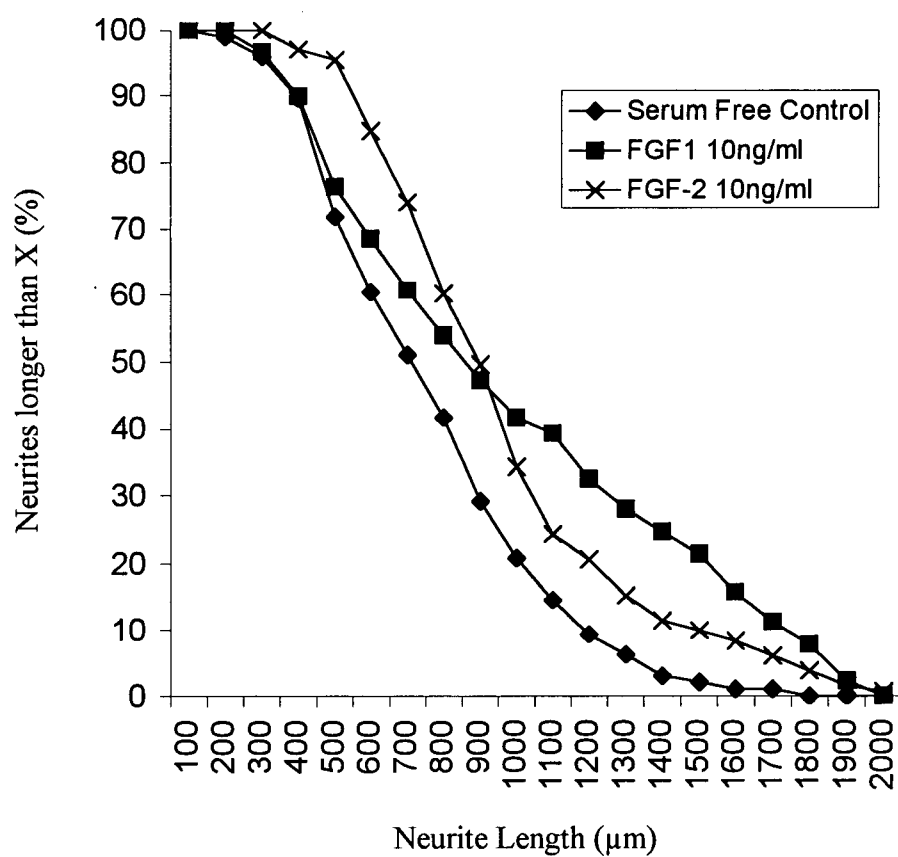


Figure 18: FGF-1 and FGF-2 effects on reticulospinal neurite outgrowth. The distribution of neurite lengths after FGF-1 treatment showed an increase in length for the longest population of neurites, and little change for shorter neurites. FGF-2 treatment shifted the entire distribution towards longer lengths.

4.4 DISCUSSION

4.4.1 *Summary*

These experiments identified FGF-2 as a survival promoting factor for both hindbrain neurons and vestibulospinal neurons in dissociated cell culture. FGF-9 was also effective at promoting vestibulospinal neuron survival, but not neurite outgrowth. Both FGF-1 and FGF-2 effectively promoted vestibulospinal neurite outgrowth as well as reticulospinal neurite outgrowth. FGF-5 was largely ineffective in all test assays. Thus I observed differential effects of FGFs on survival versus neurite outgrowth for bulbospinal neurons.

4.4.2 *FGFs stimulate bulbospinal neuron survival*

As with the neurotrophins, the FGFs are highly conserved across vertebrate species. Between chick and human FGF-1 for example, only 14 (of 155) amino acid differences exist (Figure 19) and only 12 (of 146) amino acid differences exist for human and chick FGF-2. The experiments described in this chapter use recombinant human FGFs, as many of the chick FGFs are not readily available.

```

MAEGEITTF T ALTEKFNLP P GNYKKPKLLY CSNGGHFLRI LPDGTVDGTR
MAEGEITTF T ALTERFGLPL GNYKKPKLLY CSNGGHFLRI LPDGKVDGTR

DRSDQHIQLQ LSAESVGEVY IKSTETGQYL AMDTDGLLYG SQTPNEECLF
DRSDQHIQLQ LSAEDVGEVY IKSTASGQYL AMDTNGLLYG SQLPGEECLF

LERLEENHYN TYISKKHAEK NWFVGLKKNG SCKRGPRTHY GQKAILFLPL
LERLEENHYN TYISKKHADK NWFVGLKKNG NSKLGPRTHY GQKAILFLPL

PVSSD -- human
PVSAD -- chick

```

Figure 19: FGF-1 amino acid sequence similarity between human (top, accession #NM_000800) and chick (accession #U31863). Differences are indicated in **bold**, known heparin binding regions in the human sequence are underlined.

FGF-1 and FGF-2 expression is not tightly regulated by development. Both are present in relatively high quantities throughout the developing and adult CNS in both neurons and glia (Eckenstein et al, 1991; Emoto et al, 1989; Gomez-Pinilla et al, 1992; Gospodarowicz et al, 1984; Pettman et al, 1985; Woodward et al, 1992). Each of these factors is expressed in brainstem regions with direct projections to the spinal cord (Kuzis et al, 1995; Matsuyama et al, 1992). Both factors are also expressed in the spinal cord itself (Kuzis et al, 1995), therefore both the neuronal cell body and the growth cones/terminals may have access to endogenous FGF-1 and FGF-2. The exact roles for these factors in the brainstem and spinal cord are still largely undefined. I found that FGF-2 (but not FGF-1, even in the presence of heparin), stimulates bulbospinal neuron survival.

There are numerous examples in the literature describing increased survival for a variety of neuronal populations under the influence of FGF-2 (Abe et al, 1990; Matsuda et al, 1990). Neuronal populations responding with increased survival after FGF-2 treatment include mesopontine cholinergic neurons (Garcia-Rill et al, 1991), cerebellar

neurons (Abe et al, 1991), septal cholinergic neurons (Kushima et al, 1992), spinal cord neurons (Peulve et al, 1994), and basal forebrain cholinergic neurons (Perkins and Cain, 1995). The survival of dopaminergic neurons in the mammalian ventral midbrain is also stimulated by FGF-2 (Beck et al, 1993; Burvenich et al, 1998; Ferrari et al, 1991; Mayer et al, 1993). Krieglestein and colleagues (1998) demonstrated that this effect was indirect; FGF-2 stimulated the production of TGF- β , which produced the direct trophic action on the neurons. There is no data currently available on FGF effects upon bulbospinal neurons.

The survival effects of FGF-1 on CNS neurons are less well studied than FGF-2. Specific populations of mammalian brain cells show increased survival with FGF-1 treatment, for example cortical and nigral neurons, but not septal neurons (Hisajima et al, 1991). FGF-1 expression in the brain appears in specific neuronal populations, notably reticular formation neurons (Stock et al, 1992), but its role in the development or maintenance of the CNS is still largely unexplained.

In my experiments, FGF-1 did not promote the survival of bulbospinal neurons. However, in my dissociated whole hindbrain survival assay, any growth factor effect on a particular subset of the neuronal population could be masked by the unresponsiveness of the majority of bulbospinal neurons. It is still possible that FGF-1 stimulated the survival of smaller populations of bulbospinal neurons but this assay may not have detected it. This emphasizes the need for the individual study of specific nuclei, and somewhat abrogates the usefulness of this assay for determining growth factor responsiveness of the entire bulbospinal neuron population. Thus, no additional growth factors were tested

using this assay, instead efforts were directed towards the study of specific populations of bulbospinal neurons, beginning with the vestibulospinal neurons.

4.4.3 FGF effects on vestibulospinal neurons

The dissociated vestibulospinal complex assay, a much more specific assay than whole hindbrain dissociates, also responded with increased survival with exogenous FGF-2 but not FGF-1. FGF-1 may be a stimulant for other aspects of neuronal differentiation, or it may be involved in the maintenance of a differentiated phenotype. For example, FGF-1 stimulates increases in somal size and choline acetyltransferase (ChAT) activity but not survival of ciliary ganglion neurons (Hill et al, 1991). FGF-1 increases choline acetyltransferase activity by 57%, glutamic acid decarboxylase activity by 58%, and aspartate aminotransferase activity by 65% in spinal cord neuron cultures (Sweetnam et al, 1991). Thus although FGF-1 effects were not detected here in dissociated hindbrain or vestibular nucleus cultures, the possibility exists that other aspects of the neuronal phenotype can be altered with this factor.

Sweetnam and co-workers (1991) also noted differential effects of FGF-1 and FGF-2 on neurotransmitter phenotype. FGF-2 increased choline acetyltransferase activity by 73% and glutamic acid decarboxylase activity by 200% but decreased aspartate aminotransferase activity by 40%. The rat C6 glioma cell line produced glial-derived neurotrophic factor (GDNF) in response to FGF-2 but not FGF-1 (Suter-Crazzolara and Unsicker, 1996). It is also interesting to note that the effects described by Sweetnam et al (1991) were heparin-independent, as I also did not observe a heparin

dependance in these experiments. It is possible that endogenous heparin, most likely in the form of heparan sulphate proteoglycans, is present in sufficient quantity to interact with the exogenously applied FGFs. In summary, these data suggest that FGF-1 and FGF-2 influence specific and distinct populations of cells in the CNS. FGF-2 functions as a survival factor for a wide variety of neurons, while FGF-1 may be more important for differentiation and/or maintenance of a differentiated neuronal phenotype. The data obtained with the bulbospinal and vestibulospinal survival assays also support a role for FGF-2 in neuronal survival, but FGF-1 effects were not detected in this assay.

FGF-9 also effectively increased vestibulospinal neuron survival. FGF-9 was originally isolated from the conditioned medium of a human glioblastoma cell line and named glia activating factor based on its mitogenic effects (Miyamoto et al, 1993). Using immunocytochemical techniques, it localizes mainly to a diverse range of neurons in the mammalian brain, including cortical, hippocampal, nigral, rubral, reticular and motor neurons (Nakamura et al, 1997; Todo et al, 1998). Detection of the FGF-9 mRNA using *in situ* hybridization corroborates its neuronal localization in these regions as well as in the vestibular nucleus (Tagashira et al, 1995). Its expression in the vestibular nucleus combined with its effects on survival described here suggest that FGF-9 may act as a survival factor for vestibulospinal neurons.

Several of the FGF family members lack consensus signal sequences for secretion through the endoplasmic reticulum (ER) - golgi pathway, including FGF-1, FGF-2 and FGF-9 (reviewed in Szebenyi and Fallon, 1999). Alternative upstream translation initiation sites used during the synthesis of these FGFs produce extended isoforms containing nuclear localization signals (Florkiewicz and Sommer, 1989; Prats et al,

1989). Some cell types synthesizing these so-called high molecular weight (HMW) FGFs do indeed have FGF present in the nucleus (Powell and Klagsbrun, 1991; Tessler and Neufeld, 1990), while low molecular weight (LMW) FGFs tend to localize to the cytoplasm (reviewed in Szebenyi and Fallon, 1999). The function of HMW FGFs remains largely unknown, however the nuclear localization of FGF-2 may be important for its mitogenic activity (reviewed in Stachowiak et al, 1997). These data suggest an intracrine mode of action for HMW FGF-1, FGF-2 and FGF-9. Still largely uncharacterized is the extent of HMW FGF synthesis in the brain, and the potential role these factors may play in neuronal cells.

Low molecular weight FGFs such as FGF-1, FGF-2 and FGF-9, lacking both nuclear localization signals and secretory signals, are found in the cytoplasm of many cell types including neurons and astrocytes (reviewed in Szebenyi and Fallon, 1999). Nonetheless, there is evidence for the release of these factors via a novel, energy-dependent exocytotic pathway (Florkiewicz et al, 1995) in some cell types. As well, FGF-1 and FGF-2 are found extracellularly in the brain and other tissues, associated with HSPGs on cell surfaces and in the extracellular matrix (Klagsbrun and Baird, 1991; Bernfield et al, 1992). The presence of cell surface tyrosine kinase FGFR (see Figure 20) also demonstrates the need for extracellular FGFs. Furthermore, for non-neuronal cells there is evidence that exogenously applied (extracellular) FGFs can gain access to the intracellular environment, indeed even the nucleus, to exert their biological effects (Hawker and Granger, 1992; Imamura et al, 1992). Much remains to be learned about the trans-membrane trafficking of FGFs and the transduction of FGF effects, especially as it applies to the nervous system.

4.4.4 FGFs enhance bulbospinal neurite outgrowth

Explant assays derived from vestibular and reticular brainstem regions were used to examine FGF effects on neurite outgrowth. FGF-1 and FGF-2 increased neurite outgrowth for these two populations of spinally-projecting neurons, but FGF-5 and FGF-9 were largely ineffective. Statistical analyses of the change in mean neurite length revealed that FGF-5 marginally stimulated neurite outgrowth ($p < 0.05$) for vestibulospinal neurons in one experiment. This proved to be an inconsistent result over several experiments, and in light of the significance for FGF-1 or FGF-2 treatment ($p < 10^{-7}$) it seems unlikely that FGF-5 is a neurite growth promotion factor for vestibulospinal neurons. Alternatively, there may be a small sub-population of vestibulospinal neurons responding to the FGF-5, and the assay was simply not sensitive enough to detect this reliably.

FGF-2 is known to stimulate neurite outgrowth from numerous neuronal populations, including GABAergic neurons of the caudate/putamen (Zhou and DiFiglia, 1993) and spinal cord neurons (Peulve et al, 1994; Sweetnam et al, 1991). Cell adhesion molecules (CAMs) such as neural CAM (NCAM), L1 and N-cadherin also promote neurite outgrowth via FGFR activation (reviewed in Saffell et al, 1995). The FGFRs contain a so-called CAM homology domain (see Figure 20), which is a short 20 amino acid stretch with high homology to one of the homophilic binding domains of the CAMs. In the outgrowth assays described here, there is little opportunity for FGFR-CAM

binding and CAM-stimulated neurite outgrowth. Many of the axons extend in isolation ahead of the zone of non-neuronal cell migration and with little fasciculation; their major contact is with the laminin substratum and the exogenously applied FGFs. Thus, the neurite outgrowth response suggests that the bulbospinal neurons themselves express FGFR. This will be tested in the following chapter.

The choice of laminin as a substrate for these experiments deserves some consideration. Laminin is a large extracellular matrix protein that binds integrin receptors on many cell types including neurons, leading to the activation of secondary messenger cascades including the mitogen activated protein kinase (MAPK) pathway (reviewed in Aplin et al, 1998). FGFs also activate intracellular secondary messenger cascades via the FGF receptor tyrosine kinases, leading to activation of MAPK among others. The potential for downstream convergence of signaling from growth factor receptors and integrins should be acknowledged. However, large gaps in our knowledge of secondary messenger systems still exist, and the significance of signaling convergence in neurons remains largely unknown. Future experiments should examine growth factor effectiveness with varying ECM molecule substrates.

4.4.5 Differential effects of FGFs

The major finding of these experiments was the differential effects of FGFs. FGF-1 promoted vestibulospinal neurite outgrowth but not survival, but FGF-9 did the reverse. FGF-2 stimulated both neurite outgrowth and survival. The basis for these

effects may be found by examining the known functions of the FGFRs. A more detailed discussion of FGFR can be found in the following chapter.

