"No Restrictions"

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## Addendum

Prefix page i: Add after paragraph 1:

A poster presentation "Gibbons A.S. and Bailey K.A. The effect on survival of motor neurons supplied with a constant source of exogenous neurotrophins" based on the work contained in Chapter 3 and Chapter 4 has been presented at the joint meeting of the Australian Neuroscience Society and the International Society for Developmental Neuroscience, Sydney, 2002. A research paper "Gibbons A.S., Wreford N.G., Pankhurst J. and Bailey K.A. Constant supply of the neurotrophins BDNF and NT-3 improve chick motor neuron survival *in vivo*" has been accepted for publication in the International Journal of Developmental Neuroscience (publication of paper in progress at the time of printing).

*Page 6 Add after paragraph 1:*

**Motor Pools and their Fate Specification**

Motoneurons within the spinal cord are localised into discrete regions defined by the specific target tissues they innervate. Those that supply the muscles of the trunk are contained within the medial motor column (MMC), which extends the length of the spinal cord. The lateral motor column (LMC) is restricted to the cervical and lumbar regions and contains those motoneurons that innervate the limbs. The LMC is further segregated into the medial ($LMC_M$) and lateral LMC ($LMC_L$) motoneuron populations, which project to ventrally and dorsally derived limb muscles respectively. Across these regions, motoneurons are divided into two major subdivisions: the smaller fusimotor γ-motoneurons, which supply the muscle spindles, and the larger skeletomotor α-motoneurons, which supply the skeletal muscle fibres. The α-motoneurons can be further divided depending on whether they innervate fast twitch or slow twitch muscle fibres. Motoneurons that innervate the same target field are grouped into distinct pools. The motoneurons within these pools are electrically coupled to co-ordinate synaptic firing of functionally related neurons and receive synaptic input from the proprioceptive sensory neurons that supply the same target tissue (Landmesser, 1978a; Landmesser, 1978b; Smith and Frank, 1987; Brenowitz *et al*, 1983).

Within the CNS, the differentiation of neuronal precursor cells into distinct neuronal types involves an interplay between two regimes of signalling that define cell patterning along the dorso-ventral and rostro-caudal axes of the neural tube. The identity and concentration of these molecular signals across both axes specifies the fate of the progenitor cells. Sonic hedgehog (Shh) provides a major cue for dorso-ventral patterning in the spinal cord. Its secretion from the notochord establishes a concentration gradient across the dorso-ventral axis of the neural tube. Specification of the neuronal progenitors into motoneuronal and interneuronal fates is determined by the concentration of Shh along the axis (Roelink *et al*, 1994). Interpretation of the graded Shh signals is facilitated by two groups of homeodomain proteins expressed by ventral progenitor cells. Expression of Class I proteins, which include Pax6, Pax7, Dbx1, Dbx2 and Irx3, is repressed at varying threshold concentrations of Shh. In contrast, expression of Class II proteins, which includes Nkx2.2 and Nkx6.1, is induced at varying threshold concentrations of Shh (Briscoe *et al*, 2000). Therefore, as the concentration of Shh decreases towards the dorsal spinal Class II expression decreases and, in the absence of Shh mediated repression Class I protein expression increases. In this way, the combinational interaction between Class I and Class II proteins acts to define the ventral and dorsal boundaries of the neuronal progenitor domains respectively. From these progenitor domains arise the motoneurons and various ventral interneuron classes. Some neuronal progenitors within the neural tuber utilise Shh independent signalling for differentiation. In such instances, paraxial mesoderm derived retinoids and FGF facilitate the expression of Class I and II proteins (Novitch *et al*, 2003). Of the homeodomain proteins that define the motoneuron progenitor domain, Nkx6.1 has been found to direct the expression of downstream motoneuron specific factors, such as the homeodomain protein MNR2 (Tanabe *et al*, 1998). MNR2 is first expressed during the final mitosis of motoneuron progenitor cells and facilitates post-mitotic differentiation of these cells to a motoneuron fate.

Progenitor cells in the prospective spinal cord initially express cell patterning signals indicative of those seen in cells of the forebrain. Following induction of the neural plate, FGFs derived from the primitive streak, as well as signals from the paraxial mesoderm, respecify the spinal progenitors to a caudal neural fate (Reviewed in Doniach, 1993; Doniach, 1995). While FGFs appear to impose a generic caudal character on the CNS, the synthesis of retinoids by the paraxial mesoderm appears to be an important cue in specifying a spinal cord fate. Shh defines general motoneuron identity and its expression remains constant across the rostro-caudal axis of the neural tube. Within the spinal cord motoneurons form longitudinal columns on both sides of the neural tube. All motoneurons are derived from a single ventral progenitor domain and they acquire their discrete columnar identity as a function of their position along the rostro-caudal axis of the neural tube. The assignment of motoneuron identity and position along this axis of the neural tube is predominantly dependant upon signals derived from the paraxial mesoderm (Ersini *et al*, 1998).

Members of the LIM protein family represent major signalling cues for the differentiation of motoneuron types across the rostro-caudal axis (Appel *et al*, 1995). They function to define both motoneurons as a gross neuronal population as well as to specify distinct motoneuron subpopulations and are also involved in axonal migration. Isl1 is initially expressed by all cranial and spinal motoneurons. Its expression is necessary for motoneuron formation and targeted disruption of this gene in mice results in the absence of motoneurons (Pfaff et al 1996). In post-mitotic motoneurons, LIMs are expressed in various combinations to define the columnar identity of motoneurons (Tsuchida *et al*, 1994; Sharma *et al*, 2000; Thaler *et al*, 2004). Members of the Hox family of homeodomain proteins have also been implicated in the specification of motoneuron subtypes along the rostro-caudal axis. Dasen *et al* (2003) suggested that the FGF directed expression of various Hox genes facilitated the specification of motoneuron columnar identities in both the motoneuron progenitors and post mitotic cells. In the model proposed, Hox6 was implicated as a post-mitotic determinant of brachial LMC, while Hox9 acted as a determinant of column of terni neurons. Furthermore, Wahba *et al* (2001) found Hox10 was expressed in the lumbar LMC. Hox6 and Hox10 are able to induce expression of LIM1, a LIM protein involved in the specifying cells of the $LMC_L$, via the promotion of retinoid synthesis. Within the LMC, the $LMC_M$ and $LMC_L$ are generated from progenitors in the same dorso-ventral and rostro-caudal position. Motoneurons of the $LMC_L$ are born after those of the $LMC_M$ and migrate past the medial cells to their final position. $LMC_M$ motoneurons do not express LIM-1. However, they appear to induce LIM-1 expression in the later born $LMC_L$ via the synthesis of retinoids (Stockanthanan and Jessell 1998). This selective expression of LIM-1 establishes a differential dorso-ventral trajectory of LMC axons as they enter the limb facilitating correct axonal path finding of the LMC subdivisions.

Motoneurons exhibit specific pool identities by the time of axonal extension. However, the mechanisms by which individual motor pools are specified are poorly understood. Like the differentiation of the $LMC_M$ and $LMC_L$, motoneuron birth date probably plays a role in defining individual pools. Motoneuron pools can also be distinguished by the combinational expression patterns of ETS transcription factors (Coonan *et al*, 2003). ETS expression coincides with the innervation of the target field. Removal of the limb bud prior to innervation has been found to prevent ETS expression in LMC motoneurons, implicating the target field as a source of signals that define motor pool identity (Lin et al 1998). ETS have been suggested to facilitate the activity of type II cadherins. Type II cadherins are expressed in motoneurons in pool specific combinations and have been implicated in directing the segregation of discrete motor pools (Price *et al*, 2002). The selective expression of Type II cadherins is also thought to contribute to the establishment of connections between the motoneurons and the sensory neurons (Patel *et al*, 2003).

*Page 7 paragraph 1 line 22: between "...(Chu-Wang & Oppenheim, 1978)." and "The mechanisms by which neurons..." Add:*

Neuronal death during NOCD employs similar mechanisms to the apoptotic processes common to non-neuronal cells. The pro-apoptotic and anti-apoptotic transcription factors of the Bcl-2 family act to regulate the activity of caspases. In response to cell death signalling, the pro-apoptotic Bcl-2 family protein, such as Bax, induces the release of a diverse array of apoptogenic factors, including cytochrome *c*, from mitochondria (Lang Rollin *et al*, 2004). These in turn activate members of the caspase family of apoptotic proteases (Li *et al*, 1997). Caspases mediate their activity in a two step process. The initiator caspases, such as caspase 9, are activated by the mitochondria derived apoptogenic factors and cleave effector caspases, such as caspase 3. The effector caspases then act to cleave various proteins leading to morphological and biochemical features characteristic of apoptosis. The anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and $Bcl\text{-}X_L$ act to inhibit the permeability of the mitochondrial membrane preventing the release of apoptogenic factors, such as cytochrome *c*. Bcl-2 has been shown to reduce the onset of NOCD in the cervical motoneurons (Sato *et al*, 2002).

During NOCD, neurons have typically been thought to initiate there own phagocytosis. It now appears that microganglia may facilitate the process of neuronal death and corpse degradation. Marin-Teva *et al* (2004) examined the role of microglia in neuronal phagocytosis using P3 murine cerebellar slices maintained *in vitro*. In such slices, Purkinje cells normally die within 1 day *in vitro*. However by ablating the microganglia, Purkinje cell survival was maintained over this period. Neuronal death is thought to be caused by the release of superoxide following engulfment by the microglia. It is unknown whether the microglia initiate superoxide release or whether the dying neuron induces the microglia to release superoxide.

The events prior to this execution phase of neuronal apoptosis are poorly characterized. *In vitro* studies have shown that mitosis inhibitors can reduce cell death in DRG sensory neurons (Taylor *et al*, 2003). Such responses suggest that NOCD in sensory neurons may result from an abortive attempt to re-enter the cell cycle. In contrast, cell death in motoneurons is unaffected by treatment with mitosis inhibitors. Thus, it would appear that the upstream initiators of cell death differ between neuronal types.

*Page 9 Add after paragraph 1:*

The function of these neurotrophic factors does not appear to be limited to the prevention of neuronal apoptosis. Access to neurotrophic factors appears to be required for the normal development of neurons. In *bax* knockout mice, an increased number of motoneurons survive due to the absence of Bax mediated cell death. While these motoneurons innervate targets, only a subpopulation receive sufficient target derived support to grow and sustain target innervation postnatally. Addition supplementation with exogenous neurotrophic factors is required for the normal development of the all motoneurons in the *bax* knockouts (Sun *et al*, 2003).