The actions of a particular growth factor are largely dependent upon the presence of the appropriate receptor and secondary messenger signalling pathway. For the FGF family, there are 3 known tyrosine kinase receptors expressed in the chick brain: cek1, cek2 and cek3 (Pasquale and Singer, 1989; Pasquale, 1990). These receptors are homologous to the mammalian FGFR1, FGFR3 and FGFR2 respectively. However, unlike the trk receptors, there is no clear ligand-receptor specificity aiding a functional analysis.

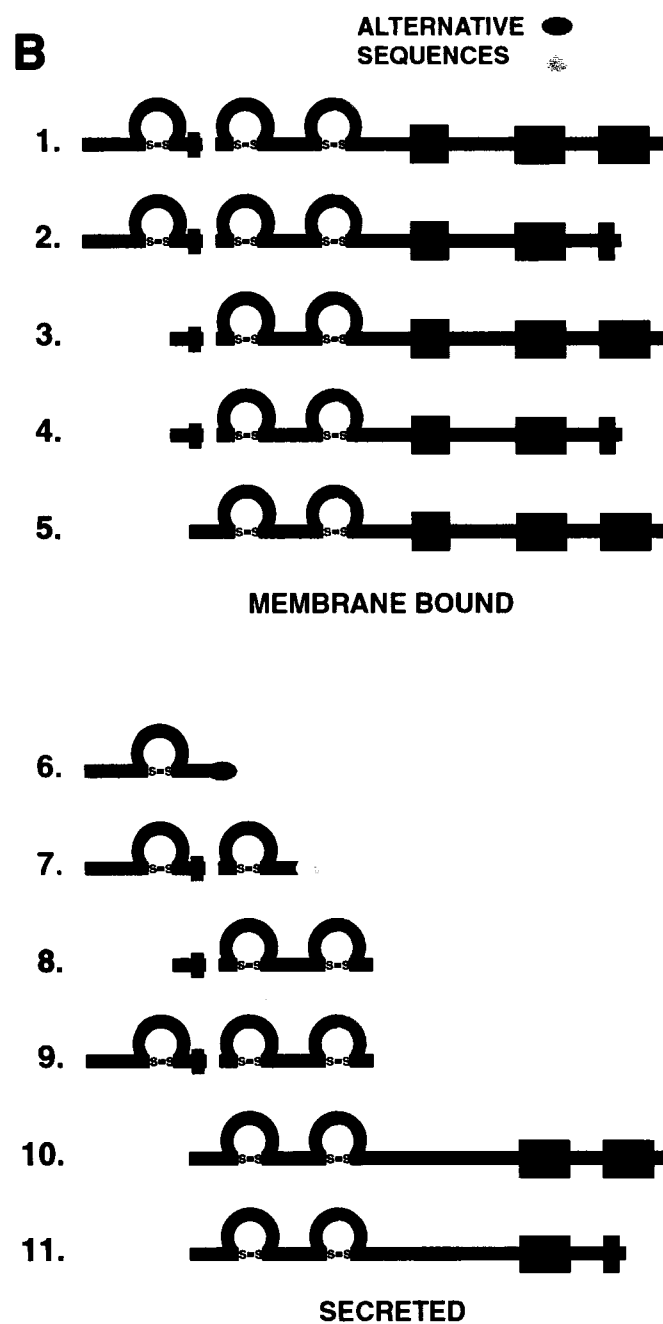
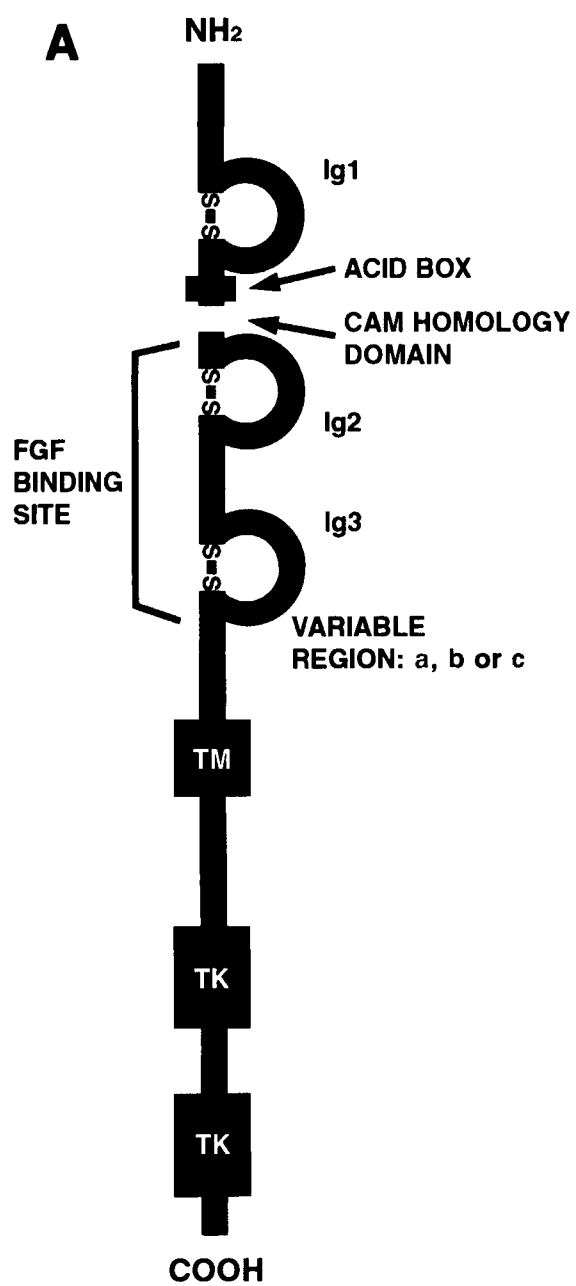
The expression of FGFR is complicated by the numerous mRNA splice variants or truncations produced for each member of the FGFR family (Figure 20). The genetic structure of the three FGFR is very similar, so the possible isoforms listed in the figure are equally applicable to each receptor. There may also exist receptors with more than one of the variations present (for example an insertion and a truncation), so in theory there could be well over 100 different possible combinations of FGFR. Fortunately, from a functional standpoint it is most important to consider the variations in the second and third Ig domains for receptors that contain an intracellular kinase domain.

The binding site for FGFs is comprised of regions in the Ig II and Ig III loops (Zimmer et al, 1993). The specificity of the receptor is at least partially determined by the variably spliced regions within Ig II and Ig III, which generate 3 isoforms referred to as the IIIa, IIIb or IIIc receptors (Johnson et al, 1991). The IIIa splice variant actually codes for a secreted (and presumably non-signaling) receptor (Johnson et al, 1991). The presence of "b" or "c" isoforms confers some selectivity for FGF binding, as FGF-9

efficiently activates the "c" splice forms of FGFR2 and FGFR3. Both FGF-1 and FGF-9 bind to and activate the "b" splice form of FGFR3 (Santos-O'Campo et al, 1996). These data suggest that perhaps vestibulospinal neurons express FGFR2IIIc, FGFR3IIIc or FGFR3IIIb to mediate the FGF-9 survival effect reported here.

FGF-1 specifically enhanced bulbospinal neurite outgrowth, but not survival, unlike FGF-2, which stimulates both neurite outgrowth and survival. This suggests that these two factors act through different receptors, or distinct receptor mechanisms. FGF-1 does activate the "IIIb" splice form of FGFR3 (Santos-O'Campo et al, 1996), suggesting that perhaps this receptor is involved in mediating the neurite outgrowth response. However, FGF-9 can also activate this receptor, but produces a survival response in vestibulospinal neurons with no effect on neurite outgrowth. The mechanism for distinguishing FGF-1 from FGF-9 at the receptor level is not well understood. The explanation for FGF specificity based on receptor expression is still problematic, much needs to be learned about sites of expression and mechanisms of FGF receptor activation to satisfy the functional data that are beginning to emerge.

Figure 20: Alternative splicing of the FGFR mRNA generates multiple isoforms of the receptors. The structure of the FGFR (A) includes 3 immunoglobulin (Ig) domains, a transmembrane domain (TM), and a divided tyrosine kinase domain (TK). Alternative mRNA splicing generates numerous structures (B) including truncated carboxy termini (2,4,11) and missing Ig domains (3-8, 10-11), the acid box (5,10,11), or the TM domain (10,11). Additional variability is possible, including "a", "b" or "c" variants in the Variable region. (adapted from Green et al, 1996)



4.4.6 Conclusions

In conclusion, the data presented here support the hypothesis that the appropriate FGFs will enhance survival or neurite outgrowth of bulbospinal neurons. FGF-2 emerged as a general factor for stimulating bulbospinal neuron survival and neurite outgrowth. These characteristics make it a promising candidate for *in vivo* trials after spinal cord injury. Similarly, FGF-1 promotes neurite outgrowth suggesting it too is a good candidate for stimulating regeneration of injured bulbospinal neurons.

- CHAPTER 5 - MECHANISM OF FGF EFFECTS ON BULBOSPINAL NEURONS

5.1 INTRODUCTION

5.1.1 Summary

In the previous Chapters, the development and application of novel *in vitro* assays for examining growth factor effects on specific populations of bulbospinal neurons was described. The survival and growth-promoting effects of neurotrophins and FGFs on bulbospinal neurons were investigated. In this chapter, the mechanism by which FGFs exert their effects is examined.

5.1.2 FGFs and their receptors

The FGFs transduce neuronal growth signals via a family of tyrosine kinase FGF receptors (FGFR). In the chick, there are three known members comprising the genetically related FGFR family, cek1, cek2 and cek3, which are homologous to the mammalian FGFR1, FGFR3 and FGFR2, respectively (Avivi et al, 1991; Partanen et al, 1991; Pasquale, 1990). These proteins were originally isolated from a chicken expressed kinase library by Pasquale and Singer (1989) and later identified to be a family of signal-transducing receptors for FGFs. All three receptors share a common structure comprised of an intracellular tyrosine kinase domain linked via a transmembrane region to the FGF-

binding extracellular domain consisting of several Ig repeats (reviewed in Green et al, 1996).

In spite of the many possible splice variants (as discussed in Chapter 4), the presence of an intracellular tyrosine kinase domain is required to transduce the FGF signal. Thus, the isoforms lacking this domain cannot transduce signals from bound FGFs and are thought to be non-functional. Participation in the physiology of FGFs may still occur even for these "non-functional" isoforms via interactions with the FGFs that alter ligand presentation or availability for the signal transducing FGFR. Nonetheless, it is thought that functional pathways including these truncated receptors must eventually converge upon signal transducing FGFR to produce a functional outcome. This provides a means to test for functional FGFR pathways using complementary oligonucleotide probes that detect regions of the mRNA encoding the tyrosine kinase domain.

The hypothesis to be tested is that:

Bulbospinal neurons will express signal transducing, tyrosine kinase domain-containing FGFR.

To test this, oligonucleotide probes were designed to recognize sequences specific to each of the intracellular tyrosine kinase domains for cek1, cek2 and cek3. In this Chapter, *in situ* hybridization was used to examine the expression of FGFR both in tissue sections from embryonic animals and in cultured brainstem cells.

After determining patterns of FGFR expression in bulbospinal neurons, subsequent experiments will be directed at examining the mechanism of FGF action in

greater detail using the dissociated hindbrain DiI-labeled bulbospinal neuron survival assay. If the DiI-labeled neurons express FGFR, then additional experiments will be done to address the hypothesis that FGFs act directly on bulbospinal neurons to signal their survival. If the DiI-labeled neurons are found not to express detectable levels of FGFR, but other cells in the brainstem do, then experiments will address the hypothesis that non-neuronal cells influence bulbospinal neuron survival.

5.2 MATERIALS AND METHODS

All chemicals were purchased from BDH Laboratory Supply (Dorset, England) unless otherwise indicated.

5.2.1 *In situ hybridization*

Bulbospinal neurons were DiI-labeled *in ovo* as described in Chapter 2, and prepared for *in situ* hybridization using standard procedures (Verge et al, 1992). Briefly, the brainstems of E8 (age at start of culture procedure), E10 (approximate equivalent age at end of culture period) or E17 chicks were quickly dissected and frozen on dry ice, and sectioned at a thickness of 14 or 20 μ m on a Zeiss Microm cryostat. For E8 and E10 brainstems, sections were collected every 100 μ m from the spino-medullary junction to the rostral pons, and alternately mounted between 3 sets of slides. This produced 3 sets of sections with 300 μ m spacings between each section within a set. For E17 brainstems, sections were only collected from the vicinity of the vestibular complex at intervals as described above. This procedure provided one set of sections for each of the FGFR probes. Sections were stored at -80°C until hybridization.

Dissociated hindbrain cultures containing DiI-labeled bulbospinal neurons were prepared as described in Chapter 2. Cells were grown in either serum free-, serum supplemented (10%), or FGF-2 (10ng/ml) treated medium, then fixed with freshly hydrolyzed 4% paraformaldehyde, followed by 3 rinses in PBS. All solutions for treating tissue or sections for this procedure were prepared from RNase-free ingredients.

Prior to hybridization, images were captured of tissue sections containing DiI-labeled bulbospinal neurons using a Zeiss Axioskop epifluorescence microscope equipped with a video CCD camera and Northern Exposure image capture/analysis system (Empix Imaging, ON). Similarly, images were obtained of 61 DiI-labeled cultured neurons that appeared not to contact or overly the cytoplasm of neighboring cells. This ensured that silver grains observed over the DiI-labeled neurons were not a result of expression in underlying cells.

The 50-mer oligonucleotide probes for the three chick FGFR were designed to hybridize with sequences in the tyrosine kinase domain of each receptor mRNA, *cek1*, *cek2* and *cek3*. No similarities were found for other molecular sequences with a 75% cutoff level in a BLASTN database search at the National Center for Biotechnology Information (Altschul et al, 1990). The *cek1* probe was complementary to bases 437-388: 5'–

CTACTTCTCCGTCAACGTCTCAGACGCACTCCCTTCTGCAGAGGATGATG–3',

the *cek2* probe was complementary to bases 486-437: 5'–

ATGTGTCATATGACGATTCGGGGCTGTACAGTTGCAAGCCAAGGCATTCC–3'

and the *cek3* probe was complementary to bases 327-278: 5'–

CCACTTGAGTTGCGCTGTCAATTGAAAGACGCCGTCATGATCAGTTGGAC–3'.

The Nucleic Acid Protein Synthesis (NAPS) Unit at the University of British Columbia synthesized the probes, and the concentration was determined using absorbance of the stock solution at 260nm illumination.

The probes were end-labeled using deoxynucleotide terminal transferase and ³⁵S-dATP (New England Nuclear, Boston, MA) according to a standard protocol (Ausubel et

al, 1987). Unlabeled dATP was removed by gravity-fed column chromatography and a small aliquot of the labeling reaction withdrawn for scintillation counting. The specific activity was calculated to judge the success of the labeling reaction.

Previously published protocols for *in situ* hybridization (Verge et al, 1992) were followed with minor modification. The tissue sections were dehydrated in an ascending alcohol series (50%-70%-95%-100% ethanol) then allowed to dry in air. Pre-hybridization solution (50% deionized formamide, 5x SSPE (sodium chloride, sodium phosphate and ethylene diamine tetraacetic acid), 10% (w/v) dextran sulfate, 5x Denhardt's solution, 0.5% sodium dodecyl sulfate) containing 200 mM dithiothreitol, 200 µg/ml salmon sperm DNA and 200 µg/ml yeast tRNA was applied for two hours at 42°C, then replaced by the hybridization buffer (same as pre-hybridization buffer, but containing the oligonucleotide probe). Sections collected from E8 DiI labeled hindbrains and slides containing dissociated cell cultures were hybridized to 1.2×10^6 CPM of probe in 100 µl of hybridization mixture overnight at 42°C. Post-hybridization rinses were performed at increasing stringency, beginning with 5x SSC at room temperature and progressing through a series of rinses with descending concentrations of SSC to 0.1x SSC at 55°C. The slides were then allowed to air dry and exposed to x-ray film overnight prior to autoradiographic processing.

Slides were dipped into NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, NY) using standard autoradiography techniques. The stock emulsion was warmed to 37°C and diluted 1:1 with water. Slides were dipped slowly into the emulsion, excess allowed to run off and then laid flat to dry in a light-proof environment. After several hours, the slides were collected and placed in a light-proof box containing a

desiccant and exposed 0.5-2 weeks (6-8 weeks for cultured cells). Some of the sectioned material was exposed for extended times (3-4 weeks) to produce an intentional over-exposure in an effort to detect cck expression in bulbospinal neurons. The photographic emulsion was developed using D-19 (Eastman Kodak Co.), and fixed with Kodafix (Eastman Kodak Co.), then the silver grains imaged as above using darkfield optics. The previously captured fluorescence images were then compared with the same field of view under darkfield to determine the expression of FGF receptors in bulbospinal neurons.

5.2.2 Astrocyte conditioned medium preparation

To examine the influence of non-neuronal cells on bulbospinal neurons, astrocyte conditioned medium (ACM, generous gift from Dr. S Kim, University of British Columbia, Vancouver, BC) was prepared from 99% pure human fetal cortical astrocyte cultures exposed to serum free DMEM for three days. Experimental media consisted of the ACM at various dilutions (neat, or diluted with DMEM to 30% and 10% ACM), or DMEM supplemented with 10% FCS (positive control), or serum free DMEM (negative control) in the dissociated whole hindbrain survival assay. The limited quantity of ACM available precluded the possibility of testing ACM effects on neurite outgrowth.

5.2.3 Determination of FGF-2 in ACM

To determine if FGF-2 was present in the ACM, the survival assay was repeated using a rhFGF-2 function-blocking antibody (R&D Systems Inc.). First, test cultures

were used to titre the effect of the antibody. Increasing concentrations of antibody were used to block the effect of 10ng/ml FGF-2 (R&D Systems Inc., a concentration of FGF-2 that stimulates a robust survival response from bulbospinal neurons) under the experimental conditions described above. Thus, the DMEM containing FGF-2 (at 10ng/ml) was pre-incubated with the function-blocking FGF-2 antibody for one hour at 37°C to deplete the available FGF-2, and this medium tested in the survival assay. The highest concentration to significantly block the survival effect only weakly achieved significance ($p < 0.05$), so to ensure a complete block of function, the next higher concentration of FGF-2 antibody tested was similarly used to deplete the ACM prior to use in the survival assay.

5.2.4 Measurement of FGF-2 concentration in ACM by ELISA

The concentration of FGF-2 in ACM was tested using a double antibody enzyme linked immunosorbent assay (ELISA). First, ELISA plates (Nunc Inc.) were coated with 5mg/ml goat rhFGF-2 antibody overnight at 4°C. After three water rinses, the antigens (FGF-1, FGF-2, FGF-4, FGF-5, and FGF-9, all at various concentrations from 0.5-500ng/ml; or ACM, undiluted or 1:1 with PBS) were then applied, then three water rinses were followed by incubation with a rabbit FGF-2 antibody (5mg/ml, R&D Systems). After rinsing, an alkaline phosphatase (AP)-conjugated goat anti-rabbit antibody was applied, followed by three water rinses, then incubation with the AP substrate p-nitrophenyl phosphate for 1-2 hours. The absorbance of the FGF-2 treated wells (at 420

nm wavelength) was used to generate a standard curve to determine the concentration of FGF-2 in the ACM treated wells.

5.3 RESULTS

5.3.1 *FGF Receptor (FGFR) Expression*

To determine whether the FGFs can act directly upon bulbo-spinal neurons, 3 oligonucleotide probes (50-mer) were designed to detect tryosine kinase domain-containing mRNA transcripts of the three chick FGFR genes, *cek1*, *cek2* and *cek3*. Expression of FGFR mRNA was examined in dissociated cell cultures or tissue sections containing DiI-labeled bulbo-spinal neurons. No difference was observed between the E8 and E10 FGFR mRNA expression profiles, hence the results are presented for early (E8/E10) or late (E17) stage embryos.

All three FGFR mRNA were robustly expressed by ependymal cells lining the fourth ventricle of early stage embryos. Within the brainstem parenchyma itself, small clusters of grains were apparent that appeared more numerous closer to the ventricular surface. These small clusters were only seen over cells (presumably non-neuronal cells) surrounding DiI-labeled bulbo-spinal neurons, and were most evident for *cek2* mRNA (Figure 21). The expression of *cek1* and *cek3* mRNA was not as robust as *cek2*, but followed a similar pattern (data not shown). Even after an extended exposure, FGFR expression was not detectable in either DiI-labeled spinally projecting neurons within brainstem tissue sections (Figure 21) or within DiI-labeled brainstem-spinal neurons in dissociated cultures (data not shown).

In late stage embryos, *cek1* mRNA expression was observed in some (but not all) vestibulospinal neurons (Figure 22). Larger clusters of grains were also observed over

cells lacking DiI staining. *cek2* and *cek3* mRNA expression appeared mainly in small clusters of grains exclusive of DiI labeled vestibulospinal neurons (data not shown).

5.3.2 *Survival effects mediated by Astrocyte-Conditioned Medium*

Non-neuronal cells in the dissociated cultures may mediate some of the effects of growth factors on bulbospinal neurons. In particular, astrocytes are known to synthesize a variety of trophic factors. Therefore, the effects of astrocyte-conditioned medium (ACM) on survival of bulbospinal neurons was tested with the hindbrain dissociated survival assay. The highest concentration of ACM (neat) resulted in a $43 \pm 11\%$ ($p < 0.005$) increase in bulbospinal neuron survival compared to serum free control treated cultures, comparable to the increase in survival mediated by 10% FCS (Figure 23).

Since astrocytes synthesize FGF-2 (Ferrara et al, 1988; Hatten et al, 1988) and FGF-2 increased bulbospinal neuron survival (see Chapter 4), I tested whether the ACM survival effect was mediated by FGF-2. First, an anti-human FGF-2 function-blocking antibody was used to deplete FGF-2 from the ACM prior to testing with the dissociated hindbrain survival assay. The anti-FGF-2 treatment did not prevent the increase in survival mediated by the ACM, nor did it alter the survival seen in control cultures where anti-FGF-2 was added to the serum free medium (Figure 24). This suggests that if non-neuronal cells (e.g. astrocytes or astrocyte progenitors) in the dissociated cultures respond to FGF-2 treatment by secreting a factor that stimulates bulbospinal neuron survival, the increased survival is not due to non-neuronal cells releasing FGF-2 into the culture medium.

Figure 21: *cek2* mRNA expression in E8 chick brainstem. DiI labeled vestibulospinal neurons (A) did not express detectable levels of *cek2* mRNA (B, C). Even with extended exposure of the photographic emulsion, as shown here, *cek* mRNA expression was below the level of detection of the *in situ* hybridization. (D) Outline (red box) indicating the area shown in (A,B,C). Scale bar, 50 μ m.

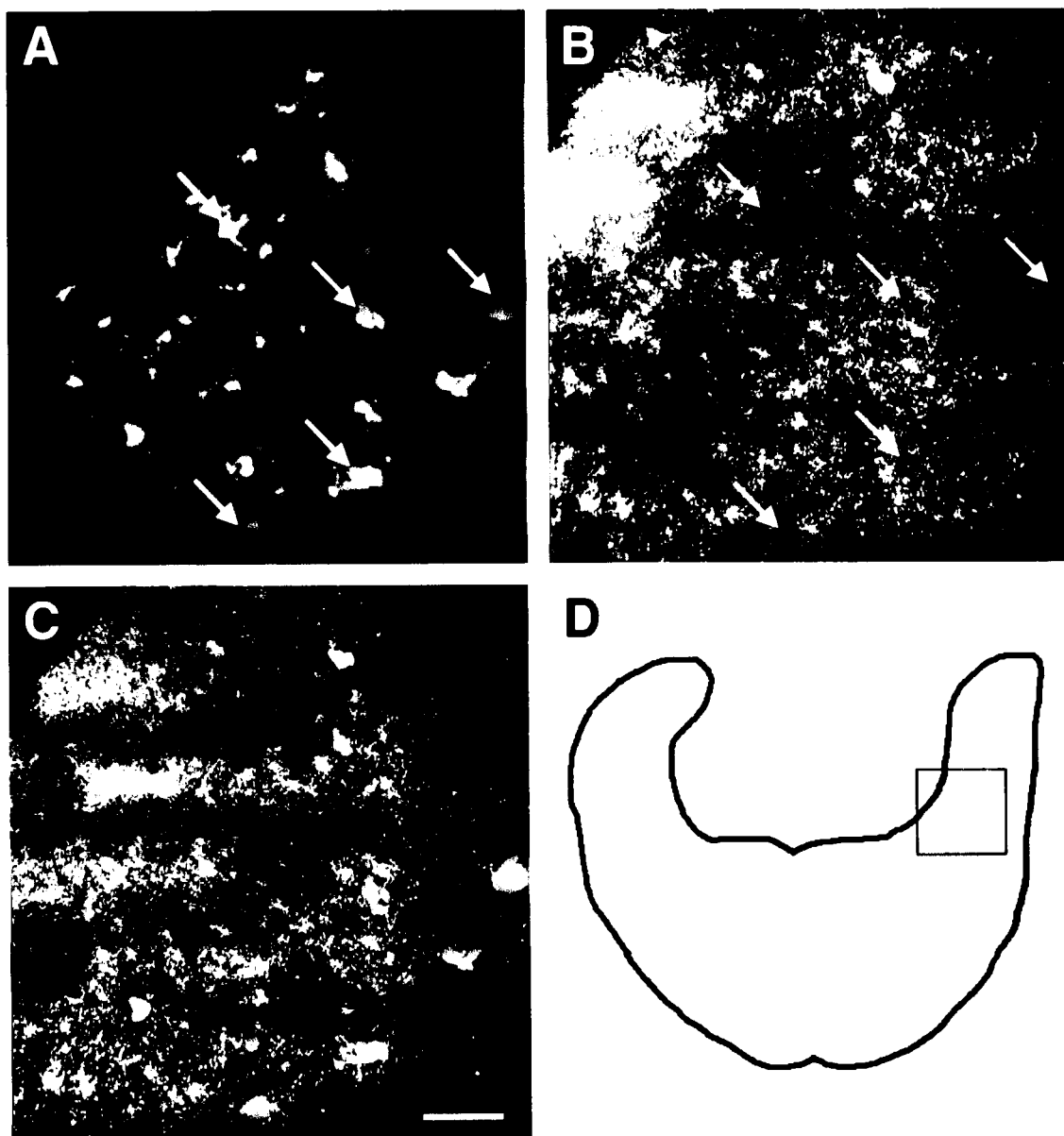
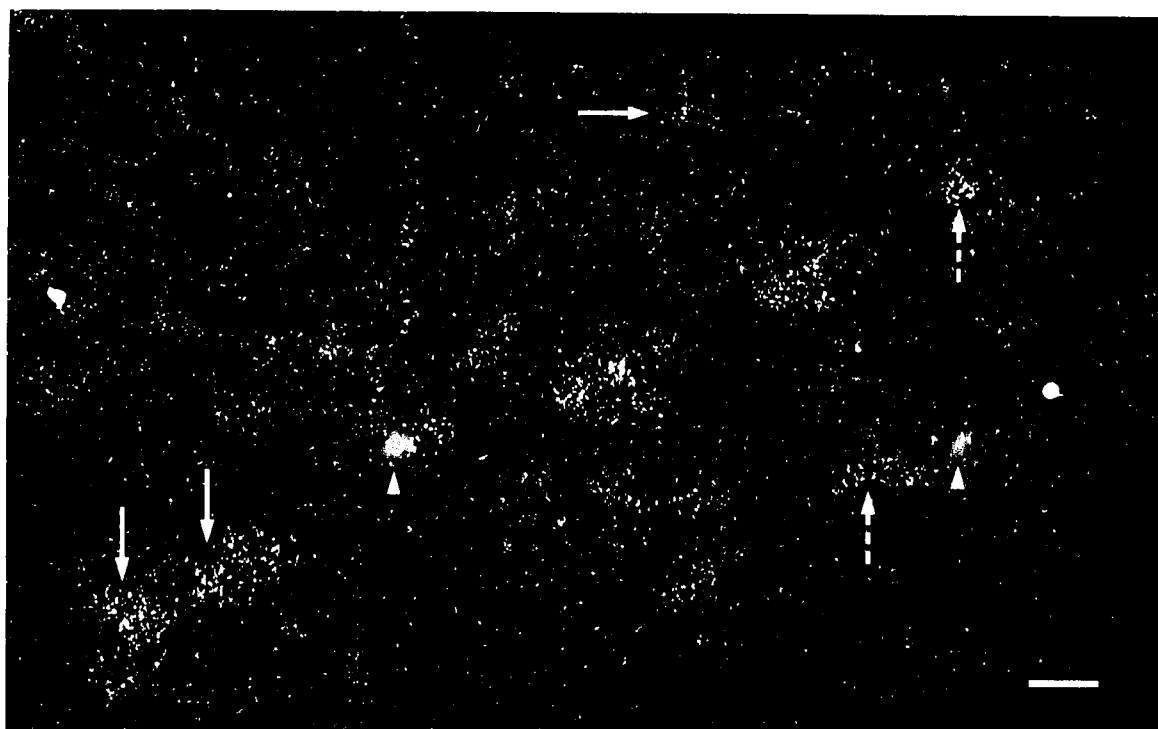


Figure 22: E17 vestibulospinal neurons expressed *cek1* mRNA. The expression of *cek1* mRNA (silver grains) was observed over DiI labeled vestibulospinal neurons (arrows) from E17 chick embryos. Vestibulospinal neurons were also visible that did not express detectable *cek1* (arrowheads). Large unlabeled cells that expressed *cek1* were evident (dashed arrows). Scale bar, 25 μ m





The concentration of FGF-2 antibody used in the blocking experiments effectively neutralized 10ng/ml of FGF-2 in control experiments. In the unlikely event that there was in excess of 10ng/ml FGF-2 in the ACM, double antibody sandwich ELISA was used to measure the amount of FGF-2 present. Increasing concentrations of FGF-2 were used to develop a standard curve, and increasing concentrations of FGF-1, FGF-4, FGF-5, and FGF-9 were used to test the antibody specificity. The technique could readily detect 0.5ng/ml of FGF-2, yet the highest concentration of the other FGFs (500ng/ml) yielded no signal above background (data not shown). Using this technique, the amount of FGF-2 present in the ACM was below the detection limit of the assay (data not shown).

To summarize, the data did not support the hypothesis that FGFs act directly on early embryonic bulbospinal neurons via tyrosine kinase-containing FGFR. Neighbouring non-neuronal cells did express *cek1*, *cek2* and *cek3* mRNA, which may have provided an indirect pathway for FGFs to influence neuronal survival. Conditioned medium from one type of non-neuronal cell, astrocytes, did support the survival of bulbospinal neurons, and this effect was not dependent upon FGF-2 in the conditioned medium.