*Page 35 Add after paragraph 1:*

**Expression of Trk receptors with Motor Pools**

The correlation between Trk expression and specific motoneuron pool types is ambiguous. Neurotrophic theory suggests that Trks act in a pool specific manner. The expression of distinct combinations of neurotrophic factors by the target fields facilitates the innervation of these targets by the appropriate motoneuron pools. The receptors of neurotrophic factors are expressed heterogeneously across the spinal cord. Within the spinal cord, the individual motor pools appear to display a more homogenous pattern of receptor expression with distinct combinations of neurotrophic factors being expressed by all of a pool's motoneurons (Gould and Oppenheim, 2004). How Trk expression is specified in these pools remains largely undetermined. The expression patterns of Trks and their specific ligands do not appear to correlate to distinct motoneuronal subtypes. There appears to be no difference in the gross expression levels of BDNF and NT-3, nor their respective Trk receptors, amongst fast and slow motoneurons or between the motoneurons of the $LMC_M$ and $LMC_L$ (Cospray and Kernell, 2000; Vernon *et al*, 2004). However, TrkC is more intensely expressed in γ-motoneurons compared to α-motoneurons (Cospray and Kernell, 2000). Therefore, the observed patterns of Trk expression in spinal motoneurons (Tsoulfas *et al*, 1993) appears to represent currently unknown parameters of functional motoneuron specialisation. Amongst the cells that associate with motor pools, TrkC is known to be essential in the survival of the proprioceptive sensory neurons. NT-3 is also thought to play a role in establishment of Ia/motoneuron functional connectivity (Seebach *et al*, 1999). TrkC-/- knockouts are devoid of group Ia sensory neurons (Klein *et al* 1994). Removal of the limb buds prior to target innervation results in the drastic loss of the group 1a sensory neurons. Supplying of NT-3 is also capable of rescuing group Ia sensory neurons from the large scale cell death that results from the removal of the limb bud prior to target innervation. In contrast, limb bud removal has little effect on TrkA+ and TrkB+ sensory neurons in the lumbar and thoracic regions (Oakley *et al*, 1997).

*Page 151 paragraph 2 line 3: between "...transcripts throughout the body." and "However, β-actin transcripts..." add:*

β-actin is routinely employed as an internal standard for RNA quantification for both neuronal and non-neuronal tissues (Becker *et al*, 1998; Bernard *et al*, 1999; Kasprzycka *et al*, 2002; Liu *et al*, 2003; Bordukalo-Niksic *et* al, 2004). Few internal standards are without inherent problems and the varying expression across tissue types and different ages can affect the reliability of a RNA quantification using internal standards. However, this study examined the same tissue type within a 7 day range of the subjects. As such any such problems associated with β-actin as an internal standard should not arise. β-actin has been previously employed by Dr Bailey's research group and as such we believed it to be the most appropriate standard to use.

# THE EFFECTS OF SUPPLYING SPINAL MOTONEURONS WITH A CONSTANT SOURCE OF EXOGENOUS NEUROTROPHINS

A thesis submitted for the Degree

of Doctor of Philosophy

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## DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other Degree or Diploma in any university or institute. To the best of my knowledge this thesis contains no material previously published or written by another person except where due reference is made in the text.

Andrew S. Gibbons

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The candidate was the recipient of the Australian Postgraduate Award.



## ABBREVIATIONS

| | |
|---|---|
| **AMV** | avian myeloblastosis virus reverse transcriptase |
| **ANOVA** | analysis of variance |
| **ALS** | amyotrophic lateral sclerosis |
| **BDNF** | brain derived neurotrophic factor |
| **CNS** | central nervous system |
| **CNTF** | ciliary neurotrophic factor |
| **CSF** | cerebrospinal fluid |
| **DMEM** | Dulbecco's modified Eagles medium |
| **DRG** | dorsal root ganglia |
| **E** | embryonic day |
| **FCS** | fetal calf serum |
| **FGF** | fibroblast growth factor |
| **GDNF** | glial derived growth factor |
| **h** | human |
| **HeBS** | HEPES buffered Saline |
| **HS** | horse serum |
| **IGF** | insulin-like growth factor |
| **IL-6** | interleukin-6 |

| | |
|---|---|
| **LIF/CDF** | leukaemia inhibitory factor/cholinergic differentiation factor |
| **LMC** | lateral motor column |
| **NGF** | nerve growth factor |
| **NOCD** | naturally occurring cell death |
| **NT** | neurotrophin |
| **P** | postnatal day |
| **PBS** | phosphate buffered saline |
| **PNS** | peripheral nervous system |
| **r** | rat |
| **x** | *Xenopus* |



## ABSTRACT

Following neurogenesis a phase of large scale neurodegeneration, termed NOCD, occurs in which neurons that have made incorrect or superfluous connections are eliminated. Neurotrophins are known to be involved in supporting motoneuron populations during this period. We have employed a novel constant expression system to examine the effect on survival of supplying a constant source of neurotrophins to LMC motoneurons during NOCD.

Using the aforementioned constant expression system, chick embryos were supplied with BDNF and NT-3, alone and in combination, and motoneuron survival was analysed at E10 and E15. When supplied individually, BDNF and NT-3 substantially increased motoneuron survival in the LMC at E10. When supplied in combination, BDNF and NT-3 promoted a greater, though not additive, level of survival at E10 compared with single factor administrations. By E15, increased motoneuron survival was still evident in BDNF supplied chicks, although the number of surviving motoneurons had decreased and a combination of BDNF and NT-3 did not improve survival over single factor supplementation.

The effect of supplying a constant source of BDNF and NT-3, alone and in combination, on motoneuron survival following a reduction in the motoneuron target field was also examined. Removal of the limb bud

resulted in the loss of 55% of LMC motoneurons normally present at E10. At E10, single factor administrations of BDNF and NT-3 to amputated embryos were able to reduce a large proportion of amputation induced LMC motoneuron loss. A combination of BDNF and NT-3 did not increase survival over that of single factor administrations. The amount of motoneuron survival achieved under all supplementation regimes was maintained between E10 and E15.

Relative quantitative RT-PCR was used to examine the expression of the neurotrophin receptors, TrkB and TrkC, in response to supplementation with BDNF or NT-3. When embryos were supplied with BDNF, a decrease in the level of TrkB mRNA in the spinal cord was observed. Interestingly, a decrease in TrkC mRNA was also seen in these embryos. Similarly, NT-3 supplementation resulted in a decrease in TrkC and TrkB mRNA in the spinal cord.

# CHAPTER 1

## LITERATURE REVIEW

# CHAPTER 1

## 1.1 Neurulation

The development of the chordate central (CNS) and peripheral nervous systems (PNS) involves the generation of neurons in excess followed by the establishment of synaptic connections and the elimination of improperly connected neurons. The establishment of all neuronal cell populations in the CNS and PNS occurs during embryonic development. The series of molecular and morphological events resulting in the establishment of mature neuronal populations begins with neurulation. Within chordates neurulation occurs early in embryogenesis and results in the formation of the neural tube (Reviewed in Smith & Schoenwolf, 1997; Colas & Schoenwolf, 2001).

Neurulation begins with the induction of the neural plate, the ectoderm-derived placode that gives rise to all neural tissue. The processes involved with the formation of the neural plate are coordinated with the movements of the primitive streak. Generation of the neural plate from the ectoderm involves apicobasal thickening of the ectoderm. Classical studies suggested neural plate formation resulted from the induction of neural plate tissue from ectodermal tissue (Waddington, 1933; Oppenheimer, 1936). Recent evidence suggests ectoderm formation results from the inhibition of a neural plate fate, while neural plate induction results from the suppression of



these inhibitory mechanisms (Hawley *et al*, 1995; Sasai *et al*, 1995; Wilson & Hammati-Brivanlou, 1995; Rathjen *et al*, 2002). Spemann's organiser, and its avian equivalent Henson's node, appears to largely control this molecular suppression of ectodermal fate (Reviewed in Kerszberg & Changeux, 1998). Following it's induction at the rostral end of the primitive streak, the neural plate continues to thicken apicobasally and elongates rostrocaudally. The neural plate exists as a pseudostratified, columnar epithelium. Formation of the neural plate in mammalian and avian embryos results from the apicobasal elongation of cells within the placode rather than an increase in the number of layers of cells. Thus, the elongated cells extend between the apex and the base of the neuroepithelium with the nuclei at varying positions within the cells, giving rise to its pseudostratified appearance (Smith & Schoenwolf, 1997; Colas & Schoenwolf, 2001).

Following its formation, the neural plate invaginates via a process known as bending. Bending is guided by three localised hinge points, two situated dorsolaterally and the third along the midline of the placode overlying the prechordal plate and the notochord (Schoenwolf & Franks, 1984). The lateral regions of the neural plate fold in on themselves, adhering and eventually fusing to form the neural tube. Within the avian system fusion of the neural tube is first seen in the rostral region of the neural plate. Fusion subsequently proceeds in a wave towards the caudal region of the neural plate. This process draws the ectoderm over the neural tube resulting in the fusion of the ectoderm along the midline and the dissociation of the

neurectoderm from the ectodermal layer (Smith & Schoenwolf, 1997; Colas & Schoenwolf, 2001). The neural crest cells are derived from those neurectodermal cells that originally flank the neural plate and come to lie in the region between the ectoderm and the separated neural tube. These pluripotent cells migrate extensively throughout the body, differentiating into a variety of neural cell types including those of the sensory and autonomic neural cells as well as non-neural cells, such as melanocytes (Reviewed in Bronner-Fraser, 1994).

Proliferation of the single layer of neuroepithelial cells that forms the neural tube is associated with the movement of cell nuclei between the lumen and the outer surface. It is this nuclear movement which results in the pseudostratified appearance of the tissue when examined histologically. The majority of cell proliferation occurs within the ventricular zone. Within the ventricular zone, cells undergoing mitosis circularise and position themselves close to the ventricular wall. Following cell division, the daughter cells extend processes to the outer wall and migrate towards the midlevel of the neuroepithelium. The cells remaining in the ventricular zone subsequently repeat the same mitotic behaviour (Sauer, 1935).

Following their division from their neuroepithelial precursors, neurons migrate away from the proliferative region of the ventricular zone to form the intermediate layer. After dividing from the neuroepithelial precursors, neurons do not undergo any further mitosis. Much of our knowledge about the processes involved in neuronal migration comes from



examining cell movement in the cerebral cortex. Within mammals, neurons migrate with an inside out gradient, such that those cells that become post mitotic first form the deepest layers of the cerebral cortex while subsequent neurons migrate past the existing layers to form the more superficial layers (Angevine & Sidman, 1961; Rakic, 1972). Within amphibian, reptilian and avian systems, later migrational events are seen to occur in an outside in pattern (Tsai *et al*, 1981; Goffinet *et al*, 1986). Migration is such that similar aged neurons migrate to the same neuronal layers. For a long time the migration of postmitotic neurons from the proliferative layer was believed to occur solely via locomotion along the processes of single radial glia that span the parenchyma (Rakic, 1972). Recent studies that have tracked individual migrating neurons have expanded our understanding of cell movement. In addition to the traditional concept of unipolar, glial guided locomotion, migrating cells have been shown to transfer between radial glial processes during glial guided locomotion. Reinstating a previously dismissed concept of neuronal migration, neurons have also been shown to migrate via somal translocation, by which neurons migrate using their own processes (Nadarajah *et al*, 2001). Which method of migration is employed by the migrating neurons does not appear to be cell-type specific. However, early-generated neurons appear to migrate only via somal translocation while those forming the outer layers of the cortex migrate initially via somal translocation before changing to glial guided locomotion (Nadarajah *et al*, 2001). These migration events result in the generation of neuronal layers within the neural tube.