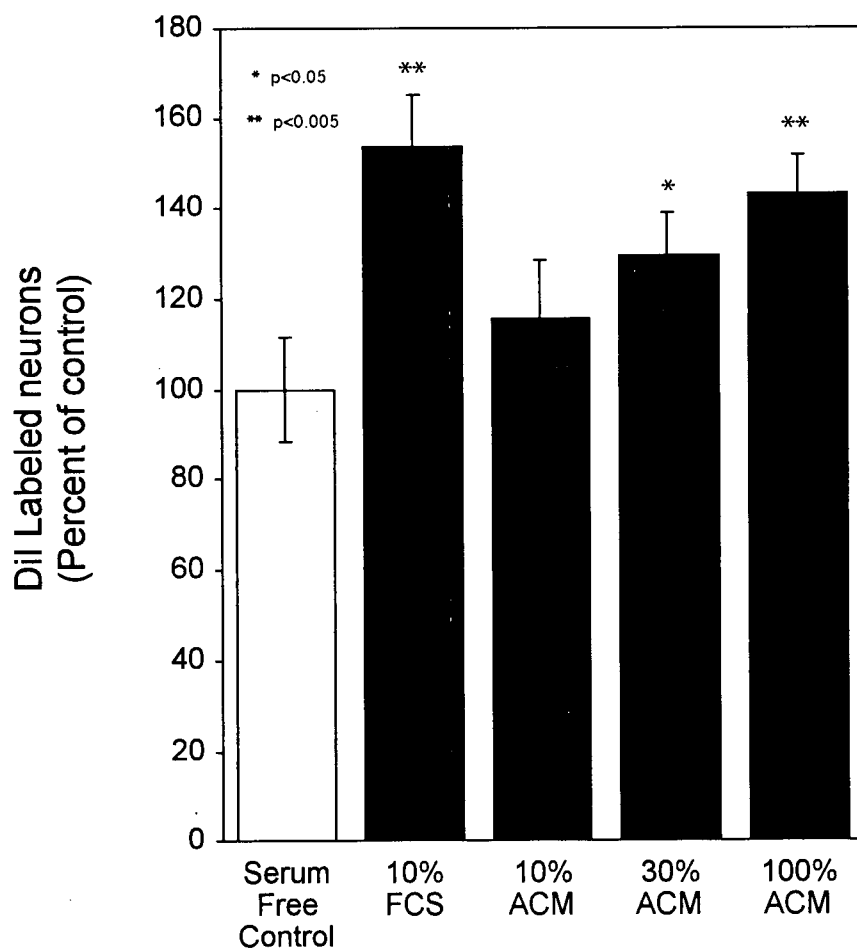


Figure 23: Astrocyte conditioned medium increased survival of bulbospinal neurons. Increasing concentrations of ACM were tested in the dissociated hindbrain survival assay. Bulbospinal neuron survival was increased $43 \pm 11\%$ ($p < 0.005$) by ACM (neat) over serum free control treated cultures.

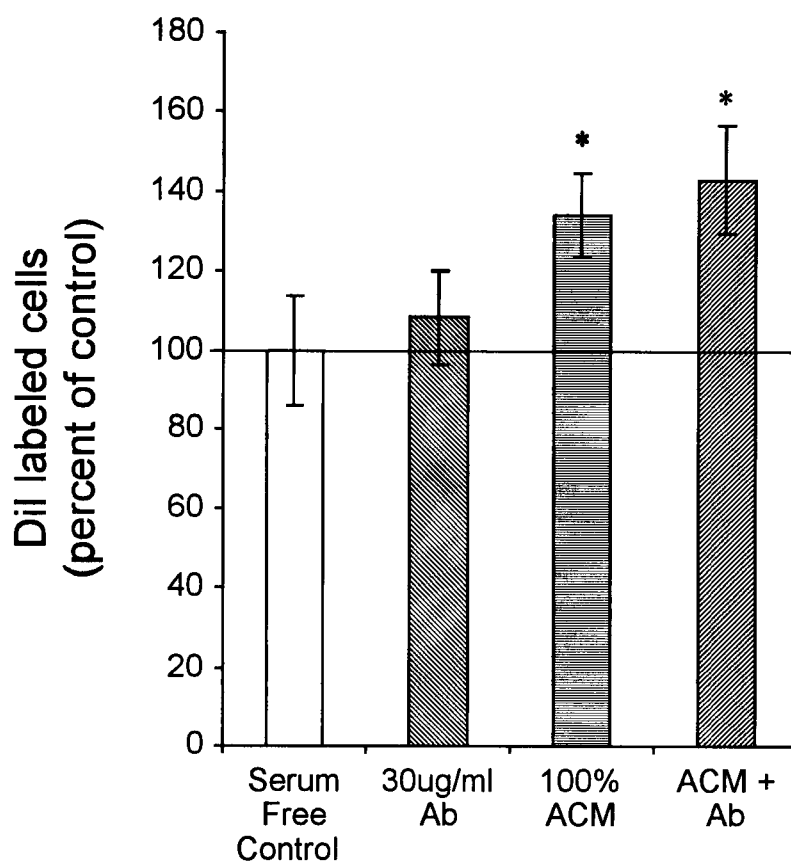


Figure 24: FGF-2 antibodies did not diminish ACM-stimulated bulbospinal neuron survival. An FGF-2 function blocking antibody (Ab) was used to deplete FGF-2 from the culture medium. At a concentration of 30 μ g/ml the antibody had no effect on basal survival in DMEM. ACM promoted bulbospinal neuron survival (100% ACM), and this effect was not blocked by the FGF-2 depletion (ACM + Ab, * $p < 0.05$).

5.4 DISCUSSION

5.4.1 Summary

In previous chapters, I described effects on survival and neurite outgrowth of bulbospinal neurons produced by FGF stimulation. To determine whether this was a direct effect of FGFs on DiI-labeled neurons, expression of the three known chick FGF receptors, cek1, cek2 and cek3 was examined using *in situ* hybridization. There is little data available on the expression of cek receptors in the chick brainstem, and less on expression of receptors specifically in bulbospinal neurons. The three chick receptors, cek1, cek2 and cek3, are homologous to mammalian FGFR-1, -3 and -2, respectively (Avivi et al, 1991; Partanen et al, 1991; Pasquale, 1990).

5.4.2 FGFR expression in embryonic chick

All three chick FGFR mRNA were expressed in the early brainstem, however cek2 mRNA appeared more abundant and cek1 mRNA appeared least abundant. All three probes labeled with similar specific activities, yet the cek2 hybridizations required the least exposure time. This decreased exposure time suggests that cek2 mRNA is more abundant than cek1 or cek3. All three FGFR mRNA were expressed in small clusters of grains, more abundant nearer the ependymal surface. This expression pattern is suggestive of a non-neuronal distribution at this early stage of development, perhaps in differentiating glia. Unfortunately, at this stage of development there is little GFAP or

myelin specific protein expressed to distinguish between astrocytic or oligodendrocytic expression.

Reports on adult mammalian FGFR distribution describe expression in both neurons and glia (Gonzalez et al, 1995; Grothe and Janet, 1995). FGFR1 mRNA was typically expressed in neurons throughout the brainstem, notably within several regions containing spinally-projecting neurons including the vestibular complex and reticular formation (Asai et al, 1993; Belluardo et al, 1997; Gonzalez et al, 1995; Peters et al, 1993; Wanaka et al, 1990; Yazaki et al, 1994). FGFR2 expression was mostly observed in small cells in the white matter, indicative of oligodendrocyte expression (Asai et al, 1993; Belluardo et al, 1997; Yazaki et al, 1994). FGFR3 mRNA was detected in a more diffuse pattern throughout the brainstem, although not in white matter, suggestive of astrocytic expression (Belluardo et al, 1997; Peters et al, 1993; Yazaki et al, 1994). *In situ* hybridization combined with retrograde DiI labeling, both in early embryonic tissue sections and in subsequently cultured cells, revealed that bulbo-spinal neurons did not express detectable levels of the three studied FGFR at this early stage of development.

The possibility exists that in early embryos, FGFR expression in bulbo-spinal neurons is below the level of detection of *in situ* hybridization. However, there may still be sufficient FGFR expressed to mediate the effects described in Chapter 4. In light of the relatively high level of FGFR expression in non-neuronal cells, the hypothesis that FGFs exert their effects via an indirect mechanism involving non-neuronal cell activation seems more likely.

Expression of FGFR was also examined in tissue from late embryonic chicks (E17). Some vestibulospinal neurons were found to express *cek1*, but not *cek2* nor *cek3*.

cek1 (and its mammalian counterpart FGFR1) expression typically is observed in adult CNS neurons (Asai et al, 1993; Belluardo et al, 1997; Gonzalez et al, 1995; Peters et al, 1993; Wanaka et al, 1990; Yazaki et al, 1994), while cek2 and cek3 expression is predominantly glial (Asai et al, 1993; Belluardo et al, 1997; Peters et al, 1993; Yazaki et al, 1994). These data suggest that the opportunity exists for FGFs to act directly on bulbospinal neurons in mature animals, future experiments in our lab will address the effects of FGFs on more mature bulbospinal neurons.

Although encoded by only 3 genes, each of the FGFR proteins can be expressed as one of many isoforms generated by alternative splicing during mRNA processing (see Figure 20). These isoforms include truncated and substituted variations that generate both soluble and transmembrane protein products from each gene. The functional significance of many of these isoforms is largely unknown, as most of the FGFR isoforms seem capable of binding most FGFs (reviewed in Green et al, 1996). However, particular combinations of exons confer some specificity for FGF binding. Much of this work was done with mammalian receptors, and therefore the mammalian nomenclature is used in the following. The variable region of Ig loop III can contain either "a", "b" or "c" exons. FGF-9 activates the "c" splice forms of FGFR2 and FGFR3, as well as the "b" splice form of FGFR3 (Santos-O'Campo et al, 1996). FGF-1 also binds and activates the "b" splice form of FGFR3 (Santos-O'Campo et al, 1996). For the most part however, it is difficult to reconcile specific FGF effects with the promiscuous nature of FGF binding to FGFR.

5.4.3 *Do FGFs act indirectly on bulbospinal neurons?*

Since the early embryonic bulbospinal neurons themselves did not express detectable levels of FGFR, I hypothesized that the observed FGF effects were the result of the paracrine production of another factor by non-neuronal cells (e.g. as described in Engele and Bohn, 1991; Gaul and Lubbert, 1992). Serum free medium conditioned by exposure to cultured astrocytes (ACM) was used in the whole hindbrain survival assay to test whether non-neuronal cell secretions promote bulbospinal neuron survival. Astrocyte conditioned medium indeed supported the survival of cultured bulbospinal neurons. The interpretation of this data could be complicated by reports of FGF-2 production by astrocytes (Ferrara et al, 1988; Hatten et al, 1988) since FGF-2 acts in the survival assay to stimulate bulbospinal neuron survival. An FGF-2 function-blocking antibody was used to deplete any FGF-2 present in the ACM, but this depleted ACM still supported bulbospinal neuron survival. The amount of antibody used to block the effect of FGF-2 was sufficient to prevent the increased survival observed with defined medium containing 10ng/ml FGF-2, a relatively high quantity. To rule out that the ACM contained a higher concentration of FGF-2 than 10ng/ml, an ELISA was used to measure the actual concentration. The ELISA procedure detected concentrations of FGF-2 as low as 0.5ng/ml, and specifically detected FGF-2 without cross-reactivity for related FGFs (FGF-1, FGF-4, FGF-5 or FGF-9) at concentrations up to 500ng/ml. No FGF-2 was detectable in the ACM using this sensitive and specific assay. This implicates other factors secreted by the astrocytes as responsible for increased bulbospinal neuron survival.

Previous analyses of trophic factor production by astrocytes (reviewed in Ridet et al, 1997) describe numerous molecules besides FGF-2 that may directly affect bulbospinal neuron survival in this assay. Astrocytes produce NGF (Houlgatte et al, 1989; Oderfeld-Nowak et al, 1992; Schwartz and Nishiyama, 1994; Yamakuni et al, 1987), BDNF (Zafra et al, 1992), NT-3 and ciliary neurotrophic factor (Rudge et al, 1992), insulin like growth factors and epidermal growth factor (Knusel et al, 1990), platelet derived growth factor (Raff et al, 1988) and midkine (Sato et al, 1993). Recently, Kriegstein et al (1998) demonstrated that FGF-2 stimulated survival of rat midbrain dopaminergic neurons was mediated by astrocytic release of transforming growth factor- β , underscoring the importance of paracrine interactions in determining neuronal survival.

The possibility exists that the differential effects of FGFs on bulbospinal survival or neurite outgrowth are the result of differential responses by non-neuronal cells. This could be addressed by studying non-neuronal cell secretions *in vitro* after specific FGF challenges. However, determining the mechanism of receptor activation for the differential FGF effects is complicated by the diversity of FGF receptors and possible interactions between receptor, ligand and heparan sulfate proteoglycans (HSPG) (Dow et al, 1994; Green et al, 1996). Alterations in the expression of heparan sequences on HSPGs can profoundly affect the specificity of FGF binding (Guimond et al, 1993; Nurcome et al, 1993).

5.4.4 Conclusions

In summary, the data described here did not support the hypothesis that FGFs act directly on early embryonic bulbo-spinal neurons. The data described here, when combined with previous data on the expression of FGFs and FGFRs in appropriate regions of the brainstem, support the hypothesis that local paracrine interactions are important in the development or maintenance of bulbo-spinal projections.

- CHAPTER 6 - GENERAL DISCUSSION

6.1 A NEW TECHNIQUE FOR STUDYING BULBOSPINAL NEURONS

A major contribution of these studies has been the creation of specific cell culture models to examine the survival/growth of selected neuronal populations. These experiments were all based on assays for defining growth factor effects on specific populations of neurons as defined by their axonal projections. The ability to isolate target neurons for these assays was dependent upon access to the developing axonal trajectory, to allow retrograde labeling with a lipophilic carbocyanine dye. The most appropriate dye for this purpose was found to be DiI, placed in crystalline form near the axonal projection trajectory of the target neurons. Dissociated cell cultures were used as a neuron survival assay while explanted tissue provided a neurite outgrowth assay.

A class of neuron important for motor control found in the brainstem, bulbospinal neurons were examined with these assays. These neurons elaborate axons into the spinal cord, carrying the majority of the motor output from the brain for spinal processing and relaying to the periphery (Holstege and Kuypers, 1984). After a spinal cord injury, volitional motor function is lost below the level of the lesion due to the interruption of these axons (Sholomenko and Steeves, 1987). Regeneration failure for brainstem-spinal projections results in permanent motor function deficits. In order to restore these motor functions, the connections between the brainstem neurons and their appropriate spinal targets must be restored.

6.2 GROWTH FACTORS AND BULBOSPINAL NEURON REGENERATION

One hypothesis regarding CNS regeneration failure proposes that mature CNS neurons themselves become less likely to produce a regenerative response after injury. Evidence to support this is found in altered gene expression after injury to mature CNS neurons compared to developing neurons (Tetzlaff et al, 1991). As well, immature neurons display a greater propensity for growth on substrates found to be inhibitory for more mature neurons (Nornes et al, 1983).

A second hypothesis proposes the development of an unfavourable (inhibitory) CNS environment blocking axonal re-growth during the maturation of the CNS. This inhibitory environment results from the progressive loss of factors conducive to axonal extension (such as growth factors) as well as the appearance of inhibitory molecules (such as myelin proteins, collapsins, CSPGs; reviewed in Schwab and Bartholdi, 1996).