As neurons reach their final destination they stop migrating and disengage from the glial processes, organising themselves into layers appropriate to their time of birth. Following the migration to their ultimate position within the nervous system, the neurons extend their axonal processes towards their target fields, where they establish synaptic connections. Axonal extension is directed by a structure at the tip of the axon known as the growth cone. Growth and redirection of the growth cone is facilitated by the extension and retraction of a series of motile, actin-filament bundles, emanating from the growth cone called filopodia (Chien *et al*, 1993; Zheng *et al*, 1996). The direction of growth cone extension is guided by a series of long and short range chemoattractive and repulsive signals. A number of conserved molecular families are known to be involved in directing axonal extension. These include both secreted proteins, such as semaphorins, Slits and netrins, as well as membrane bound proteins, such as ephrins (reviewed in Grunwald & Klein, 2002). Following innervation at the target field, neurons become dependant upon access to soluble target secreted trophic factors. Prior to the innervation of their target field, neurons are independent of the trophic factors they later come to rely on for survival.



## 1.2 Naturally Occurring Cell Death and the Neurotrophic Theory

In the period immediately following axonal migration, a phase of large-scale neuronal elimination occurs, referred to as naturally occurring cell death (NOCD) (Oppenheim, 1989). During NOCD approximately 50% of CNS and PNS neurons degenerate in order to eliminate superfluous and incorrectly connected neurons. The anatomical changes in the nervous system during NOCD have been well studied, particularly in the spinal motoneuron populations of the developing chick. The first signs of motoneuron degradation can be seen as early as the fourth embryonic day, although degrading cells within the neuronal populations are quite sparse at this early stage. The degenerating neurons undergo a rapid but progressive degradation caused by a series of autolytic changes, which eventually results in their fragmentation and disappearance. The most dramatic stage of NOCD occurs between embryonic days 6 and 9. Within the chick lateral motor column (LMC) as much as 40% of motoneurons are lost between embryonic days 6 and 9 (Chu-Wang & Oppenheim, 1978; Hamburger, 1975). Following embryonic day 9 the rate of degradation rapidly decreases. Between embryonic days 10 and 16, a further 20% to 30% of spinal motoneurons remaining at embryonic day 10 degenerate (Oppenheim, 1992). It is during this period, at around embryonic day 12, that Schwann cells begin to myelinate the LMC motoneurons. Following this period, extremely low rates of neurodegeneration can be seen to occur into the first few post-natal days (Chu-Wang & Oppenheim, 1978). The mechanisms by which neurons

survive and the sources of trophic support during this period have been an area of intense interest.

Early studies suggested neuronal support came from the target tissue itself. Hamburger and Levi-Montalcini (1949) noted that removing the developing limb buds of embryonic chicks immediately prior to the onset of NOCD resulted in a dramatic decrease in the number of LMC motoneurons at subsequent stages of development. Furthermore, when supernumerary limb buds were grafted on to the developing embryo prior to NOCD the proportion of neuronal death in the region innervating the tissue was reduced (Hollyday & Hamburger, 1976). By introducing a retrograde marker into the tissue, Chu-Wang & Oppenheim (1978) observed that neurons only succumb to NOCD after they have innervated their target tissue suggesting that trophic support of the neurons is only required after the synaptic connections have been made.

Oppenheim (1989) proposed that following innervation, neurons compete for a limiting supply of soluble neurotrophic factors secreted by the target cells. Those neurons unable to obtain sufficient trophic support ultimately die. In this way the nervous system is able to eliminate neuronal cells that innervate inappropriate targets as well as reduce superfluous connections. Neurotrophic factors are limited by both their temporal and spatial distributions. Whether neurotrophic factors are in limited supply due to their production levels, the ability of axons to access areas where the target-derived factors are present or a combination of the two mechanisms is



largely undetermined. However, for spinal motoneurons, access to neurotrophic factors appears to be the predominant limiting factor (Oppenheim, 1989). The development, survival and maintenance of the nervous system are largely dependent upon the ability of cells to obtain these neurotrophic factors. While the retrograde transport of target derived neurotrophic factors is a major source of trophic support during NOCD, support from various other sources and the ability of neurotrophins to be anteriogradely transported within the neuron has been firmly established. Significant levels of neurotrophic support are also derived from within the neuron's environment from both surrounding glial cells and neurons themselves using autocrine/paracrine mechanisms (Lindsay, 1988; Stockli *et al*, 1991; Schecterson & Bothwell, 1992; Acheson *et al*, 1995; Pruginin-Bluger *et al*, 1997; Robinson *et al*, 1996).

### 1.3 Neurotrophic Factors

Neurotrophic factors are molecules that are involved in the development and regeneration of the nervous system. A number of factors have been found to elicit a trophic response from various neuronal and non-neuronal cells within the nervous system. Many of these factors belong to diverse molecular families containing members with no known function within the nervous system. Most have been found to elicit their activity via high affinity receptors, which are often tyrosine transmembrane receptors.

### 1.31 IL-6 Family

The IL-6 family consists of various pleiotrophic cytokines including interleukin-6 (IL-6), leukaemia inhibitory factor/cholinergic differentiation factor (LIF/CDF), interleukin-11 (IL-11), oncostatin M (OSM), cardiotrophin-1 (CT-1) and ciliary neurotrophic factor (CNTF). The functions of these molecules are diverse yet often overlapping, with each factor acting via similar signal transduction pathways. While CNTF acts predominantly within the nervous system other members, such as IL-6 and LIF, illicit responses from both neural and non neural tissue. Conversely, members such as OSM and CT-1 have no known function within the nervous system. The IL-6 family receptor complex consists of a ligand specific α-subunit receptor molecule and a ubiquitously expressed, 918 amino acid common signal transducing β-subunit known as gp130. Both subunits are required for a signal transduction response to occur.

IL-6 is a single chain glycoprotein, 184 amino acid residues in length, capable of undergoing extensive post-translational modification. As a result it is produced in a variety of isoforms of variable size (21-30kDa) depending on its site of expression. It is highly conserved between different organisms and activity is mediated via the IL-6R α-subunit. IL-6 appears to play a diverse role in host defence mechanisms. Within the PNS, production of IL-6 has been observed in the sensory and sympathetic ganglia and in Schwann cells in response to neuron injury (Gadient & Otten, 1996; Grothe



*et al*, 2000). IL-6 expression levels are rapidly elevated in the nerve stumps following peripheral nerve lesion (Bolin *et al*, 1995; Reichert *et al*, 1996).

Like IL-6, LIF is also shown to prevent neural cell death caused by a variety of insults. The first report of a function within the nervous system was the observation that LIF was capable of supporting cholinergic differentiation in sympathetic neurons (Yamamori *et al*, 1989). It has since been found to affect the survival of both sensory and motoneurons (Cheema *et al*, 1994a; Cheema *et al*, 1994b). The LIF gene encodes a mature length protein of 180 amino acid residues and, like IL-6, cell population specific isoforms exist. The protein structure is typical of the IL-6-like factors consisting of 4 α-helices linked by 2 long loops and 1 short loop. As with other IL-6-like molecules binding of LIF to the receptor α-subunit LIFR facilitates the high affinity binding of gp130 through which the signal pathway is mediated.

A third neurotrophic member of the IL-6 family, CNTF, is observed to produce a trophic response in a wide variety of neuronal tissues. Initially identified by its ability to support the survival of parasympathetic neurons in the ciliary ganglion *in vitro* (Adler *et al*, 1979), CNTF was later found to enhance the survival of sensory neurons, motoneurons, cerebral neurons and hippocampal neurons *in vitro* (Scaper *et al*, 1986; Ip *et al*, 1991; Sendtner *et al*, 1992; Larkfors *et al*, 1994). Oppenheim (1991) did note that CNTF was incapable of rescuing many of these neuronal types *in vivo* during

NOCD, however CNTF was found to rescue motoneurons lost during this period of neurodegeneration. *In vitro*, CNTF was capable of supporting 60% of motoneurons purified from chick embryos prior to NOCD. CNTF is also observed to promote cholinergic differentiation of sympathetic neurons, the differentiation of glial progenitors into astrocytes and the maturation and survival of oligodendrocites. Some peripheral tissues, notably skeletal muscle, are also responsive to CNTF (Helgren *et al*, 1994). The CNTF specific receptor α-subunit CNTFR is predominantly expressed within neural tissue (Davis *et al*, 1991). Unlike other cytokine receptors CNTFR possess no cytoplasmic or transmembrane domain and is anchored to the cell surface by glycosyl phosphatidyl inositol linkage. The approximately 22kDa ligand forms a hexadimer with its receptor complex (2 CNTF: 2 CNTFR:1 LIFR: 1 gp130).

Like IL-6 and LIF, expression levels of CNTF are highest postnatally. Large quantities of CNTF are present in the adult sciatic nerve suggesting it may act to prevent motoneuron degeneration following nerve lesion (Arakawa *et* al, 1990). CNTF knockout mice are also seen to develop normally but may exhibit mild loss of motoneurons and muscle weakness in adulthood, suggesting possible cytokine redundancy or a possible lack of importance during development (Masu *et al*, 1993). Thus, there is evidence to suggest that CNTF and also IL-6 and LIF do not act as target derived support factors regulating neuronal population size but instead have a



neuroprotective role, acting primarily in the maintenance and regeneration of mature neurons.

### 1.32 FGF Family

Like the IL-6 family of factors, the fibroblast growth factors (FGF) consists of a number of pleiotrophic members only some of which have functions within the nervous system. Of these FGF-1 and FGF-2 have significance as neurotrophic factors. FGF-1 and FGF-2 share 55% sequence identity. Both FGF-1 and FGF-2 lack signal peptides and are mainly present in intracellular compartments. It is unclear as to whether these molecules are released in significant amounts. They mediate their activity via FGFR's, a family of tyrosine kinase receptors approximately 820 amino acid residues in length. Binding of the FGF ligand to FGFR requires a molecular association with a cell surface heparin proteoglycan to present the active FGF to its receptor (Klagsbrun & Baird, 1993). All FGF species can be expressed as a number of splice variants, which differ in their receptor binding affinities. Thus, the FGF's may act in a tissue specific manner based on the production of particular isoforms and the expression of specific FGFR's.

FGF-1 and FGF2 act as potential mitogens for astrocytes, oligodendrocytes and Schwann cells (Eccleston & Silverberg, 1985; Pettmann *et al*, 1985; Davies & Stroobant, 1990) They are also found to promote the survival of peripheral sympathetic neurons, parasympathetic

neurons and sensory neurons (Schubert *et al*, 1987; Unsicker *et al*, 1987; Eckenstein *et al*, 1991) as well as a variety of neuronal populations within the CNS including cerebral neurons, cortical neurons, hippocampal neurons and motoneurons. Administration of FGF-2 to *in vitro* cultures of motoneurons purified prior to NOCD shows FGF-2 capable of supporting 50% of cells. When administered in association with CNTF 100% survival of purified motoneurons is observed. (Morrison *et al*, 1986; Walicke *et al*, 1986; Sendnter *et al*, 1991). FGF-1 is expressed in high levels within sensory neurons and motoneurons and at lower levels in the substantia nigra and basal forebrain cholinergic neurons as well as several subcortical neuronal populations (Eide *et al*, 1991; Bean *et al*, 1992; Stock *et al*, 1992; Koshinaga *et al*, 1993). Within the CNS FGF-2 is expressed in astrocytes and some pyramidial cells of the hippocampus. Both FGF-1 and FGF-2 have been shown to promote the regeneration of peripheral neuron axons following lesion as well as promoting neurite outgrowth effects in injured CNS neurons (Cordiero *et al*, 1989, Ray *et al*, 1997). Gothe *et al* (2001) showed that FGF-2 was upregulated in response to peripheral neuron injury. It was also shown to upregulate the low weight isoform of IL-6 and IL-6R in Schwann cells. This suggests that FGF-2 may be responsible for the induction of the IL-6 system as an early response to neuron damage, prior to the initiation of the FGF mediated regeneration, via a paracrine autocrine mechanism involving the surrounding glia and macrophages (Meisinger & Gothe, 1997; Gothe *et al*, 2000)



### 1.33 IGF Family

Insulin-like growth factor I and II (IGF-I and IGF-II) both have roles in the nervous system. IGF-I and IGF-II are anabolic peptides, 70 and 67 amino acid residues in length respectively. Although both are expressed in most tissues during embryonic development IGF-II is more abundantly expressed *in utero* while expression levels of IGF-I peak postnatally (Stylianopoulou *et al*, 1988; Bartlett *et al*, 1991; Bartlett *et al*, 1992; Bondy & Lee, 1993; Lee *et al*, 1993) The promotion of survival appears primarily mediated through the Type I IGF receptor, a heterodimer consisting of paired, disulphide linked $\alpha$ and $\beta$ subunits sharing structural homology with the insulin receptor. IGF-I and IGF-II are expressed in discrete areas of the nervous system including the brain, spinal motoneurons and the spinal autonomic ganglia (Lund *et al*, 1986; Murphy *et al*, 1987; Rottwein *et al*, 1988; Garcia-Segura *et al*, 1991). Within these tissues, or in association with them, IGF-I and IGF-II expression has been observed in astrocytes, oligodendrocytes, skeletal muscle satellite cells and Schwann cells. Within those neurons that express IGF-I, the highest levels of expression usually occurs during periods of neuronal cell proliferation (Bartlett *et al*, 1991; Bartlett *et al*, 1992; Bondy & Lee, 1993). Expression is also evident post-natally in glial cell progenitors (Bartlett *et al*, 1992). In contrast IGF-II expression in the brain occurs predominantly in mesenchymal and neural crest derivatives during embryogenesis (Stylianopoulou *et al*, 1988). IGF-I and IGF-II have been shown to promote the survival of neurons during

NOCD as well as facilitate regeneration in injured neurons. IGF-I is capable of inducing intra muscular nerve sprouting and has been shown to increase the rate of sensory nerve regeneration following lesion of the sciatic nerve (Kanje *et al*, 1989). IGF-II has also been found to stimulate motoneuron and sensory nerve regeneration following crush injury to the sciatic nerve (Near *et al*, 1992; Glazner *et al*, 1993).

### 1.34 GDNF Family

Glial derived growth factor (GDNF) was first isolated as a growth factor capable of promoting the survival of embryonic dopaminergic neurons providing a potential therapeutic agent for Parkinson's disease, a degenerative disease of the dopaminergic neurons. GDNF has also been shown to be a potent survival factor for motoneurons and noradrenergic neurons and can regenerate sensory axons following spinal injury (Henderson *et al*, 1994; Arenas *et al*, 1995). It has been shown to be a member of a larger family, which includes neurturin, persephin and artemin. As well as promoting the survival of dopaminergic neurons and motoneurons, some of these molecules also regulate the development of enteric, sympathetic and parasympathetic neurons, as well as having functions outside of the nervous system, eg. kidneys and testis (Pichel *et al*, 1996; Heuckeroth *et al*, 1999; Meng *et al*, 2000). These factors are distant members of the transforming growth factor-β (TGF-β) super family. The GDNF family share 40-50% sequence homology. Unlike other TGF-β



members, which signal through serine threonine kinase receptors, GDNF-like factors signal via tyrosine kinase receptors (Jing *et al*, 1996). The GDNF-like factors bind specifically to members of the GFR-α family. GFR-α facilitates binding of these ligands to Ret, through which signal transduction occurs. GDNF has also been observed to trigger Ret independent signalling via its specific receptor GFR-α1.

### 1.4 Neurotrophins

The neurotrophin family is made up of a group of structurally related survival and differentiation proteins. To date six members of the family have been described: NGF, BDNF, NT-3, NT-4/5, NT-6 and NT-7. Neurotrophins regulate the growth and survival of distinct populations of peripheral and central nervous system neuronal cells, as well as some non-neuronal cell populations. They act to regulate the size and connectivity of neural populations. Neurotrophin expression is generally highest during the period of NOCD, with lower levels of expression continuing through into adulthood. The onset of NOCD often coincides with an increase in the expression levels of various neurotrophins. Following their release at the innervation sites, neurotrophins are internalised via the action of receptor molecules on the cell surface. They are then retrogradely transported to the cell body (Hendry *et al*, 1974; Schwab *et al*, 1979). The different neurotrophins and their specific Trk receptors possess both distinct and overlapping distributions in neuronal and non-neuronal cells of the CNS and

PNS. Expression of neurotrophins generally corresponds to that of their specific Trk receptor both spatially and temporally, however some tissues are known to only express mRNA for either the ligand or the receptor (Yamamoto *et al*, 1996) suggesting factors have the potential to act from a diffusible distance.

### 1.41 NGF

NGF was originally isolated from the murine sarcoma cells. Bueker (1948) observed that when murine sarcoma 180 was grafted on to the body wall of pre-NOCD chick embryos, sensory nerve fibres emerged from the adjacent dorsal root ganglia (DRG) and entered the neoplastic tissue. Further studies by Levi-Montalcini and Hamburger (1951) showed that sympathetic fibres as well as sensory fibres entered the tumor tissue. The fibres were seen to construct an extremely dense network and the sensory and sympathetic ganglia that innervated the tumor were observed to undergo a progressive increase in volume. The hypothesis that the grafted sarcoma cells were secreting a factor which elicited a neurotrophic response was confirmed when Cohen *et al* (1954) isolated a nucleoprotein fragment from these cells capable of promoting nerve growth activity *in vitro*. An attempt to isolate the protein component of this fragment utilising snake venom as a source of the nucleic acid degrading enzyme phosphodiesterase, led to the serendipitous discovery of the neurotrophic factor within snake venom itself. From the snake venom Cohen isolated a



nondialysable, heat labile protein of approximately 20kDa capable of promoting nerve growth. Administration of this factor into 6-8 day old chicken eggs for 3-5 days resulted in an increased growth in sympathetic and sensory neurons as well as excessive production of their fibres. Furthermore, treatment of neonatal mice with antisera against NGF lead to the near total disappearance of sympathetic paraganglia and prevertebral chain glia (Levi-Montalicini & Booker, 1960; Levi-Montalicini, 1964), suggesting that NGF was essential for the differentiation and survival of sympathetic nerve cells. Cultured sensory and sympathetic neurons derived from the ganglia of 8 to 11 day old chick embryos were found to require the supplementation of NGF to their culture medium (Levi-Montalicini & Angeletti, 1963)

It soon became apparent that NGF was present in a number of tissues from a variety of sources. NGF has been found to act on a number of cell populations including aforementioned neural crest derivatives, the CNS and some cells of non-neuronal origin such as mast cells. Within the central nervous system NGF has been shown to support various cholinergic neurons, such as those of the corpus striatum, basal forebrain and septum, as well as adrenergic, indoleaminergic and peptidergic neurons. NGF's specific Trk receptor is not expressed in motoneurons. However, NGF has been shown to elicit a mild trophic response from some motoneuron populations, possibly through the low affinity neurotrophin receptor p75 (Yan *et al*, 1993).

Sequencing of NGF derived from the mouse submandular gland (Hogue-Angeletti & Bradshaw 1971) showed that the NGF gene encoded for a 307 amino acid precursor peptide. Translation of the NGF gene leads to the production of two forms of precursor molecules, the long (I) and short (II) precursors, which are sequentially cleaved at the N-terminal to form a 13 kDa mature protein 118 amino acid residues in length. The NGF precursor also undergoes cleavage at the C-terminal. It is thought that the short precursor is involved in the correct folding of the molecule. NGF shows a high degree of evolutionary conservation and is active as a homodimer held together by hydrophobic bonds. NGF mRNA is short lived. Tang *et al* (1997) noted that the 3' untranslated region of NGF mRNA contained a short *cis*-acting, AU-rich sequence common to other short lived RNA's. This instability determinant motif results in a half-life of 30-60 minutes for NGF mRNA.

**1.42 BDNF**

The purification of a second related neurotrophic factor, brain derived neurotrophic factor (BDNF), led to the realisation that NGF was part of a larger family of neurotrophic factors, known as the neurotrophins. BDNF was purified from porcine brain by Barde *et al* (1982). Unlike the abundant expression levels of NGF, which facilitated its isolation, BDNF is present in low levels in the nervous system. BDNF was observed to be expressed in various neural cell populations, including neural crest and placode-derived sensory neurons, retinal ganglia, mesencephalic dopaminergic neurons,



spinal and facial motoneurons, basal forebrain cholinergic neurons, dorsal root ganglia and nodose ganglia (Ernfors & Persson, 1991; Eide *et al*, 1993; Korsching, 1993) as well as various non-neuronal cell populations (Hallböök *et al*, 1993)

Protein analysis displayed similar characteristics to NGF having a molecular weight of 13.5 kDa and an isoelectric point of 10.2 (Barde *et al*, 1982; Barde *et al*, 1987). Cloning of the gene (Leibrock *et al*, 1989) revealed a 252 amino acid precursor peptide which, like NGF, undergoes successive post translational modification to produce the 119 amino acid mature factor. Like NGF, it is active as a homodimer, a common feature of all known neurotrophins. In comparison to other neurotrophic factors BDNF shows an extremely high level of evolutionary conservation with mammalian BDNF sharing approximately 95% homology to chick and approximately 90% homology to fish (Isackson *et al*, 1991; Götz *et al*, 1992). BDNF displays approximately 50% sequence homology to NGF, including the absolute conservation of six cysteine residues that in NGF have been shown to form three disulphide bridges, and extremely high homology in the regions surrounding these residues (Leibrock *et al*, 1989).

Analysis of the sequence homology between NGF and BDNF initiated a search for further members of the neurotrophin family. Low stringency PCR, utilising degenerate primers aimed at regions of high homology, led to the cloning of a further four neurotrophins: neurotrophin-3

(NT-3) (Hohn *et al*, 1990; Maisonpierre *et al*, 1990), neurotrophin-4/5 (NT-4/5) (Hallböök *et al*, 1991), neurotrophin-6 (NT-6) (Götz *et* al, 1994) and neurotrophin-7 (NT-7) (Lai *et al*, 1998; Nilsson *et al*, 1998).