Both of these hypotheses are important for explaining the failure of CNS regeneration. Previous work with other CNS neurons showed that both of these negative influences on mature nervous system regeneration could be at least partially reversed with exposure to the appropriate growth factor. This provided the motivation for developing techniques to address growth factor effects on a functionally important class of neuron in the motor control pathway, the brainstem-spinal neuron. The assays developed here allowed testing of the hypothesis that exposure to the appropriate growth factor results in increased survival or neurite outgrowth for brainstem-spinal neurons.

The data indicate that from the neurotrophin family, NT-3 effectively promoted neurite outgrowth for reticulospinal neurons, whilst the other neurotrophins were ineffective. None of the neurotrophins detectably increased the survival of brainstem-

spinal neurons, nor neurite outgrowth of vestibulospinal neurons. Of the FGFs, FGF-2 was the most versatile, promoting survival and neurite outgrowth of all populations examined. FGF-1 increased neurite outgrowth of vestibular and reticular explants, and FGF-9 increased survival of vestibulospinal neurons. FGF-5 was largely ineffective under the conditions tested in all the assays. The differential effects observed here underscored the need for the study of individual populations of brainstem-spinal neurons. Furthermore, these data suggest that successful restoration of brainstem-spinal connectivity after spinal cord injury likely will require a tailor-made cocktail of growth factors to be effective for all populations of spinally-projecting neurons.

6.3 TOWARDS A REPAIR STRATEGY FOR SPINAL CORD INJURY

The applicability of these results for the promotion of mature injured nervous system regeneration needs to be addressed. It is possible that the conditions promoting the success of these experiments with developing neurons differ from those in the mature nervous system. Part of the difficulty in addressing this point arises from the inability to suitably address these issues *in vitro* or *in vivo* with mature tissue. It is generally accepted that with increasing tissue maturity the ability to produce viable cultured cells decreases (Goslin and Banker, 1991).

The existence of many *in vivo* barriers to regeneration as reviewed in the Introduction also makes it unlikely that growth factor administration will be the only requirement for successful CNS repair. Thus, measuring the effectiveness of *in vivo* administration of growth factors alone becomes problematic if regeneration is failing due

to a combination of events and factors in the injured CNS. However, several groups are currently studying growth factor treatment *in vivo* combined with the provision of a suitable growth environment, a peripheral nerve transplant.

Regeneration is much more successful after nerve injury in the PNS. The Schwann cells providing myelination in the PNS are thought to provide a conducive environment for regeneration. Houle and colleagues used PNS transplants (Houle, 1991) and growth factor administration (Houle and Ye, 1997; Ye and Houle, 1997) to test the effectiveness of various factors *in vivo* after injury. They recently demonstrated that BDNF and NT-3 promoted the regeneration of reticular neurons into the peripheral nerve transplant (Ye and Houle, 1997). Although an effect of BDNF on caudal pontine reticular formation neurite outgrowth was not observed in the neurite outgrowth assay described in Chapter 3, NT-3 was effective. Perhaps there are specific populations of pontine reticular formation neurons that are responsive to BDNF, but were under-represented in the explants prepared for the assays used here. Nonetheless, the peripheral nerve transplant model remains an attractive system for testing growth factor effects *in vivo*.

A second possibility is that subtle species differences in growth factor responsiveness exist between birds and mammals. However, there are many neuronal types, such as the DRG, that share common growth factor sensitivities. In both mammals and birds, similar DRG subpopulations respond to each of the neurotrophins in a similar fashion. Further work, including the development of similar assays with mammalian bulbospinal neurons, is required to resolve this issue.

Another study of gene expression changes in vestibulospinal neurons revealed that c-jun expression was upregulated with ciliary neurotrophic factor (CNTF) treatment but not FGF-2 (Houle et al, 98). This study provided the FGF-2 and CNTF at the injury site in the spinal cord, a protocol which enhanced regeneration into a peripheral nerve transplant (Houle and Ye 97). Although CNTF was not tested in my experiments, my data indicated that FGF-2 stimulated neurite outgrowth for vestibulospinal neurons. The difference in effectiveness observed for FGF-2 might be explained by considering the site of delivery of the trophic factor.

An important consideration when interpreting data from *in vivo* experiments, is the site of growth factor administration. One advantage afforded by the *in vitro* assay is the growth factor has access to both the cell body and the growth cone. Access to the cell body may be of critical importance in light of the data suggesting that FGF-2 acts via non-neuronal cells *in vitro*, thus cell body administration may effectively promote regeneration *in vivo* while injury site administration is ineffective.

Another consideration is that the *in vivo* administration of a factor such as FGF-2 at the injury site may have undesirable consequences due to its many reported effects on CNS cells. The reported effects of FGF-2 on non-neuronal CNS cells are many and varied. For example, FGF-2 treatment affected astrocytes by promoting astrogliosis (Bolego et al, 1997; Eclancher et al, 1996) and altered gene expression of Heparan Sulphate Proteoglycans, Tenascin and other proteins (Bansal et al, 1996; Reuss et al, 1998; Treichel et al, 1998; Tryoen-Toth et al, 1998). If triggered at the injury site, these changes might offset any beneficial effects of the FGF-2 treatment. The full implications of FGF-2 treatment *in vivo* still requires additional research.

FGF-1 may be more appropriate for *in vivo* use, especially in light of a recent paper that examined regeneration of supraspinal axons through implanted peripheral nerve bridges. The peripheral nerve transplants were placed into a cavity in the spinal cord such that the rostral ends contacted the severed axons in the white matter, and the caudal end was directed into the gray matter. It was thought that by directing the growth into the gray matter, the inhibition from CNS white matter could be bypassed. This resulted in enhanced regeneration of supraspinal neurons, which was improved with FGF-1 saturated peripheral nerve bridges (Cheng et al, 1996).

The results from a full evaluation of brainstem-spinal neuron growth factor responsiveness will provide an important part of future repair strategies for spinal cord injury. Additional experiments are currently underway in our lab to further characterize responses to a variety of growth factors for Raphe, LoC and other reticular populations. Considerable work lies ahead for characterizing receptor expression, mechanisms of action and potential combinatorial effects. Additionally, successful promotion of regeneration *in vivo* may be dependent upon determining the ideal administration route (cell body vs. injury site).

6.4 SIGNIFICANCE OF THE *IN VITRO* BULBOSPINAL PREPARATION

The utility of the assays developed here extends far beyond simple survival or neurite outgrowth assays. The effects of extracellular matrix molecules and CAMs can also be tested. Combinations of soluble and substrate bound molecules as well as interactions with other cell types (monolayers or explants) will provide greater insights

into the biology of bulbospinal neurons. These cultures are ideal for the application of the many available drugs to dissect the intracellular signaling mechanisms underlying growth factor receptor activation in bulbospinal neurons.

Also significant was the finding that the great majority of axons re-growing from explants were labeled with the DiI. This suggests that long projection brainstem-spinal neurons are fundamentally different from other interneurons in the explants, and may be the only neurons capable of re-growing neurites as quickly and to the long lengths observed here. This implies that similar experiments could be performed with mammalian embryos without prior retrograde tracing (an exceedingly difficult task involving intrauterine surgery). Characterizing bulbospinal neuron responses to growth factors and other treatments using a mammalian animal model would be an important step towards developing treatments for human spinal cord injury.

6.5 CONCLUSIONS

A technique based on retrograde labeling with DiI was developed to specifically study bulbospinal neurons. Dissociated cell cultures were used for a survival assay and explant cultures for a neurite outgrowth assay. The effectiveness of growth factor treatment on bulbospinal neuron survival and neurite outgrowth was examined. I observed differential effects of growth factors on survival and neurite outgrowth both within and between specific populations of brainstem-spinal neurons. For example, FGF-1 and FGF-2 promoted vestibulospinal neurite outgrowth but only FGF-2 promoted vestibulospinal neuron survival. NT-3 promoted reticulospinal but not vestibulospinal

neurite outgrowth. The effect of FGFs on brainstem-spinal neurons may involve an indirect mechanism via activation of another cell type in the cultures, such as astrocytes. These data suggest that FGF-1, FGF-2 and NT-3 are good candidates for *in vivo* experiments addressing regeneration failure after spinal cord injury.

This technique is equally applicable to any population of neurons for which there is an accessible axon trajectory for the DiI labeling, for example, retinal ganglion neurons, spinal sensory neurons, propriospinal neurons, inter-hemispheric projection neurons and others. The methods developed herein will be useful for a variety of experimental designs, including cell-cell interactions, biochemical and molecular experiments, survival and neurite outgrowth assays with soluble or substrate bound molecules.

For the future, additional experiments are required to investigate the effects of other growth factors, and address the combinatorial effect of multiple growth factor treatments. The application of these techniques to address other aspects of brainstem-spinal neuron biology will likely also prove fruitful. It is hoped that these data, and future experiments using the techniques developed here, will guide the implementation of successful spinal cord repair strategies in humans.

REFERENCES

- Abe, K., Takayanagi, M. and Saito, H. (1990) A comparison of neurotrophic effects of epidermal growth factor and basic fibroblast growth factor in primary cultured neurons from various regions of fetal rat brain. *Japanese J. Pharm.* 54:45-51.
- Abe, K., Takayanagi, M. and Saito, H. (1991) Basic fibroblast growth factor and epidermal growth factor promote survival of primary cultured cerebellar neurons from neonatal rats. *Japanese J. Pharm.* 56:113-116.
- Abercrombie, M. (1946) Estimation of nuclear population from microtome sections. *Anat. Rec.* 94:239-247.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Aplin, A.E., Howe, A., Alahari, S.K. and Juliano, R.L. (1998) Signal transduction and signal modulation by cell adhesion receptors: The role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharm. Rev.* 50:197-263.
- Armstrong, D.M., Terry, R.D., Deteresa, R.M., Bruce, G., Hersh, L.B. and Gage, F.H. (1987) Response of septal cholinergic neurons to axotomy. *J. Comp. Neurol.* 264:421-436.
- Arshavsky, Y.I., Orlovsky, G.N. and Perret, C. (1988) Activity of rubrospinal neurons during locomotion and scratching in the cat. *Behav. Brain Res.* 28:193-199.
- Asai, T., Wanaka, A., Kato, H., Masana, Y., Seo, M. and Tohyama, M. (1993) Differential expression of two members of FGF receptor gene family, FGFR-1 and FGFR-2 mRNA, in the adult rat central nervous system. *Mol. Brain Res.* 17:174-178.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, K.A. and Struhl, K. Editor (1987) *Current Protocols in Molecular Biology*. New York: Wiley-Interscience.
- Avivi, A., Zimmer, Y., Yayon, A., Yarden, Y. and Givol, D. (1991) flg-2, a new member of fibroblast growth factor receptors. *Oncogene* 6:1089-1092.
- Bandtlow, C., Zachleder, T. and Schwab, M.E. (1990) Oligodendrocytes arrest neurite growth by contact inhibition. *J. Neurosci.* 10:3837-3848.

- Bansal, R., Kumar, M., Murray, K. and Pfeiffer, S.E. (1996) Developmental and FGF-2-mediated regulation of syndecans (1-4) and glypican in oligodendrocytes. *Mol. Cell. Neurosci.* 7:276-288.
- Barnard, J.W. and Carpenter, W. (1950) Lack of regeneration in spinal cord of rat. *J. Neurophysiol.* 13:223-228.
- Barnes, C. D. (1984) Brainstem control of spinal cord function. Orlando: Academic Press Inc. 291 pages.
- Beck, K.D., Knusel, B. and Hefti, F. (1993) The nature of the trophic action of brain-derived neurotrophic factor, des(1-3)-insulin-like growth factor-1, and basic fibroblast growth factor on mesencephalic dopaminergic neurons developing in culture. *Neurosci.* 52:855-866.
- Belluardo, N., Wu, G., Mudo, G., Hansson, A.C., Pettersson, R. and Fuxe, K. (1997) Comparative localization of fibroblast growth factor receptor-1, -2, and -3 mRNAs in the rat brain: in situ hybridization analysis. *J. Comp. Neurol.* 379:226-246.
- Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L. and Lose, E.J. (1992) Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* 8:365-393.
- Berry, M., Carlile, J. and Hunter, A. (1996) Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. *J. Neurocyt.* 25:147-170.
- Bignami, A. and Dahl, D. (1975) Astroglial protein in the developing spinal cord of the chick embryo. *Dev. Biol.* 44:204-209.
- Bisby, M.A. and Tetzlaff, W. (1992) Changes in cytoskeletal protein synthesis following axon injury and during axon regeneration. *Mol. Neurobiol.* 6:107-123.
- Bjorklund, A., Katzman, R., Stenevi, U. and West, K.A. (1971) Development and growth of axonal sprouts from noradrenaline and 5-hydroxytryptamine neurons in the rat spinal cord. *Brain Res.* 31:21-33.
- Bolego, C., Ceruti, S., Brambilla, R., Puglisi, L., Cattabeni, F., Burnstock, G. and Abbracchio, M.P. (1997) Characterization of the signalling pathways involved in ATP and basic fibroblast growth factor-induced astrogliosis. *Brit. J. Pharm.* 121:1692-1699.
- Boyle, R. and Pompeiano, O. (1980) Responses of vestibulospinal neurons to sinusoidal rotation of neck. *J. Neurophys.* 44:633-649.

- Brodal, A., Taber, E. and Walberg, F. (1960) The raphe nuclei of the brain stem in the cat. II. Efferent connections. *J. Comp. Neurol* 114:239-260.
- Burrus, L.W. and Olwin, B.B. (1989) Isolation of a receptor for acidic and basic fibroblast growth factor from embryonic chick. *J. Biol. Chem.* 264:18647-18653.
- Burvenich, S., Unsicker, K. and Kriegstein, K. (1998) Calcitonin gene-related peptide promotes differentiation, but not survival, of rat mesencephalic dopaminergic neurons in vitro. *Neurosci.* 86:1165-1172.
- Cabot, J.B., Wild, J.M. and Cohen, D.H. (1979) Raphe inhibition of sympathetic preganglionic neurons. *Science* 203:184-186.
- Cabot, J.B., Reiner, A. and Bogan, N. (1982) Avian bulbospinal pathways: anterograde and retrograde studies of cells of origin, funicular trajectories and laminar terminations. H. G. J. M. Kuypers and G. F. Martin: *Anatomy of Descending Pathways to the Spinal Cord*. Prog. Brain Res. Amsterdam: Elsevier, Vol. 57, pp. 79-108
- Cai, D., Shen, Y., De Bellard, M., Tang, S. and Filbin, M.T. (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* 22:89-101.
- Carleton, S.C. and Carpenter, M.B. (1983) Afferent and efferent connections of the medial, inferior and lateral vestibular nuclei in the cat and monkey. *Brain Res.* 278:29-51.
- Caroni, P. and Schwab, M.E. (1988) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* 1:85-96.
- Casaccia-Bonnel, P., Gu, C., Khursigara, G. and Chao, M.V. (1999) p75 neurotrophin receptor as a modulator of survival and death decisions. *Microscopy Res. Tech.* 45:217-224.
- Chen, D.F., Jhaveri, S. and Schneider, G.E. (1995) Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. *Proc. Natl. Acad. Sci. USA* 92:7287-7291.
- Chen, D.F., Schneider, G.E., Martinou, J.C. and Tonegawa, S. (1997) Bcl-2 promotes regeneration of severed axons in mammalian CNS [see comments]. *Nature* 385:434-439.
- Cheng, H., Cao, Y. and Olson, L. (1996) Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function [see comments]. *Science* 273:510-513.