### 1.43 NT-3

NT-3 was cloned independently by two research groups in 1990 (Hohn *et al*, 1990; Maisonpierre *et al*, 1990). Like NGF and BDNF it is a small (~13 kDa), highly basic protein, existing in an active form as a homodimer (Alter *et al*, 1994). Translation leads to the production of two forms of precursor molecules, however, unlike the NGF precursor, NT-3 and BDNF undergo no proteolytic modification at the C-terminal. The mature NT-3 protein is 119 amino acid residues in length (Hohn *et. al.*, 1990) and possesses ~55% homology with both the NGF and BDNF protein sequences (Ernfors & Persson, 1991). Between the three species, there are 54 conserved amino acids, including the six cysteine residues.

In comparison to NGF and BDNF, expression levels of NT-3 are extremely low. NT-3 mediates a range of trophic effects, including proliferation, survival and maintenance of distinct cell populations, depending upon the target population it acts on. In migrating neural crest cells NT-3 acts as a mitogen, allowing targeted cells to proliferate (Kalcheim *et al*,1992; Chalazonitis *et al*, 1994). Proliferation is especially enhanced in the presence of somites, suggesting signals of somitic origin may assist in modulating the



NT-3 response. Within the developing CNS, NT-3 primarily aids survival and maintenance of neurons, whereas in the mature and aging nervous system, NT-3 plays a major role in neuron maintenance. NT-3 has also been associated with the induction and maintenance of enhanced synaptic activity (Kim *et al*, 1994). NT-3 has been shown to provide support for a number of cell types both *in vivo* and *in vitro*. Within the PNS it is capable of supporting dorsal root, cranial and sympathetic ganglia (Maisonpierre *et al*, 1990; Rosenthal *et al*, 1990; Gaese *et al*, 1994). Within the CNS, NT-3 has been shown to promote survival of spinal motoneurons (Hughes *et al*, 1993) as well as various regions of the brain, including the neurons of the cerebellum, hippocampus, striatum, substantia nigra as well as cortical neurons (Cheng & Mattson 1994; Hyman *et al*, 1994; Morfini *et al*, 1994; Gao *et al*, 1995; Ventimiglia *et al*, 1995).

### 1.44 NT4/5

The discovery of the first three neurotrophins was followed by the characterisation of the Xenopus derived neurotrophic factor, NT-4 (Hallböök *et al*, 1991). xNT-4 appeared to share similar neuronal specificity to BDNF as well as mediate activity via the same kinase receptor as BDNF. Cloning of NT-4 from rat and human sources revealed significantly lower evolutionary conservation when compared with the sequence homology of NGF, BDNF and NT-3 between amphibians and mammals. An independent study cloned the mammalian homologue proposing that the mammalian sequence

represented a novel neurotrophin designated NT-5 and not a homologue of xNT-4 arguing that, in contrast to xNT-4, rNT-5 and hNT-5 were capable of efficiently binding to the NGF receptor TrkA (Berkemeier *et al*, 1991). Further analysis by Ip *et al* (1993) showed xNT-4 and the mammalian NT-5 homologues were incapable of binding to TrkA in the restrictive environment of neuronal cells. Other similarities, such as the ability to elicit neurotrophic response at identical concentrations, further supported the theory that these factors were indeed homologues. Chromosomal mapping of xNT-4 and hNT-5 enabled the recognition of these factors as homologues of the same neurotrophin. Thus, the mammalian neurotrophin species was renamed NT-4/5 reflecting its relationship to xNT-4 (Eide *et al*, 1993). NT-4/5 is a much more evolutionarily divergent molecule when compared with other members of the neurotrophin family. No homologue has yet been found in fish or birds. Despite the absence of an avian homologue, xNT-4 appears to elicit a response in avian neurons, although hNT-5 displays no such activity (Fandl *et al*, 1994). In contrast, NGF, BDNF and NT-3 display quite high levels of interspecies cross reactivity, demonstrating the greater degree of sequence divergence amongst NT-4/5 homologues. While NT-4/5 and BDNF bind to the same receptor, the regions of the ligands thought responsible for receptor binding show little homology suggesting these factors bind to partially distinct epitopes within the TrkB receptor. Evidence suggests the low affinity neurotrophin receptor p75 may also play a part in determining TrkB specificity (Rydèn *et al*, 1995).



NT-4/5 is expressed at extremely low levels. It is expressed in a wide array of neuronal and non-neuronal tissues and appears to be less influenced by environmental signals compared to other neurotrophins (Timmusk *et al*, 1993). As with other neurotrophins, embryonic expression levels are seen to correlate with the onset of NOCD. However, following a decrease in expression after NOCD, NT-4/5 expression is seen to increase again postnatally.

**1.45 NT-6 and NT-7**

A fifth member of the neurotrophin family of factors was reported in 1994 (Götz *et al*, 1994). Cloned from the teleost fish *Xiphophorus maculatus*, NT-6 showed similarity in expression to NGF, however its activity was not eliminated by anti-NGF antibodies. As with other neurotrophins, the NT-6 gene encodes a sequentially cleaved 283 amino acid precursor, from which a 143 amino acid mature protein is produced of approximately 16 kDa with an isoelectric point of 10.8. However, while it shows evolutionary conservation of 61% with fish NGF, including the six cysteine residues common to all neurotrophins, a distinguishing feature of this molecule is the presence of a 22 residue insert between the second and the third cysteine containing domain. This insert contains a motif with the characteristics of a heparin binding domain. It was noted that heparin was required for the processing of the precursor *in vitro*. While this suggests NT-6 binds to cell surface heparin sulphate proteoglycans, the reasons for such an association

remains unclear as does the role, if any, such proteoglycans play in the biological activity of NT-6 (Götz *et al,* 1994).

Northern analysis revealed the onset of NT-6 expression in 8 day old fish, during organogenesis, and the continuing expression of this factor in the adult brain. Within the embryo, expression was also detected in the valvual cerebelli, a rostral protrusion of the cerebellum of teleost fish. Within the adult, expression was noted in the gills, eye and liver with low levels of expression in the skin, spleen, heart and skeletal muscle. Like NGF, NT-6 shows survival activity in the sympathetic and sensory DRG and lacks activity in ciliary and nodose neurons. However, NT-6 appears to be a neurotrophin specific to teleost fish with no homologous factors isolated from other vertebrates (Götz *et al,* 1994).

Attempts to isolate NT-6 homologues in fish other than platyfish led to the isolation of a sixth member of the neurotrophin family from carp, capable of promoting neurite outgrowth in chick DRG. Using primers directed towards a conserved region of platyfish NT-6, a partial gene sequence encoding a mature 133 amino acid protein was cloned from the *Cyprinus carpio* (Lai *et al*, 1998). Soon after the characterisation of the carp neurotrophin NT-7, the zebrafish homologue zNT7 was independently described (Nilsson *et al*, 1998). NT-7 was found to possess 66% sequence homology to NGF and NT-6 and contained the six cysteine residues and nearby conserved regions characteristic of neurotrophins as well as the R-X-



K/R-R proteolytic cleavage motif at which the mature neurotrophin protein is cleaved from its precursor. The NT-7 protein contained a 15 residue insert between the second and the third cysteine at a position corresponding to the 22 residue insert of NT-6. However, this insert did not show high homology to the NT-6 insert. Northern blot analysis showed that NT-7 was predominantly expressed in the skin and heart although weak expression was also seen in brain and intestinal tissue. This is in contrast to NT-6, which predominantly displayed high levels of expression in the brain and low expression in the skin and heart (Götz *et al,* 1994). Despite being capable of promoting cell survival in the avian and mammalian nervous systems (Götz *et al,* 1994; Lai *et al*, 1998; Nilsson *et al*, 1998), homologues of NT-6 and NT-7 have not been characterised in higher vertebrate species to date. Thus, these factors probably represent members of an NGF subfamily that evolved following the divergence of higher vertebrates from fish.

### 1.5 Neurotrophin Receptors

Neurotrophins bind to two classes of receptor molecule that differ in both their binding affinity and their biological properties: the Trk family of receptors and the p75 receptor.

### 1.51 Trk Family

An understanding of the molecular mechanisms by which neurotrophins mediate their activity was, for a number of decades, hindered by the lack of information about their signalling receptors. In 1986, Martin-Zanca *et al* (1986) isolated a chimeric oncoprotein from a human colon carcinoma, designated Trk. It was not until 1991 that the physiological role of Trk, as a signal receptor for NGF, was realised (Kaplan *et al*, 1991). The discovery of an NGF receptor led to the search for similar receptors specific to the other neurotrophin ligands, resulting in the characterisation of two more Trk receptor members; TrkB (Klein *et al*, 1991; Klein *et al*, 1992) and TrkC (Lamballe *et al*, 1991). The Trk oncoprotein was given the nomenclature of TrkA and, like its ligand, is often treated as the model Trk receptor partially due to the facilitation of its study by the scientifically important cell type, PC12, which expresses Trk A but does not express any other members of the Trk family of ligands. Neurotrophins bind specifically and with high affinity to the Trk receptor family. Neurotrophin activity is primarily mediated through these molecules. Thus, NGF binds to the TrkA receptor (Kaplin *et al*, 1991), BDNF and NT-4/5 bind to TrkB (Klein *et al*, 1991; Klein *et al*, 1992) and NT-3 binds to TrkC (Lamballe *et al*, 1991), with the potential for lower affinity binding to TrkA and TrkB. Ip *et al*, (1993) suggested that the restrictive neuronal environment may not facilitate *in vivo* association of NT-3 to TrkA. Whether NT-3's ability to bind to Trk B is physiologically relevant *in vivo* is unclear. Like their ligands Trk receptors



display both high sequence homology between members as well as high evolutionary conservation. The expression of specific neurotrophins generally correlates to the expression of their corresponding Trk receptors possessing similar distinct and overlapping regional and temporal distributions (Tsoulfas *et al*, 1993).

### 1.52 Trk A

The TrkA receptor gene encodes two tyrosine protein kinase isoforms, differing in the presence of a 6 amino acid residue sequence located in the extracellular domain in close proximity to the transmembrane domain. While the two TrkA isoforms appear to possess similar activities, the larger 796 residue isoform is primarily expressed in neuronal tissue while the shorter molecule is usually found in cells of non-neuronal origin (Barker *et al*, 1993; Horigome *et al*, 1993). While both isoforms bind to NGF with equal affinity, isoforms lacking the insert show reduced NT-3 binding (Clary & Reichhart, 1994). As with the other known Trk members, TrkA is active as a homodimer (Alter *et al*, 1994). The Trk family of neurotrophin receptors are structurally very similar. Full length isoforms of the three Trk receptors possess an extracellular domain, consisting of a signal peptide, two cysteine clusters separated by a leucine motif and two immunoglobulin C2 type motifs, a transmembrane domain, a juxtamembrane domain believed to interact with proteins that modulate receptor activity, and a tyrosine kinase

domain through which signal transduction is primarily mediated (Schneider & Schweiger, 1991; Ultsch *et al*, 1999).

### 1.53 TrkA Signal Transduction

Trk receptors are activated by a two-step process involving ligand mediated oligomerisation followed by autophosphorylation of the tyrosine residues (Ullrich & Schlessinger, 1990; Schlessinger & Ullrich, 1992). Phosphorylated tyrosine residues in the cytoplasmic tyrosine kinase domain serve as anchors for binding downstream signalling elements. Amongst these proteins is the small GTP-binding protein Ras. Ras interacts directly to mediate signal transduction through both the PI-3K/Akt survival pathway (Vaillant *et al*, 1999) and the MEK/MAP kinase pathway (Bonni *et al*, 1999). The PI-3K/Akt pathway is the primary pathway involved in NGF-mediated survival. The downstream serine/threonine kinase, Akt, suppresses apoptosis by phosphorylating various apoptotic proteins: these include p53, as well as other proteins in the p53 pathway; pro-caspase-9, a precursor of the pro-apoptotic caspase-9; Forkhead, a transcription factor that induces apoptosis by increasing levels of Fas ligand; and BAD, an inhibitor of the pro-apoptotic Bcl-2 (Datta *et al*, 1997; del Peso *et al*, 1997, Brunet *et al*, 1999). As well as targeting Akt, PI-3K also targets the IAP family of caspase inhibitors (Weise *et al*, 1999).



The MEK/MAPK pathway has a number of roles in neuronal populations including synaptic plasticity and long term potentiation. However, there is conflicting evidence for a role in neuronal survival. While NGF can induce activation of MAPK in sympathetic neurons and PC12 cells, the low effect MEK inhibition has on NGF-mediated, neuronal survival suggests the MEK/MAPK pathway is not necessary for survival (Creedon *et al*, 1996; Virdee & Tolkovsky, 1996; Kleese *et al*, 1999; Mazzoni *et al*, 1999). Instead, it appears that the major role that the MEK/MAPK pathway plays may be in the protection of neurons from death resulting from injury or toxicity (Anderson *et al*, 1999; Hetmann *et al*, 1999; Skaper *et al*, 1996). In contrast to PI-3K, MEK acts to stimulate the activity or expression of anti-apoptotic transcription factors, including Bcl-2 and CREB (Liu *et al*, 1999; Riccio *et al*, 1999). Amongst the transcription factors known to bind to the tyrosine kinase domain of Trk receptors, protein tyrosine phosphatases (PTP) appear to be involved in the inhibition of Trk activity. Inhibition of PTP activity has been shown to increase survival in nigrostriatal neurons following axotomy (Lu *et al*, 2002). In PC12 cells, the PTP SHP-1 binds to TrkA and is tyrosine phosphorylated in response to NGF (Vambutas *et al*, 1995). SHP-1 acts to dephosphorylate the tyrosine kinase domain of Trk inhibiting neurotrophin induced inhibition of apoptosis, in effect regulating the survival effects of excess neurotrophins.

### 1.54 TrkB

TrkB, the primary ligand for both BDNF and NT-4/5, is a heavily glycosilated molecule, 821 amino acid residues in length. The extracellular domain of TrkA and TrkB share 57% homology. Most of the residue similarity is located within the second and third leucine rich domains and particularly within the second Ig-like domain suggesting an importance of these domains for ligand binding. Like TrkA, expression of TrkB results in the generation of two isoforms differing in the presence or absence of a short insert in the extracellular domain. While TrkB variants lacking this sequence show normal binding to BDNF, the binding affinity of TrkB's alternate ligand, NT-4/5, as well as the promiscuous NT-3 is decreased (Strohmaier *et al*, 1996). In contrast to TrkA, alternative splicing of the TrkB receptor also results in a series of isoforms differing in their intracellular tyrosine kinase domain. Two TrkB isoforms are known which lack the tyrosine kinase domain: TrkB.T1 and TrkB.T2. The intracellular domain of these two isoforms is replaced by carboxyl terminals 23 and 21 amino acid residues in length respectively. Of the two intracellular domains the final 11 residues of TrkB.T1 and 9 residues of TrkB.T2 are unique. Baxter *et al* (1997) showed that the TrkB.T1 and TrkB.T2 were still capable of producing a signal transduction response whereas a recombinant TrkB void of an extracellular domain showed no such response. In contrast to theories implying Trk isoforms which lack a tyrosine kinase act to sequester factor, such evidence suggests these isoforms may serve a more active role mediating neurotrophin activity.



Despite the ability to bind to NT-3 *in vitro* the expression patterns of TrkB follow similar expression patterns to BDNF and NT-4/5 rather than to NT-3. TrkB transcripts are first detected at E4 in the developing chick system (McKay *et al*, 1996), where expression occurs in a wide variety of cells, including the forebrain, caudal midbrain, hindbrain, spinal cord, the trigeminal ganglion and the differentiating neural crest cells which later form the dorsal root ganglion. By midgestation TrkB expression is seen across the developing CNS and PNS network, an expression pattern which is maintained during late foetal development. In adults TrkB transcripts are present at lower levels within the brain and spinal cord. Within the brain expression is seen in most regions of the cerebellum, various areas of the mid brain, such as the medium and large neurons of the substantia nigra, superior colliculus and central gray matter, as well as regions of the brain stem (Merlio *et al*, 1992).

### 1.55 TrkC

The NT-3 receptor molecule, TrkC, is expressed as a number of different isoforms which vary in their catalytic activity. The TrkC isoforms can be broadly divided into two classes: the catalytic $TrkC^{TK+}$ isoforms and the non-catalytic $TrkC^{TK-}$ isoforms, differentiated by the presence or absence of the tyrosine kinase domain. NT-3 binds to both classes of TrkC with similar affinity. Of the $TrkC^{TK+}$ class, four isoforms are known. The full length

transcript, TrkC.K1, was first isolated from a porcine brain cDNA library. In contrast to TrkC.K1, other TrkC$^{TK+}$ isoforms differ by the presence of 14 (TrkC.K14), 25 (TrkC.K25) and 39 (TrkC.K39) amino acid inserts within the tyrosine kinase domain. The 39 amino acid insert consists of both the 14 and 25 amino acid sequence. The inserts are encoded by alternatively spliced exons (Tsoulfas *et al*, 1993; Valenzuela *et al*, 1993). While TrkC.K1 is known to activate PLC and PI-3K, TrkC.K14, TrkC.K25 and TrkC.K39 do not. This may be due to comformational differences, caused by the additional amino acids, preventing interactions between molecules. It may be possible for other molecules in the signal transduction pathway to interact with TrkC.K14, TrkC.K25 and TrkC.K39 receptors.

The TrkC$^{TK-}$ class molecules possess the same transmembrane and extracellular domain structures as the full length TrkC.K1 receptor, however, they lack the tyrosine kinase domain (Tsoulfas *et al*, 1993; Valenzuela *et al*, 1993). Four isoforms have been described, designated TrkC$^{TK-158}$, TrkC$^{TK-143}$, TrkC$^{TK-113}$ and TrkC$^{TK-108}$ based on the number of cytoplasmic residues possessed by the isoform. The sequences starting from residue 529 are derived from combinations of four distinct, alternatively spliced exons. The relevance, if any, of the tyrosine kinase lacking TrkB and TrkC receptor isoforms *in vivo* is not entirely clear. They may act as dominant, negative inhibitors to the full length receptor, sequestering factor to minimise trophic effect. Alternatively, they may induce non-tyrosine kinase mediated signal transduction.



A high correlation exists between regions of NT-3 expression and regions of TrkC expression (Alter *et al*, 1994; Lamballe *et al*, 1994). During embryonic development TrkC mRNA expression is first seen in the gastrulating embryo correlating highly with that of NT-3 (Tessarollo *et al*, 1993). TrkC mRNA is expressed in the dorsal root ganglia, forebrain and spinal cord (Lamballe *et al*, 1994). By midgestation, TrkC mRNA is present throughout the neural network. Between E11.5 and E13.5 in mice, TrkC is found in the auditory system, correlating with the migration of the otic ganglion cells from the auditory ganglion to the otic vesicle. Within the CNS, TrkC is expressed in the ventral spinal cord, the cerebellum (Purkinje cells), the cerebral cortex and the tectum of the CNS (Alter *et al*, 1994; Lamballe *et al*, 1994). Between E15.5 and E17.5 in murine development, TrkC is observed in the pyramidal cells of Ammon's horn and the germinal trigone of the cerebellum (Lamballe *et al*, 1994; Tessarollo *et al*, 1993). Similar patterns of expression are seen in the developing quail (Yoa *et al*, 1994) and chicken (Hallböök *et al*, 1993) systems.

**1.56 p75**

The dimeric transmembrane glycoprotein p75 is a common receptor to all neurotrophins. The mature neurotrophin ligands bind with a low affinity to the p75, although recent studies suggest the true high affinity ligands for p75 are actually the neurotrophin precursors (Lee *et al*, 2001). p75 belongs to a family of diversely functioned proteins, among whose

members include two tumour necrosis factor receptors, the Fas antigen, the T-cell OX40 and Mu4-IBB antigens, and B-cell antigens. The extracellular domain of the mature 399 amino acid molecule contains four negatively charged, cysteine rich repeats responsible for ligand binding, a common feature amongst TNFR members (Yan & Chao, 1991; Baldwin *et al*, 1992). The intracellular region of p75 contains several domains which may facilitate signal transduction: including the juxtamembrane domain; a type II death domain, an approximately 80 residue association module common to a number of related TNFR members; and a carboxyl-tripeptide, the PDZ domain binding site which facilitates the binding of PDZ containing proteins (Chao *et al*, 1986; Johnson *et al*, 1986; Radeke *et al*, 1987).

p75 appears to possess dual roles as the potentiator of both survival and apoptosis. p75 appears to mediate cell death and survival via independent pathways. For example, the signal transduction molecule NFκβ can promote survival for both P75 and Trk, via IKK. Conversely, p75 can be pro-apoptotic via jun (Casaccia-Bonnefil *et al*, 1996; Yoon *et al*, 1998). Adaptor proteins, such as TRAF and RIP2, are believed to mediate these dual roles of p75. RIP2, for example, interacts with p75 to up regulate NFκβ leading to the inhibition of NGF induced death by blocking the death signals through NFκβ (Khursigara *et al*, 2001). Evidence also suggests p75 may function as a presentation receptor, concentrating neurotrophins in close proximity to Trk molecules or may actually facilitate the binding of neurotrophins to their Trk receptors. Rydèn *et al* (1995) observed that the



loss of p75 results affected TrkB receptor activation by NT-4/5 but not BDNF, suggesting that p75 may aid in the discrimination between neurotrophins. Evidence also suggests the interrelationship and coregulation of p75 and Trk receptor signal transduction pathways. Roux *et al* (2001) found that p75 can augment serine phosphorylation of Akt. p75 is also able to enhance phosphorylation of SHC, possibly via a mechanism of either stabilising or presenting the molecule to TrkA by forming a receptor complex with the TrkA dimer.