- Clarke, P.G. and Oppenheim, R.W. (1995) Neuron death in vertebrate development: in vitro methods. *Meth. Cell Biol.* 46:277-321.
- Cohen, J., Woodhams, P.L. and Balazs, R. (1979) Preparation of viable astrocytes from the developing cerebellum. *Brain Res.* 161:503-514.
- Condé, F. (1988) Cerebellar projections to the red nucleus of the cat. *Behav. Brain Res.* 28:65-68.
- Coote, J.H. and MacLeod, V.H. (1974) The influence of bulbospinal monoaminergic pathways on sympathetic nerve activity. *J. Physiol. (Lond)* 241:453-475.
- Cummings, B.J., Yee, G.J. and Cotman, C.W. (1992) bFGF promotes the survival of entorhinal layer II neurons after perforant path axotomy. *Brain Res.* 591:271-276.
- Dahl, D., Rueger, D.C., Bignami, A., Weber, K. and Osborn, M. (1981) Vimentin, the 57 000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. *Eur. J. Cell Biol.* 24:191-196.
- Dahl, D. (1981) The vimentin-GFA protein transition in rat neuroglia cytoskeleton occurs at the time of myelination. *J. Neurosci. Res.* 6:741-748.
- Daniel, H., Billard, J.M., Angaut, P. and Batini, C. (1987) The interposito-rubrospinal system. Anatomical tracing of a motor control pathway in the rat. *Neurosci. Res.* 5:87-112.
- Davis, G.R., Jr., Troxel, M.T., Kohler, V.J., Grossmann, E.M. and McClellan, A.D. (1993a) Time course of locomotor recovery and functional regeneration in spinal-transected lamprey: kinematics and electromyography. *Exp. Brain Res.* 97:83-95.
- Davis, G.R., Jr. and McClellan, A.D. (1993b) Time course of anatomical regeneration of descending brainstem neurons and behavioral recovery in spinal-transected lamprey. *Brain Res.* 602:131-137.
- De la Monte, S.M., Federoff, H.J., Ng, S.C., Grabczyk, E. and Fishman, M.C. (1989) GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system. *Dev. Brain Res.* 46:161-168.
- Doster, S.K., Lozano, A.M., Aguayo, A.J. and Willard, M.B. (1991) Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. *Neuron* 6:635-647.
- Dow, K.E., Ethell, D.W., Steeves, J.D. and Riopelle, R.J. (1994) Molecular correlates of spinal cord repair in the embryonic chick: heparan sulfate and chondroitin sulfate proteoglycans. *Exp. Neurol.* 128:233-238.

- Duggan, A.W. and Griersmith, B.T. (1979) Inhibition of the spinal transmission of nociceptive information by supraspinal stimulation in the cat. *Pain* 6:
- Dusart, I., Airaksinen, M.S. and Sotelo, C. (1997) Purkinje cell survival and axonal regeneration are age dependent: an in vitro study. *J. Neurosci.* 17:3710-3726.
- Dyer, J.K., Bourque, J.A. and Steeves, J.D. (1998) Regeneration of brainstem-spinal axons after lesion and immunological disruption of myelin in adult rat. *Exp. Neurol.* 154:12-22.
- Eccles, R.M. and Lundberg, A. (1959) Supraspinal control of interneurons mediating spinal reflexes. *J. Physiol. (Lond)* 147:565-584.
- Eckenstein, F.P., Shipley, G.D. and Nishi, R. (1991) Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central and peripheral nerves. *J. Neurosci.* 11:412-419.
- Eclancher, F., Kehrli, P., Labourdette, G. and Sensenbrenner, M. (1996) Basic fibroblast growth factor (bFGF) injection activates the glial reaction in the injured adult rat brain. *Brain Res.* 737:201-214.
- Eidelberg, E. (1981) Consequences of spinal cord lesions upon motor function with special reference to locomotor activity. *Prog. Neurobiol.* 17:185-202.
- Emoto, N., Gonzales, A.M., Walicke, P.A., Wada, E., Simons, D.M., Shimasaki, S. and Baird, A. (1989) Identification of specific loci of basic fibroblast growth factor synthesis in the rat brain. *Growth Factors* 2:21-29.
- Engberg, I., Lundberg, A. and Ryall, R.W. (1968) Is the tonic decerebrate inhibition of reflex paths mediated by monoaminergic pathways? *Acta Physiol. Scand.* 72:123-133.
- Engle, J. and Bohn, M.C. (1991) The neurotrophic effects of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. *J. Neurosci.* 11:
- Faissner, A. and Kruse, J. (1990) J1/tenascin is a repulsive substrate for central nervous system neurons. *Neuron* 5:627-637.
- Fernandes, K.J.L., Fan, D.-P., Tsui, B.J., Cassar, S.L. and Tetzlaff, W. (1999) Influence of the axotomy to cell body distance in rat rubrospinal and spinal motoneurons: differential regulation of GAP-43, tubulins, and neurofilament-M. *J. Comp. Neurol.* 414:495-510.

- Ferrara, N., Ousley, F. and Gospodarowicz, D. (1988) Bovine brain astrocytes express basic fibroblast growth factor, a neurotropic and angiogenic mitogen. *Brain Res.* 462:223-232.
- Ferrari, G., Toffano, G. and Skaper, S.D. (1991) Epidermal growth factor exerts neuronotrophic effects on dopaminergic and GABAergic CNS neurons: comparison with basic fibroblast growth factor. *J. Neurosci. Res.* 30:493-497.
- Filbin, M.T. (1995) Myelin-associated glycoprotein: a role in myelination and in the inhibition of axonal regeneration? *Curr. Op. Neurobiol.* 5:588-595.
- Florkiewicz, R.Z. and Sommer, A. (1989) Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc. Natl. Acad. Sci. USA* 86:3978-3981.
- Friedman, W.J., Ibanez, C.F., Hallbook, F., Persson, H., Cain, L.D., Dreyfus, C.F. and Black, I.B. (1993) Differential actions of neurotrophins in the locus coeruleus and basal forebrain. *Exp. Neurol.* 119:72-78.
- Galzie, Z., Kinsella, A.R. and Smith, J.A. (1997) Fibroblast growth factors and their receptors. *Biochem. Cell. Biol.* 75:669-685.
- Garcia-Rill, E., Davies, D.L., Skinner, R.D., Biedermann, J.A. and McHalfey, C. (1991) Fibroblast growth factor-induced increased survival of cholinergic mesopontine neurons in culture. *Dev. Brain Res.* 60:267-270.
- Gaul, G. and Lubbert, H. (1992) Cortical astrocytes activated by basic fibroblast growth factor secrete molecules that stimulate differentiation of mesencephalic dopaminergic neurons. *Proc. Roy. Soc. Lond.* 249:57-63.
- Gibbs, R.B. and Pfaff, D.W. (1994) In situ hybridization detection of trkA mRNA in brain: distribution, colocalization with p75NGFR and up-regulation by nerve growth factor. *J. Comp. Neurol.* 341:324-339.
- Giehl, K.M. and Tetzlaff, W. (1996) BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. *Eur. J. Neuro.* 8:1167-1175.
- Giuffrida, R., Li Volsi, G. and Perciavalle, V. (1988) Influences of cerebral cortex and cerebellum on the red nucleus of the rat. *Behav. Brain Res.* 28:109-111.
- Glover, J.C. (1993) The development of brain stem projections to the spinal cord in the chicken embryo. *Brain Res. Bull.* 30:265-271.
- Gomez-Pinilla, F., Lee, J.W. and Cotman, C.W. (1992) Basic FGF in adult rat brain: cellular distribution and response to entorhinal lesion and fimbria-fornix transection. *J. Neurosci.* 12:345-355.

- Gomez-Pinilla, F. and Cotman, C.W. (1993) Distribution of fibroblast growth factor 5 mRNA in the rat brain: an in situ hybridization study. *Brain Res.* 606:79-86.
- Gonzalez, A.M., Berry, M., Maher, P.A., Logan, A. and Baird, A. (1995) A comprehensive analysis of the distribution of FGF-2 and FGFR1 in the rat brain. *Brain Res.* 701:201-226.
- Goslin, K. and Banker, G. (1991) Primary dissociated cell cultures of neural tissue. K. Goslin and G. Banker: *Culturing Nerve Cells*. Cambridge: The MIT Press.
- Gospodarowicz, D. (1974) Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature* 249:123-127.
- Gospodarowicz, D., Cheng, J., Lui, G., Baird, A. and Bohlen, P. (1984) Isolation of brain fibroblast growth factor by heparin-sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* 81:6963-6967.
- Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986) Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. *Cell. Differ.* 19:1-17.
- Grant, P. and Tseng, Y. (1986) Embryonic and regenerating *Xenopus* retinal fibers are intrinsically different. *Dev. Biol.* 114: 475-491.
- Green, P.J., Walsh, F.S. and Doherty, P. (1996) Promiscuity of fibroblast growth factor receptors. *Bioessays* 18:639-646.
- Grillner, S. and Wallen, P. (1985) Central pattern generators for locomotion, with special reference to vertebrates. *Ann. Rev. Neurosci.* 8:233-261.
- Grothe, C. and Janet, T. (1995) Expression of FGF-2 and FGF receptor type 1 in the adult rat brainstem: effect of colchicine. *J. Comp. Neurol.* 353:18-24.
- Guilbaud, G., Oliveras, J.L., Giesler, G. and Besson, J.M. (1977) Effects induced by stimulation of the central inferior nucleus of the raphe on dorsal horn interneurons in cat's spinal cord. *Brain Res.* 61:417-422.
- Guimond, S., Maccarana, M., Olwin, B.B., Lindahl, U. and Rapraeger, A.C. (1993) Activating and inhibitory heparin sequences for FGF-2 (basic FGF). *J. Biol. Chem.* 268:23906-23914.
- Guo, M., Dow, K.E., Kisilevsky, R. and Riopelle, R.J. (1993) Novel neurite growth-inhibitory properties of an astrocyte proteoglycan. *J. Chem. Neuroanat.* 6:239-245.

- Halfter, W., Reckhaus, W. and Kroger, S. (1987) Nondirected axonal growth on basal lamina from avian embryonic neural retina. *J. Neurosci.* 7:3712-3722.
- Hamburger, V. and Hamilton, H.L. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49-92.
- Hasan, S.J., Keirstead, H.S., Muir, G.D. and Steeves, J.D. (1993) Axonal regeneration contributes to repair of injured brainstem-spinal neurons in embryonic chick. *J. Neurosci.* 13:492-507.
- Hatten, M.E., Lynch, M., Rydel, R.E., Sanchez, J., Joseph-Silverstein, J., Moscatelli, D. and Rifkin, D.B. (1988) In vitro neurite extension by granule neurons is dependent upon astroglial-derived fibroblast growth factor. *Dev. Biol.* 125
- Haub, O. and Goldfarb, M. (1991) Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development* 112:397-406.
- Hawker, J.R. and Granger, H.J. (1992) Internalized basic fibroblast growth factor translocates to nuclei of venular endothelial cells. *Am. J. Physiol.* 262:1525-1537.
- Heacock, A.M. and Agranoff, B.W. (1977) Clockwise growth of neurites from retinal explants. *Science* 198:64-66.
- Henry, J.L. and Calaresu, R.R. (1974) Excitatory and inhibitory inputs from medullary nuclei projecting to spinal cardioacceleratory neurons in the cat. *Exp. Brain Res.* 20:485-504.
- Hill, C.E., Belford, D.A., Godovac-Zimmermann, J. and Hendry, I.A. (1991) Class I heparin binding growth factor promotes the differentiation but not the survival of ciliary neurones in vivo. *Dev. Brain Res.* 63:13-19.
- Hirsch, S. and Bahr, M. (1999) Immunocytochemical characterization of reactive optic nerve astrocytes and meningeal cells. *Glia* 26:36-46.
- Hisajima, H., Miyagawa, T., Saito, H. and Nishiyama, N. (1991) Human acidic fibroblast growth factor has trophic effects on cultured neurons from multiple regions of brain and retina. *Japanese J. Pharmacol.* 56:495-503.
- Hofer, M.M. and Barde, Y.A. (1988) Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature* 331:261-262.
- Holstege G. and Kuypers H.G. (1982) The anatomy of brain stem pathways to the spinal cord in cat. A labeled amino acid tracing study. *Prog. Brain Res.* 57:145-75.

- Holstege, G., Kuypers, H.G. and Boer, R.C. (1979) Anatomical evidence for direct brain stem projections to the somatic motoneuronal cell groups and autonomic preganglionic cell groups in cat spinal cord. *Brain Res.* 171:329-333.
- Holtzman, D.M., Kilbridge, J., Li, Y., Cunningham, E.T., Jr., Lenn, N.J., Clary, D.O., Reichardt, L.F. and Mobley, W.C. (1995) TrkA expression in the CNS: evidence for the existence of several novel NGF-responsive CNS neurons. *J. Neurosci.* 15:1567-1576.
- Houle, J.D. (1991) Demonstration of the potential for chronically injured neurons to regenerate axons into intraspinal peripheral nerve grafts. *Exp. Neurol.* 113:1-9.
- Houle, J.D. and Ye, J.H. (1997) Changes occur in the ability to promote axonal regeneration as the post-injury period increases. *Neuroreport* 8:751-755.
- Houle, J.D., Schramm, P. and Herdegen, T. (1998) Trophic factor modulation of c-Jun expression in supraspinal neurons after chronic spinal cord injury. *Exp. Neurol.* 154:602-611.
- Houlgatte, R., Mallat, M., P., B. and Prochiantz, A. (1989) Secretion of nerve growth factor in cultures of glial cells and neurons derived from different regions of the mouse brain. *J. Neurosci. Res.* 24:143-152.
- Imamura, T., Tokita, Y. and Mitsui, Y. (1992) Identification of a heparin-binding growth factor-1 nuclear translocation sequence by deletion mutation analysis. *J. Biol. Chem.* 267:
- Ip, N.Y. and Yancopoulos, G.D. (1996) The neurotrophins and CNTF: two families of collaborative neurotrophic factors. *Ann. Rev. of Neurosci.* 19:491-515.
- Isackson, P.J., Towner, M.D. and Huntsman, M.M. (1991) Comparison of mammalian, chicken and *Xenopus* brain-derived neurotrophic factor coding sequences. *FEBS Lett.* 285:260-264.
- Iwamoto, G.A., Tyu, H. and Wagman, I.H. (1980) Effects of stimulation of the caudal brain stem on late ventral root reflex discharge elicited by high threshold sural nerve afferents. *Brain Res.* 183:193-199.
- Jacobson, R.D., Virag, I. and Skene, J.H. (1986) A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. *J. Neurosci.* 6:1843-1855.
- Jarrat, H. and Hyland, B. (1999) Neuronal activity in rat red nucleus during forelimb reach-to-grasp movements. *Neurosci.* 88:629-642.
- Johnson, D., Lu, J., Chen, H., Werner, S. and Williams, L.T. (1991) The human fibroblast

growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol. Cell Biol.* 11:4627-4634.