### 1.6 Neurodegenerative Diseases

The degeneration of neurons observed in various neurodegenerative diseases in adults parallels many of the processes involved in NOCD in the embryo. As has been discussed previously in this chapter, numerous neurotrophic factors, including the neurotrophins, have been shown to affect the maintenance, proliferation and regeneration of neural cells in CNS and PNS of the embryo as well as the PNS of the adult. The dramatic rise and fall of neurotrophin expression spanning the period of NOCD in various neurons has drawn attention to them as potential therapeutic agents for neurodegenerative diseases of the CNS. Thus, it is hoped that research into neurotrophins may lead to not only a better understanding of neuron degeneration but also to therapy for neurodegenerative diseases, such as motoneuron disease. While neurons of the adult CNS display an inability to regenerate following trauma or

neurodegeneration, many of these cell populations affected by such damage still express neurotrophin receptors and are thus potentially responsive to neurotrophins. It has been suggested that adult cells undergoing neurodegeneration succumb due to a lack of sufficient cell repair response via neurotrophins or other neurotrophic factors. Various neurotrophins have been shown to affect, with varying degrees, the neuronal populations degenerated during the progression of motoneuron diseases (Sendtner *et al*, 1992; Koliatsos *et al*, 1993; Mitsumoto *et al*, 1994) and BDNF has already entered clinical trials as a therapeutic agent. However, despite some clinically observable short term effects, Phase III trials using BDNF as therapeutic agent against amyotrophic lateral sclerosis (ALS) had limited success.

Motoneuron diseases, such as ALS, spinal muscular atrophy and post-polio syndrome, are caused by varying amounts of degeneration of the upper and/or lower motoneurons. Such diseases are terminal with little effective treatment currently available. Studies have found that cultured motoneurons are responsive to the neurotrophins BDNF, NT-3 and NT-4/5, but not NGF (Henderson *et al*, 1993; Hughes *et al*, 1993; Becker *et al*, 1998). Further support for the role of neurotrophins in motoneuron survival is provided by the expression of the TrkB, TrkC and p75 receptors in motoneurons (Eckenstein, 1988; Merlio *et al*, 1992; Henderson *et al*, 1993, McKay *et al*, 1996). Other neurotrophic factors, such as CNTF, have also



been found to have a trophic effect on motoneuron populations (Oppenheim, 1991; Sendtner *et al*, 1992).

BDNF and NT-3 have been found to rescue cranial and spinal cord motoneurons from neuronal death and are known to be normal regulators of spinal motoneurons (Oppenheim *et al*, 1992; Sendtner *et al*, 1992; Yan *et al*, 1992; Henderson *et al*, 1993 Hughes *et al*, 1993; Yan *et al*, 1993; Li *et al*, 1994; Sayer *et al*, 2002). Following motoneuron damage, an increase in neurotrophin-mediated retrograde transport is seen (Curtis *et al*, 1998). Altered $Cu^{2+}/ZN^{2+}$ superoxide dismutase is often seen associated with ALS cases (Rosen *et al*, 1993). This suggests a role for free radicals in motoneuron injury. BDNF can also serve a neuroprotective role, reducing motoneuronal cell damage caused by such free radicals (Spina *et al*, 1992). NT-4/5 has been shown to serve a neuroprotective role in *in* vitro models of neurodegeneration (Corse *et al*, 1999; van Westerlaak *et al*, 2000). Thus there is considerable weight behind the possibility that neurotrophin therapy may assist in the treatment of neurodegenerative diseases.

Despite the experimental evidence suggesting neurotrophins have therapeutic value for the treatment of various motoneuron diseases, clinical trials using BDNF in the treatment of ALS have not been as promising as expected. Phase I and II clinical trails showed a significant decrease in neuronal deterioration following the daily administration of BDNF to ALS patients up to12 months after the commencement of treatment. The results

also implied that long term administration appeared safe and well tolerated with no serious side effects (Bradley *et al*, 1995; Ochs *et al*, 1998). In contrast, phase III trials failed to observe a significant effect of daily administered BDNF in ALS patients after 9 months (The BDNF Study Group (Phase III) 1999). However, post hoc subgroup analysis showed that BDNF endowed a survival advantage for patients who were at a greater risk of dying during the trial period suggesting the gross lumping of patients at various stages of ALS may have diluted the apparent survival affect. None the less, clinical trials have not shown BDNF to be the potent survival factor that experimental data suggests.

### 1.7 Constant Supplementation of Neurotrophins

While studies that have investigated the effects of supplying motoneurons with various neurotrophins *in vivo* during the period of NOCD have noted a significant increase in the neuronal survival rate, no study has yet reported the ability of any single factor to rescue all motoneurons during this period. Such experiments have relied on the intermittent supplementation of factor to the developing embryo. Sendtner *et al* (1992) noted that when axotomised rat facial motoneurons were suppled daily with BDNF or NT-3, the surviving cells were found to be abnormally small compared to the unlesioned control rats, implying that such treatment does not provide complete trophic support to factor responsive neurons.



The primary difficulty experienced in such factor supplementation studies lies with their inability to supply a constant source of factor. Intravenous injection of NGF has been shown to have an elimination half-life of 2-2½ hours while subcutaneous injections of the same factor have only a slightly longer elimination half-life of ~4½ hours (Tria *et al*, 1994). The intervals between factor administrations in previous studies usually exceed a day, far in excess of the factors' elimination half-lives. Such a temporal gap between the factor half-life and the interval of administration creates a window within which neuronal populations do not have access to the factor. Given the high structural similarity and sequence homology between NGF and other neurotrophins it is highly likely that such short half-lives are also displayed by other neurotrophins. Within sympathetic superior cervical ganglion neurons, the deprivation of NGF results in the detectable atrophy of approximately 50% of neuron soma after 19 hours. By 36 hours, 95% of neuronal cells atrophy. While factor deprivation of up to 12 hours causes no consistent degeneration of neuron soma or neurites (Deckwerth & Johnson 1993), it is unclear as to when cells reach an irreversible point of cell death in comparison to when the neuron atrophy is detectable. Therefore, studies that have employed intermittent factor supplementation to analyse the effects of neurotrophins on neurons cannot conclude whether or not the lack of complete neuronal survival evidenced is the result of a neuronal population unresponsive to the supplied factor or whether a responsive population has died as a result of factor deprivation. Thus, an analysis of the effects of providing a constant source of neurotrophin to neuronal populations must be

carried out to adequately characterise neuronal responsiveness to neurotrophins.

In recent years several studies have attempted to address the limitations of intermittent factor administration by employing various methods to administer a continuous supply of factor. Giehl and Tetzlaff (1996) used an osmotic pump to deliver NGF, BDNF and NT-3 via a cannula implanted into the brains of post-natal rats, showing that BDNF and NT-3 could rescue corticospinal neurons following axotomy. While mechanical delivery of factor into the CNS is effective, such methods require access to highly purified factor and are technically tedious, requiring ongoing maintenance and restricting movement in the subject. Several studies have attempted to overcome this problem using various constant expression systems. Gravel *et al* (1997) employed adenoviral transfer to examine the ability of CNTF and BDNF to support motoneurons following sciatic nerve axotomy. Detectable expression was observed for 3 weeks following delivery to the proximal nerve stump. Motoneuron survival was observed for the 5 week period of examination although the level of survival was found to decrease over this period.

The use of cell grafts, genetically modified to secrete the factors of interest, is becoming an increasingly popular method of constant factor delivery as it overcomes the technical difficulties associated with prolonged administration. Such cells grafts generally employ cell lines derived from



neural cell types to facilitate graft acceptance into the site of implantation (Strömberg *et al*, 1990; Kilpatrick *et al*, 1994; Grill *et al*, 1997a; Grill *et al*, 1997b).

Strömberg *et al* (1990) examined NGF-promoted cell survival in basal forebrain cholinergic neurons following axotomy. The murine fibroblast derived 3T3 cell line was stably modified to secrete NGF and grown in a three-dimensional rat collagen gel matrix. Implantation of the gel into the fimbria-fornix pathway of adult rats, following lesion to the area, prevented axotomy induced cell death in basal forebrain cholinergic neurons. Support of cholinergic neurons was observed to persist for over six weeks post lesion in immuno-suppressed animals.

Such factor secreting cell grafts have also been successful in reducing cell loss in models of spinal injury. Grill *et al* (1997a, 1997b) modified fibroblast cells to produce either NGF or NT-3. Three months after chronic lesion to the adult rat spinal cord, the factor secreting cells were grafted on to the lesion site. Constant supplementation of NGF was shown to augment axonal growth following injury. Under the same regime, NT-3 produced a greater response in corticospinal axon growth following spinal lesion when compared to previously published data for single dose treatment. In both instances the augmentation of neuronal support following axotomy of the mid-thoracic spinal cord continued for over three months. Thus, it is apparent that the cumulative effects of factor deprivation during

long term factor administration is an issue which must be examined before assumptions about the effectiveness of factor supplementation can be made.

The following study employs the transient transfection of the mammalian expression vector pcDNA3 (*figure 1.1*) into the 2.3D cell line to deliver factor to the embryonic spinal cord. The 2.3D cell line was derived from neuroepithelial cells isolated from the neural tube of E10 mouse embryos and immortalised with the *c-myc* oncogene using a murine retrovirus delivery system. The morphology and antigenic phenotype of the 2.3D cell line is characteristic of normal E10 neuroepithelium and while the cells do not spontaneously differentiate they have been shown to produce identifiable glial and neuronal cells in the presence of FGF-1 and FGF-2 (Bartlett *et al*, 1988). *In situ*, 2.3D cells have been found to successfully implant into the neonatal rat brain, subsequently committing to a variety of cell fates. Kilpatrick *et al* (1994) found that approximately 0.5% of the cells originally grafted were detectable after three months post implantation. Whether this low figure was due to poor graft acceptance or the down regulation of the reporter gene was unclear.



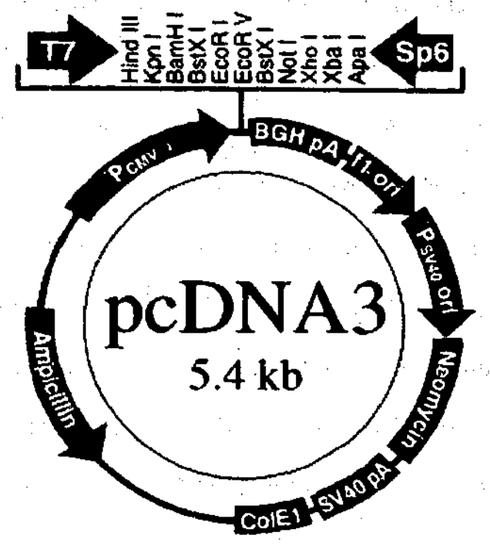



Figure 1.1: pcDNA3 mammalian expression vector (Invitrogen). In this study neurotrophin secreting cell grafts were generated by transiently transfecting 2.3D cells with pcDNA3 vectors containing neurotrophin cDNA inserts. Expression is driven by the CMV major intermediate early promoter/enhancer region and the resultant transcript is polyadenylated of the BGH polyadenylation signal. Ampicillin and neomycin resistance markers facilitate antibiotic selection in bacterial and mammalian cells respectively.