- Kalcheim, C. and Neufeld, G. (1990) Expression of basic fibroblast growth factor in the nervous system of early avian embryos. *Development* 109:203-215.
- Kan, M., Wang, F., Xu, J., Crabb, J.W., Hou, J. and McKeehan, W.L. (1993) An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science* 259:1918-1921.
- Kaplan, D.R. (1998) Studying signal transduction in neuronal cells: the Trk/NGF system. *Prog. in Brain Res.* 117:35-46.
- Keirstead, H.S., Hasan, S.J., Muir, G.D. and Steeves, J.D. (1992) Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. *Proc. Nat. Acad. Sci. USA* 89:11664-11668.
- Keirstead, H.S., Dyer, J.K., Sholomenko, G.N., McGraw, J., Delaney, K.R. and Steeves, J.D. (1995) Axonal regeneration and physiological activity following transection and immunological disruption of myelin within the hatchling chick spinal cord. *J. Neurosci.* 15:6963-6974.
- Keirstead, H.S., Pataky, D.M., McGraw, J. and Steeves, J.D. (1997) In vivo immunological suppression of spinal cord myelin development. *Brain Res. Bull.* 44:727-734.
- King, V.R., Michael, G.J., Joshi, R.K. and Priestley, J.V. (1999) trkA, trkB, and trkC messenger RNA expression by bulbospinal cells of the rat. *Neurosci.* 92:935-944.
- Klagsbrun, M. and Baird, A. (1991) A dual receptor system is required for basic fibroblast growth factor activity. *Cell* 67:229-231.
- Knusel, B., Michel, P.P., Schwaber, J.S. and Hefti, F. (1990) Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J. Neurosci.* 10:558-570.
- Kobayashi, N.R., Fan, D.P., Giehl, K.M., Bedard, A.M., Wiegand, S.J. and Tetzlaff, W. (1997) BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and α -tubulin mRNA expression, and promote axonal regeneration. *J. Neurosci.* 17:9583-9595.
- Koistinaho, J., Hicks, K.J. and Sagar, S.M. (1993) Long-term induction of c-jun mRNA and Jun protein in rabbit retinal ganglion cells following axotomy or colchicine treatment. *J. Neurosci. Res.* 34:250-255.

- Korsching, S. (1993) The neurotrophic factor concept: a reexamination. *J. Neurosci.* 13:2739-2748.
- Kriegstein, K., Reuss, B., Maysinger, D. and Unsicker, K. (1998) Short communication: transforming growth factor-beta mediates the neurotrophic effect of fibroblast growth factor-2 on midbrain dopaminergic neurons. *Eur. J. Neurosci.* 10:2746-2750.
- Kuhn, T.B., Brown, M.D., Wilcox, C.L., Raper, J.A. and Bamburg, J.R. (1999) Myelin and collapsin-1 induce motor neuron growth cone collapse through different pathways: inhibition of collapse by opposing mutants of rac1. *J. Neurosci.* 19:1965-1975.
- Kushima, Y., Nishio, C., Nonomura, T. and Hatanaka, H. (1992) Effects of nerve growth factor and basic fibroblast growth factor on survival of cultured septal cholinergic neurons from adult rats. *Brain Res.* 598:264-270.
- Kuypers, H.G. and Lawrence, D.G. (1967) Cortical projections to the red nucleus and the brain stem in the Rhesus monkey. *Brain Res.* 4:151-188.
- Kuypers, H.G.J.M. and Martin, G.F. (1982) *Anatomy of Descending Pathways to the Spinal Cord*. Progress in Brain Res.. Amsterdam: Elsevier, Vol. 57, pp.
- Kuzis, K., Reed, S., Cherry, N.J., Woodward, W.R. and Eckenstein, F.P. (1995) Developmental time course of acidic and basic fibroblast growth factors' expression in distinct cellular populations of the rat central nervous system. *J. Comp. Neurol.* 358:142-153.
- Lamballe, F., Smeyne, R.J. and Barbacid, M. (1994) Developmental expression of trkC, the neurotrophin-3 receptor, in the mammalian nervous system. *J. Neurosci.* 14:14-28.
- Landmesser, L. (1978) The distribution of motoneurons supplying chick hind limb muscles. *J. Physiol.* 284:371-389.
- Lawrence, D.G. and Kuypers, H.G. (1968) The functional organization of the motor system in the monkey. I. The effects of bilateral pyramidal lesions. *Brain* 91:1-14.
- Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.A. (1989) Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* 341:149-152.
- Levi-Montalcini, R. (1966) The nerve growth factor: its mode of action on sensory and sympathetic nerve cells. *The Harvey Lectures*. New York: Academic Press, Vol. 60, pp. 217-259

- Levine, J.M. (1994) Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. *J. Neurosci.* 14:4716-4730.
- Li, D., Field, P.M. and Raisman, G. (1995) Failure of axon regeneration in postnatal rat entorhinohippocampal slice coculture is due to maturation of the axon, not that of the pathway or target. *Eur. J. Neurosci.* 7:1164-1171.
- Lindsay, R.M., Thoenen, H. and Barde, Y.A. (1985) Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev. Biol.* 112:319-328.
- Liu, Y., Kim, D.H., Himes, B.T., Chow, S.Y., Schallert, T., Murray, M., Tessler, A. and Fischer, I. (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. *J. Neurosci.* 19:4370-4387.
- Lowenstein, D.H. and Arsenault, L. (1996) The effects of growth factors on the survival and differentiation of cultured dentate gyrus neurons. *J. Neurosci.* 16:1759-1769.
- Lund, S. and Pompeiano, O. (1965) Descending pathways with monosynaptic action on motoneurons. *Experientia* 21:602-603.
- Lund, S. and Pompeiano, O. (1968) Monosynaptic excitation of alpha-motoneurons from supraspinal structures in the cat. *Acta Physiol. Scand.* 73:1-21.
- Lurie, D.I. and Selzer, M.E. (1991) Preferential regeneration of spinal axons through the scar in hemisected lamprey spinal cord. *J. Comp. Neurol.* 313:669-679.
- Maisonpierre, P.C., Le Beau, M.M., Espinosa, R.d., Ip, N.Y., Belluscio, L., de la Monte, S.M., Squinto, S., Furth, M.E. and Yancopoulos, G.D. (1991) Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. *Genomics* 10:558-568.
- Martin, G.F., Humbertson, A.O., Jr., Laxson, L.C., Panneton, W.M. and Tschismadia, I. (1979a) Spinal projections from the mesencephalic and pontine reticular formation in the North American Opossum: a study using axonal transport techniques. *J. Comp. Neurol.* 187:373-399.
- Martin, G.F., Humbertson, A.O., Laxson, C. and Panneton, W.M. (1979b) Evidence for direct bulbospinal projections to laminae IX, X and the intermediolateral cell column. Studies using axonal transport techniques in the North American opossum. *Brain Res.* 170:165-171.

- Martin, G.F., Cabana, T., Humbertson, A.O., Jr., Laxson, L.C. and Panneton, W.M. (1981) Spinal projections from the medullary reticular formation of the North American opossum: heterogeneity. *J. Comp. Neurol.* 196:663-682.
- Martin, G.F. and Xu, X.M. (1988) Evidence for developmental plasticity of the rubrospinal tract. Studies using the North American opossum. *Brain Res.* 467:303-308.
- Matsuda, S., Saito, H. and Nishiyama, N. (1990) Effect of basic fibroblast growth factor on neurons cultured from various regions of postnatal rat brain. *Brain Res.* 520:310-316.
- Matsuyama, A., Iwata, H., Okumura, N., Yoshida, S., Imaizumi, K., Lee, Y., Shiraishi, S. and Shiosaka, S. (1992) Localization of basic fibroblast growth factor-like immunoreactivity in the rat brain. *Brain Res.* 587:49-65.
- Mayer, E., Dunnett, S.B., Pellitteri, R. and Fawcett, J.W. (1993) Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons--I. Effects in vitro. *Neurosci.* 56:379-388.
- Mayer, E., Dunnett, S.B., Pellitteri, R. and Fawcett, J.W. (1993) Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons--I. Effects in vitro. *Neurosci.* 56:379-388.
- McClellan, A.D. (1988) Functional regeneration of descending brainstem command pathways for locomotion demonstrated in the in vitro lamprey CNS. *Brain Res.* 448:339-345.
- McClellan, A.D. (1990) Locomotor recovery in spinal-transected lamprey: role of functional regeneration of descending axons from brainstem locomotor command neurons. *Neurosci.* 37:781-798.
- McConnell, J.A. and Sechrist, J. (1980) Identification of early neurons in the brainstem and spinal cord: an autoradiographic study in the chick. *J. Comp. Neurol.* 192:769-783.
- McKeon, R.J., Schreiber, R.C., Rudge, J.S. and Silver, J. (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J. Neurosci.* 11:3398-3411.
- McKeon, R.J., Hoke, A. and Silver, J. (1995) Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp. Neurol.* 136:32-43.

- McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J. and Braun, P.E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* 13:805-811.
- Meakin, S.O. and Shooter, E.M. (1992) The nerve growth factor family of receptors. *TINS* 15:323-331.
- Merry, D.E., Veis, D.J., Hickey, W.F. and Korsmeyer, S.J. (1994) bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* 120:301-311.
- Miller, F.D., Naus, C.C., Durand, M., Bloom, F.E. and Milner, R.J. (1987) Isotypes of alpha-tubulin are differentially regulated during neuronal maturation. *J. Cell Biol.* 105:3065-3073.
- Miller, F.D., Tetzlaff, W., Bisby, M.A., Fawcett, J.W. and Milner, R.J. (1989) Rapid induction of the major embryonic alpha-tubulin mRNA, T alpha 1, during nerve regeneration in adult rats. *J. Neurosci.* 9:1452-1463.
- Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T. and Kurokawa, T. (1993) Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Mol. Cell Biol.* 13:4251-4259.
- Moscatelli, D. (1987) High and low affinity-binding sites for basic fibroblast growth factor on cultured cells: Absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell Physiol.* 131:123-130.
- Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R. and Filbin, M.T. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* 13:757-767.
- Nakamura, S., Todo, T., Haga, S., Aizawa, T., Motoi, Y., Ueki, A., Kurokawa, T. and Ikeda, K. (1997) Motor neurons in human and rat spinal cord synthesize fibroblast growth factor-9. *Neurosci. Lett.* 221:181-184.
- Nornes, H., Bjorklund, A. and Stenevi, U. (1983) Reinnervation of the denervated adult spinal cord of rats by intraspinal transplants of embryonic brain stem neurons. *Cell Tissue Res.* 230:15-35.
- Nurcome, V., Ford, M.D., Wildschut, J.A. and Bartlett, P.F. (1993) Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulphate proteoglycan. *Science* 260:103-106.

- O'Donovan, M., Sernagor, E., Sholomenko, G., Ho, S., Antal, M. and Yee, W. (1992) Development of spinal motor networks in the chick embryo. *J. Exp. Zool.* 261:261-273.
- Oblinger, M.M. and Kost, S.A. (1994) Coordinate regulation of tubulin and microtubule associated protein genes during development of hamster brain. *Dev. Brain Res.* 77:45-54.
- Oderfeld-Nowak, B., Bacia, A., Gradkowska, M., Fusco, M., Vantini, G., Leon, A. and Aloe, L. (1992) In vivo activated brain astrocytes may produce and secrete nerve growth factor-like molecules. *Neurochem. Int.* 21:455-461.
- Oh, Y.J., Swarzenski, B.C. and O'Malley, K.L. (1996) Overexpression of Bcl-2 in a murine dopaminergic neuronal cell line leads to neurite outgrowth. *Neurosci. Lett.* 202:161-164.
- Okado, N. and Oppenheim, R.W. (1985) The onset and development of descending pathways to the spinal cord in the chick embryo. *J. Comp. Neurol.* 232:143-161.
- Ozawa, K., Saida, T., Saida, K., Nishitani, H. and Kameyama, M. (1989) In vivo CNS demyelination mediated by anti-galactocerebroside antibody. *Acta Neuropathol.* 77:621-628.
- Ozawa, K., Uruno, T., Miyakawa, K., Seo, M. and Imamura, T. (1996) Expression of the fibroblast growth factor family and their receptor family genes during mouse brain development. *Mol. Brain Res.* 41:279-288.
- Pantoliano, M.W., Horlick, R.A., Springer, B.A., Van Dyk, D.E., Tobery, T., Wetmore, D.R., Lear, J.D., Nahapetian, A.T., Bradley, J.D. and Sisk, W.P. (1994) Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerisation. *Biochem.* 33:10229-10248.
- Parent, A., ed. (1996) *Carpenter's Human Neuroanatomy*. Baltimore: Williams and Wilkins. 1011 pages.
- Partanen, J., Makela, T.P., Berola, B., Korhonen, J., Hirvonen, H., Claesson-Welsh, L. and Alitalo, K. (1991) FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO J.* 10:1347-1354.
- Pasquale, E.B. and Singer, S.J. (1989) Identification of a developmentally regulated protein-tyrosine kinase by using anti-phosphotyrosine antibodies to screen a cDNA expression library. *Proc. Nat. Acad. Sci. USA* 86:5449-5453.
- Pasquale, E.B. (1990) A distinctive family of embryonic protein-tyrosine kinase receptors. *Proc. Nat. Acad. of Sci. USA* 87:5812-5816.

- Perkins, L.A. and Cain, L.D. (1995) Basic fibroblast growth factor (bFGF) increases the survival of embryonic and postnatal basal forebrain cholinergic neurons in primary culture. *International J. Dev. Neurosci.* 13:51-61.
- Peters, K., Ornitz, D., Werner, S. and Williams, L. (1993) Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev. Biol.* 155:423-430.
- Pettman, B., Weibel, M., Sensenbrenner, M. and Labourdette, G. (1985) Purification of two astroglial growth factors from bovine brain. *FEBS Lett.* 189:102-108.
- Peulve, P., Laquerriere, A., Hemet, J. and Tadie, M. (1994) Comparative effect of alpha-MSH and b-FGF on neurite extension of fetal rat spinal cord neurons in culture. *Brain Res.* 654:319-323.
- Powell, P.P. and Klagsbrun, M. (1991) Three forms of rat basic fibroblast growth factor are made from a single messenger RNA and localize to the nucleus. *J. Cell. Physiol.* 148:202-210.
- Powell, S.K. and Kleinman, H.K. (1997) Neuronal laminins and their cellular receptors. *Int. J. Biochem. & Cell Biol.* 29:401-414.
- Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A. and Caput, D. (1989) High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc. Natl. Acad. Sci. USA* 86:836-840.
- Rabacchi, S.A., Solowska, J.M., Kruk, B., Luo, Y., Raper, J.A. and Baird, D.H. (1999) Collapsin-1/semaphorin-III/D is regulated developmentally in Purkinje cells and collapses pontocerebellar mossy fiber neuronal growth cones. *J. Neurosci.* 19:4437-4448.
- Raff, M.C., Lillien, L.E., Richardson, W.D., Burne, J.F. and Noble, M.D. (1988) Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* 333:562-565.
- Raivich, G., Bohatschek, M., Kloss, C.U., Werner, A., Jones, L.L. and Kreutzberg, G.W. (1999) Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Rev.* 30:77-105.
- Ramon y Cajal, S. (1928) *Degeneration and Regeneration in the Nervous System.* London: Oxford University Press. 484-516 pages.
- Reh, T.A., Tetzlaff, W., Ertlmaier, A. and Zwiers, H. (1993) Developmental study of the expression of B50/GAP-43 in rat retina. *J. Neurobiology* 24:949-958.

- Reuss, B., Dermietzel, R. and Unsicker, K. (1998) Fibroblast growth factor 2 (FGF-2) differentially regulates connexin (cx) 43 expression and function in astroglial cells from distinct brain regions. *Glia* 22:19-30.
- Richardson, P.M., McGuinness, U.M. and Aguayo, A.J. (1980) Axons from CNS neurons regenerate into PNS grafts. *Nature* 284:264-265.
- Ridet, J.L., Malhotra, S.K., Privat, A. and Gage, F.H. (1997) Reactive astrocytes: cellular and molecular cues to biological function. *TINS* 20:570-577.
- Ringstedt, T., Lagercrantz, H. and Persson, H. (1993) Expression of members of the trk family in the developing postnatal rat brain. *Dev. Brain Res.* 72:119-131.
- Robinson, G.A. (1994) Immediate early gene expression in axotomized and regenerating retinal ganglion cells of the adult rat. *Brain Res.. Molecular Brain Res.* 24:43-54.
- Romanes, G. (1964) The motor pools of the spinal cord. J. Eccles: *Organization of the spinal cord*. New York: Elsevier, Vol. 11, pp. 93-119
- Rosenthal, A., Goeddel, D.V., Nguyen, T., Martin, E., Burton, L.E., Shih, A., Laramée, G.R., Wurm, F., Mason, A., Nikolics, K. and et al. (1991) Primary structure and biological activity of human brain-derived neurotrophic factor. *Endocrinology* 129:1289-1294.
- Rudge, J.S., Alderson, R.F., Pasnikowski, E., McClain, J., Ip, N.Y. and Lindsay, R.M. (1992) Expression of ciliary neurotrophic factor and the neurotrophins - nerve growth factor, brain-derived growth factor and neurotrophin 3 - in cultured rat hippocampal astrocytes. *Eur. J. Neurosci.* 4:459-471.
- Rudland, P.S., Seifert, W.E. and Gospodarowicz, D. (1974) Growth control and mitogenic response in cultured fibroblasts: induction of the pleiotypic and mitogenic by a purified growth factor. *Proc. Natl. Acad. Sci. USA* 71:2600-2604.
- Saffell, J.L., Williams, E.J., Doherty, P. and Walsh, F.S. (1995) Axonal growth mediated by cell adhesion molecules requires activation of fibroblast growth factor receptors. *Biochem. Soc. Trans.* 23:469-470.
- Sandberg, D.E. and Segal, M. (1978) Pharmacological analysis of analgesia and self-stimulation elicited by electrical stimulation of catecholamine nuclei in the rat brain. *Brain Res.* 152:529-542.
- Santos-Ocampo, S., Colvin, J.S., Chellaiah, A. and Ornitz, D.M. (1996) Expression and biological activity of mouse fibroblast growth factor-9. *Journal of Biological Chemistry* 271:1726-1731.

- Sato-Yoshitake, R., Shiomura, Y., Miyasaka, H. and Hirokawa, N. (1989) Microtubule-associated protein 1B: molecular structure, localization, and phosphorylation-dependent expression in developing neurons. *Neuron* 3:229-238.
- Satoh, J.-I., Muramatsu, H., Moretto, G., Muramatsu, T., Chang, h.J., Kim, S.T., Cho, J.M. and Kim, S.U. (1993) Midkine that promotes survival of fetal human neurons is produced by fetal human astrocytes in culture. *Dev. Brain Res.* 75:201-205.
- Schnell, L., Schneider, R., Kolbeck, R., Barde, Y.A. and Schwab, M.E. (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion [see comments]. *Nature* 367:170-173.
- Schnell, L. and Schwab, M.E. (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* 343:269-272.
- Schnell, L. and Schwab, M.E. (1993) Sprouting and regeneration of lesioned corticospinal tract fibres in the adult rat spinal cord. *Eur. J. Neurosci.* 5:1156-1171.
- Schwab, M.E. and Bartholdi, D. (1996) Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol. Rev.* 76:319-370.
- Schwab, M.E. and Caroni, P. (1988) Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *J. Neurosci.* 8:2381-2393.
- Schwartz, J.P. and Nishiyama, N. (1994) Neurotrophic factor gene expression in astrocytes during development and following injury. *Brain Res. Bulletin* 35:403-407.
- Segal, M. and Sandberg, D. (1977) Analgesia produced by electrical stimulation of catecholamine nuclei in the rat brain. *Brain Res.* 123:369-372.
- Sergott, R.C., Brown, M.J., Silberberg, D.H. and Lisak, R.P. (1984) Antigalactocerebroside serum demyelinates optic nerve in vivo. *J. the Neurological Sciences* 64:297-303.
- Shepherd, I., Luo, Y., Raper, J.A. and Chang, S. (1996) The distribution of collapsin-1 mRNA in the developing chick nervous system. *Developmental Biology (Orlando)* 173:185-199.
- Shiga, T., Kunzi, R. and Oppenheim, R.W. (1991) Axonal projections and synaptogenesis by supraspinal descending neurons in the spinal cord of the chick embryo. *J. Comp. Neurol.* 305:83-95.

- Shimizu, I., Oppenheim, R.W., O'Brien, M. and Shneiderman, A. (1990) Anatomical and functional recovery following spinal cord transection in the chick embryo. *J. Neurobiol.* 21:918-937.
- Sholomenko, G.N. and Steeves, J.D. (1987) Effects of selective spinal cord lesions on hind limb locomotion in birds. *Exp. Neurol.* 95:403-418.
- Sholomenko, G.N. and O'Donovan, M.J. (1995) Development and characterization of pathways descending to the spinal cord in the embryonic chick. *J. Neurophys.* 73:1223-1233.
- Sklair-Tavron, L. and Nestler, E.J. (1995) Opposing effects of morphine and the neurotrophins, NT-3, NT-4 and BDNF, on locus coeruleus neurons in vitro. *Brain Res.* 702:117-125.
- Snyder, S.E., Li, J. and Salton, S.R. (1997) Comparison of VGF and trk mRNA distributions in the developing and adult rat nervous systems. *Brain Res. Molecular Brain Res.* 49:307-311.
- Stachowiak, M.K., Moffett, J., Maher, P., Tucholski, J. and Stachowiak, E.K. (1997) Growth factor regulation of cell growth and proliferation in the nervous system. *Mol. Neurobiol.* 15:257-283.
- Steeves, J.D., Sholomenko, G.N. and Webster, D.M. (1987) Stimulation of the pontomedullary reticular formation initiates locomotion in decerebrate birds. *Brain Res.* 401:205-212.
- Stock, A., Kuzis, K., Woodward, W.R., Nishi, R. and Eckenstein, F.P. (1992) Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. *J. Neurosci.* 12:4688-4700.
- Strahlendorf, J.C., Strahlendorf, H.K., Kingsley, R.E., Gintautas, J. and Barnes, C.D. (1980) Facilitation of the lumbar monosynaptic reflexes by locus coeruleus stimulation. *Neuropharmacology* 19:225-230.
- Suter-Crazzolara, C. and Unsicker, K. (1996) GDNF mRNA levels are induced by FGF-2 in rat C6 glioblastoma cells. *Brain Res. Molecular Brain Res.* 41:175-182.
- Sweetnam, P.M., Sanon, H.R., White, L.A., Brass, B.J., Jaye, M. and Whittemore, S.R. (1991) Differential effects of acidic and basic fibroblast growth factors on spinal cord cholinergic, GABAergic, and glutamatergic neurons. *J. Neurochemistry* 57:237-249.
- Szebenyi, G. and Fallon, J.F. (1999) Fibroblast growth factors as multifunctional signaling factors. *Int. Rev. Cytology* 185:45-106.

- Tagashira, S., Ozaki, K., Ohta, M. and Itoh, N. (1995) Localization of fibroblast growth factor-9 mRNA in the rat brain. *Brain Res. Molecular Brain Res.* 30:233-241.
- Tapscott, S.J., Bennett, G.S., Toyama, Y., Kleinbart, F. and Holtzer, H. (1981) Intermediate filament proteins in the developing chick spinal cord. *Dev. Biol.* 86:40-54.
- Tessler, S. and Neufeld, G. (1990) Basic fibroblast growth factor accumulates in the nuclei of various bFGF producing cell types. *J. Cell Physiol.* 145:310-317.
- Tetzlaff, W., Alexander, S.W., Miller, F.D. and Bisby, M.A. (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. *J. Neurosci.* 11:2528-2544.
- Tetzlaff, W., Kobayashi, N.R., Giehl, K.M., Tsui, B.J., Cassar, S.L. and Bedard, A.M. (1994) Response of rubrospinal and corticospinal neurons to injury and neurotrophins. *Progress in Brain Res.* 103:271-286.
- Todo, T., Kondo, T., Nakamura, S., Kirino, T., Kurokawa, T. and Ikeda, K. (1998) Neuronal localization of fibroblast growth factor-9 immunoreactivity in human and rat brain. *Brain Res.* 783:179-187.
- Tohyama, M., Sakai, K., Touret, M., Salvert, D. and Jouvett, M. (1979a) Spinal projections from the lower brain stem in the cat as demonstrated by the horseradish peroxidase technique. II. Projections from the dorsolateral pontine tegmentum and raphe nuclei. *Brain Res.* 176:215-231.
- Tohyama, M., Sakai, K., Salvert, D., Touret, M. and Jouvett, M. (1979b) Spinal projections from the lower brain stem in the cat as demonstrated by the horseradish peroxidase technique. I. Origins of the reticulospinal tracts and their funicular trajectories. *Brain Res.* 173:383-403.
- Treichel, J.A., Reddington, M. and Kreutzberg, G.W. (1998) Regulation of plasminogen activator inhibitor-1 mRNA accumulation by basic fibroblast growth factor and transforming growth factor-beta1 in cultured rat astrocytes. *J. Neurochemistry* 71:1944-1952.
- Tryoen-Toth, P., Gaveriaux-Ruff, C., Maderspach, K. and Labourdette, G. (1998) Regulation of kappa-opioid receptor mRNA level by cyclic AMP and growth factors in cultured rat glial cells. *Brain Res. Molecular Brain Res.* 55:141-150.
- Tuttle, R. and O'Leary, D.D. (1998) Neurotrophins rapidly modulate growth cone response to the axon guidance molecule, collapsin-1. *Molecular & Cellular Neurosciences* 11:1-8.

- Valenzuela, J.I., Hasan, S.J. and Steeves, J.D. (1990) Stimulation of the brainstem reticular formation evokes locomotor activity in embryonic chicken (in ovo). *Dev. Brain Res.* 56:13-18.
- Varga, Z.M., Fernandez, J., Blackshaw, S., Martin, A.R., Muller, K.J., Adams, W.B. and Nicholls, J.G. (1996) Neurite outgrowth through lesions of neonatal opossum spinal cord in culture. *J. Comp. Neurol.* 366:600-612.
- Verge, V.M., Merlio, J.P., Grondin, J., Ernfors, P., Persson, H., Riopelle, R.J., Hokfelt, T. and Richardson, P.M. (1992) Colocalization of NGF binding sites, trk mRNA and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. *J. Neurosci.* 12:4011-4022.
- von Bartheld, C.S., Schober, A., Kinoshita, Y., Willioams, R., Ebendal, T. and Bothwell, M. (1995) Noradrenergic neurons in the locus coeruleus of birds express trkA, transport NGF, and respond to NGF. *J. Neurosci.* 15:2225-2239.
- Wanaka, A., Johnson Jr., E.M. and Milbrandt, J. (1990) Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization. *Neuron* 5:267-281.
- Wang, X.M., Qin, Y.Q., Xu, X.M. and Martin, G.F. (1994) Developmental plasticity of reticulospinal and vestibulospinal axons in the north American opossum, *Didelphis virginiana*. *J. Comp. Neurol.* 349:288-302.
- Wang, X.M., Qin, Y.Q., Xu, X.M. and Martin, G.F. (1994) Developmental plasticity of reticulospinal and vestibulospinal axons in the north American opossum, *Didelphis virginiana*. *J. Comp. Neurol.* 349:288-302.
- Wang, X.M., Terman, J.R. and Martin, G.F. (1996) Evidence for growth of supraspinal axons through the lesion after transection of the thoracic spinal cord in the developing opossum *Didelphis virginiana*. *J. Comp. Neurol.* 371:104-115.
- Wang, X.M., Terman, J.R. and Martin, G.F. (1998) Regeneration of supraspinal axons after transection of the thoracic spinal cord in the developing opossum, *Didelphis virginiana*. *J. Comp. Neurol.* 398:83-97.
- Wang, X.M., Basso, D.M., Terman, J.R., Bresnahan, J.C. and Martin, G.F. (1998) Adult opossums (*Didelphis virginiana*) demonstrate near normal locomotion after spinal cord transection as neonates. *Exp. Neurol.* 151:50-69.
- Webster, D.M. and Steeves, J.D. (1988) Origins of brainstem-spinal projections in the duck and goose. *J. Comp. Neurol.* 273:573-583.

- Webster, D.M., Rogers, L.J., Pettigrew, J.D. and Steeves, J.D. (1990) Origins of descending spinal pathways in prehensile birds: do parrots have a homologue to the corticospinal tract of mammals? *Brain, Behavior & Evolution* 36:216-226.
- Weibel, D., Cadelli, D. and Schwab, M.E. (1994) Regeneration of lesioned rat optic nerve fibers is improved after neutralization of myelin-associated neurite growth inhibitors. *Brain Res.* 642:259-266.
- Woodward, W.R., Nishi, R., Meshul, C.K., Williams, T.E., Coulombe, M. and Eckenstein, F.P. (1992) Nuclear and cytoplasmic localization of basic fibroblast growth factor in astrocytes and CA2 hippocampal neurons. *J. Neurosci.* 12:142-152.
- Xu, X.M. and Martin, G.F. (1989) Developmental plasticity of the rubrospinal tract in the North American opossum. *J. Comp. Neurol.* 279:368-381.
- Xu, X.M. and Martin, G.F. (1990) The response of rubrospinal neurons to axotomy in the adult opossum, *Didelphis virginiana*. *Exp. Neurol.* 108:46-54.
- Xu, X.M. and Martin, G.F. (1991) Evidence for new growth and regeneration of cut axons in developmental plasticity of the rubrospinal tract in the North American opossum. *J. Comp. Neurol.* 313:103-112.
- Yamakuni, T., Azawa, F., Hishinuma, F., Kuwano, R., Takahashi, Y. and Amano, T. (1987) Expression of beta-nerve growth factor mRNA in rat glioma cells and astrocytes from rat brain. *FEBS Lett.* 223:117-121.
- Yamamoto, H. and Gurney, M.E. (1990) Human platelets contain brain-derived neurotrophic factor. *J. Neurosci.* 10:3469-3478.
- Yazaki, N., Hosoi, Y., Kawabata, K., Miyake, A., Minami, M., Satoh, M., Ohta, M., Kawasaki, T. and Itoh, N. (1994) Differential expression patterns of mRNAs for members of the fibroblast growth factor receptor family, FGFR-1-FGFR-4, in rat brain. *J. Neurosci. Research* 37:445-452.
- Yazaki, N.H., Y., Kawabata, K., Miyake, A., Minami, M., Satoh, M., Ohta, M., Kawasaki, T. and Itoh, N. (1994) Differential Expression patterns of mRNAs for members of the fibroblast growth factor receptor family, FGFR-1 - FGFR-4, in rat brain. *J. Neurosci. Res.* 37:445-452.
- Ye, J.H. and Houle, J.D. (1997) Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. *Exp. Neurol.* 143:70-81.

- Zafra, F., Lindholm, D., Castren, E., Hartikka, J. and Thoenen, H. (1992) Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J. Neurosci.* 12:4793-4799.
- Zhou, D. and DiFiglia, M. (1993) Basic fibroblast growth factor enhances the growth of postnatal neostriatal GABAergic neurons in vitro. *Exp. Neurol.* 122:171-188.
- Zimmer, Y., Givol, D. and Yayon, A. (1993) Multiple structural elements determine ligand binding of fibroblast growth factor receptors. *J. Biol. Chem.* 268:7899-7903.