## 1.8 Unbiased Stereological Methods: The Optical Disector/Fractionator Combination

The estimation of cell numbers is essential to assess changes in cell population density resulting from NOCD and neuronal injury under normal and experimental conditions. While serial-section reconstructions provide an accurate method of determining cell numbers within a given structure, such methods are largely impractical where efficient counting regimes are required. As such, the need for efficient, unbiased methods of estimation has been of paramount importance when determining cell numbers. Prior to the 1980's, the available methods of counting tissue particles, countable 3-dimension structures such as cell nuclei, etc, were generally associated with a high degree of bias. Many required the estimations of parameters, such as mean nuclear diameter, and assumptions regarding nuclear shape, size, distribution and orientation (Abercrombie, 1946). It was the biased nature of these assumptions that created bias in particle number estimates. The increasing intensity of research into the survival effects of neurotrophic factors has had the fortune to coincide with the advent of a number of new stereological methodologies (Reviewed in Gunderson, 1986; Gunderson & Jenson, 1987; Gunderson *et al*, 1988a; Gunderson *et al*, 1988b; Bertram & Nurcombe, 1992). This new generation of counting paradigms has provided an effective method of minimising the bias, the systematic deviation from the true value, normally associated with traditional methods of cell counting.



Traditionally, estimations of cell numbers have been obtained by counting the number of cell profiles in randomly sampled sections from the tissue of interest. The total number of cells present in the original tissue is then estimated from the number of profiles per section and multiplied by the frequency with which the sections were sampled. Such estimates are flawed as the number of profiles in a section bears no simple relationship to the number of cells in the section. This is because the number of profiles in a section depends not only upon the number of cells but also upon the size, shape and orientation of the cells within the section. As a result techniques were devised to factor in estimates of cell geometry in order to overcome the bias caused by inferring cell counts from profile count data. Amongst the most widely used was the methodology adapted by Abercrombie from Linderstrøm-Lang *et al*, (1935) (Abercrombie, 1946). Abercrombie's equation effectively multiplies the profile count (n) for a section by the expected proportion of cells whose central point lies within the section and expresses this value as a fraction of the expected total number of cells that transect the section. Accurate estimation of the number of cells transecting the section has proved difficult and, as such, bias is introduced into the resultant counts. Furthermore, the accidental omission from counting of small segments that are barely within or have fallen out of the two surfaces of the section, termed lost caps, can result in a less than actual count for n (Reviewed by Hedreen, 1998). Counting methods, such as the empirical method of cell estimation (Coggeshall *et al*, 1984), that have attempted to address the bias of

Aberbrombie's equation have done so at the expense of efficiency and have therefore not gained wide favour (Hedreen, 1998).

At around the same time as the empirical method was developing, the introduction of a novel array of stereological techniques eliminated the need for assumptions of profile geometry by allowing the efficient estimation of particle numbers, and thus the unbiased cell counts. Amongst these techniques, the disector involved producing a series of paired sections, parallel to each other and separated by a known one dimensional distance. Over these sections was laid an unbiased sampling frame. Estimations of cell number were based on the sum of those particles intersected by the plane of the first section but not the second. The disector had the disadvantage of being practically rather labour intensive. The optical disector facilitated a more efficient use of many of these new stereological techniques. The advantage of the optical disector lays in its ability to use relatively thick tissue sections which could be divided into a series of conceptualised parallel planes as thin optical sections within the thick section, essentially combining several steps of the disector. Such optical sectioning can be carried out on plastic embedded tissue sections using a conventional light microscope modified with a motorised stage and an electronic microcator for measuring movements of the stage in the z-axis. Thus, accurately calibrated movements of the field of view can be carried out along all planes (Gunderson, 1986; Gunderson & Jenson, 1987; Gunderson *et al*, 1988a).



The fractionator is amongst the simplest sampling schemes and for that reason is also amongst the most powerful. Except by deliberately misappropriating data, the fractionator cannot be biased (Gunderson 1988a). The basis behind the fractionator is the estimation of the total particle number based on the number of particles in a known fraction of the item being sampled. This way factors affecting tissue volume such as shrinkage and expansion of the tissue duing the embedding process become irrelevant. Tissues are sectioned exhaustively and sampled at fixed intervals from a random starting point. The area to be counted on each section is subdivided into discrete sampling fields within which an unbiased sampling grid is included. Moving through the z-axis of the section, particles in each of the sampling fields are counted as they come into focus. Within each sampling field an estimation of the total particle number is a function of the number of particles counted by the fraction of the sample field area ($A_{X,Y}$) that is covered by the sample frame($A_f$). Therefore, an estimate for the total particle count of the section can be derived from:

$$N = Q^- \cdot \frac{A_{X,Y}}{A_f} \cdot \frac{h}{t}$$

where h/t is the proportion of the z-axis sampled.

As the sections are sampled at known intervals, the total number of particles (N) within a given tissue is:

$$N = \sum Q^{-} \cdot \frac{A_{X,Y}}{A_f} \cdot \frac{h}{t} \cdot R$$

where R is equal to the probability that any given section is sampled or the inversion of the sampling frequency. Repeated estimates of the number of particles within the tissue will provide counts varying around the true value. The efficiency of the fractionator in estimating this true value depends upon the homogeneity of the particle density within the tissue, any random variation in the size of the particle and the number of sections which are sampled. The efficiency of the fractionator can therefore be improved by systematically sampling evenly distributed sections from the tissue, enabling a reliable estimate of the nature of the entire tissue (Gunderson, 1986; Gunderson & Jenson, 1987; Gunderson *et al*, 1988a). The optical disector/fractionator combination has proved a simple yet effective technique for obtaining unbiased counts in a variety of neural cell types (Avendano & Lagares, 1996; Helsey *et al*, 1997; Lowry *et al*, 2000; Mallard *et al*, 2000; Bye *et al*, 2001; Lowry *et al*, 2001).



## 1.9 Conclusion

A desire to halt the neurodegenerative processes occurring during the progression of motoneuron disease has led to an increased interest in the means by which the nervous system supports motoneurons during NOCD. Evidence from *in vitro* and *in vivo* studies support the importance of the role neurotrophins play in promoting motoneuron survival during NOCD.

Within the developing spinal cord BDNF and NT-3 but not NGF have been shown to support LMC motoneurons. While administration of these factors to developing motoneurons does substantially increase the number of surviving motoneurons within the LMC, no single factor has been shown to rescue all motoneurons lost during NOCD. The expression of both TrkB and TrkC in the developing spinal cord supports the concept that neuronal subpopulations based on their factor responsiveness exist within gross neuronal population and that multiple factors may be necessary for the survival of all motoneurons (reviewed by Rae, 1994).

Further complicating the lack of total factor response evident in the published data, is the possibility that the short half-life of neurotrophins may potentially result in a loss of neurons caused by factor deprivation rather than unresponsiveness. The investigation of such a possibility is crucial considering the lack of success observed in therapeutic trials of BDNF for the treatment of ALS. Should the lack of neuron survival evidenced in *in vivo*

studies result from factor deprivation between administrations, the method of factor delivery during therapeutic treatment may need to be reconsidered. The following thesis outlines the results of experiments examining the effects of supplying a constant source of BDNF and NT-3 alone and in combination, to embryonic motoneurons during the period of NOCD.

# CHAPTER 2

# MATERIALS AND METHODS

# CHAPTER 2

## 2.1 Animals

The embryonated eggs of outbred white leghorn chickens were used in this study. Embryos were sacrificed via decapitation following removal from the eggs.

## 2.2 Cell Line

The 2.3D cell line was used to generate the transiently transfected cell graft employed in this study. The 2.3D cell line is a murine cell line derived from E10 neuroepithelial cells (Bartlett *et al*, 1988). Preparation of all cell lines was carried out by Dr Karen Bailey, School of Biological Sciences, Monash University. Constructs of the pcDNA3 murine expression vector (*figure 1.1*) containing cDNA of the pre-pro-neurotrophin gene sequence (obtained from Karen Bailey) were transiently transfected into the 2.3D cell line to generate the factor secreting cell grafts.

## 2.3 Transient Transfection of 2.3D Cell line

A calcium phosphate method was used to transiently transfect the murine 2.3D cell line with the pcDNA vector containing either the full length BDNF cDNA, full length NT-3 cDNA or no insert (control). The 2.3D cells were grown to an almost confluent concentration then split with a 1:15 ratio



on the day prior to transfection. The cells were plated in 5ml DMEM/10% FCS in 25cm$^2$ tissue culture flasks. 10μg of vector DNA was precipitated in ethanol on the day prior to transfection. This allowed the DNA to be sterilised as well as resuspended in the correct volume. On the day of transfection the ethanol precipitated DNA was centrifuged, washed with 70% ethanol and the pellet air dried in a laminar flow hood. The pellet was resuspended in 450μl sterile water and the $CaCl_2$ concentration adjusted to 0.25M by the addition of 50μl of 2.5M $CaCl_2$. 500μl of 2 X HeBS (0.28M NaCl, 50mM HEPES, 1.5mM $Na_2HPO_4$, pH 7.05) was added to a sterile 15ml conical tube and the DNA/$CaCl_2$ solution slowly added dropwise to the tube. A mechanical pipettor with a 1ml pipette attached was used to blow air through the HeBS while the DNA/$CaCl_2$ was being added to ensure constant mixing of solutions. After addition of all the DNA/$CaCl_2$ the sample was vortexed then incubated 20 minutes at room temperature. The DNA precipitate solution was added to a culture flask containing 2.3D cells and incubated at 37°C in a humidifed incubator for 16 hours. After 16 hours, the media was removed and the cells washed with 5ml of 1X PBS. 5ml of fresh DMEM/10%FCS was added and the cells were incubated for a further 2 days.

Transfected cells were harvested by removal of the media and washing the cells with 5ml of PBS. Cells were treated with 2.5ml of 0.25% Trypsin in 1X PBS for 5 minutes at 37°C to detach them from the plate. The tryspin was inhibited by the addition of 0.5ml of FCS and the cells centrifuged at 160xg for 5 minutes. The cells were then washed twice with 5ml of 1X

PBS, resuspended in 3ml 1X PBS and a cell count obtained using a Neubauer haemocytometer. The cell concentration was adjusted to the required density with 1X PBS.

### 2.4 *In Ovo* Embryo Manipulation

All embryo manipulation was carried out in a laminar flow cabinet using sterile equipment. White Leghorn chicken eggs were incubated at 39.5°C with the blunt end facing up until embryonic day 5 (E5). At E5 the surface of the egg was sterilised by flooding with 70% ethanol. The blunt end of a pair of forceps was used to make a small hole in the blunt side of the egg. The shell was carefully broken away to produce an approximately $1cm^2$ aperture above the air sac. 1ml of sterile 1X PBS was added into the aperture to wet the outer membrane before drawing off the excess liquid with a micropipette. The outer membrane was removed using forceps and discarded. Fine forceps were used to manipulate the embryo into a position such that the optic tectum was accessible. Care was taken to avoid any haemorrhaging of the blood vessels surrounding the embryo. A 1ml syringe fitted with a 26 gauge needle was used to inject 100µl of cell suspension (~20,000 cells) into the optic ventricle. Recombinant cells containing the pcDNA3 vector, the pcDNA3/BDNF construct, the pcDNA3/NT-3 construct or a mixed population of cells containing the BDNF and the NT-3 constructs, were pressure injected into the optic tectum of the embryo. Following surgery the aperture was sealed with tape (Scotch Magic Tape, 3M) and the eggs incubated at 39.5°C.



### 2.5 Limb Bud Amputation

Limb bud amputation was carried out at E5. White Leghorn chicken eggs were incubated at 39°C with the blunt end facing up until embryonic day 5 (E5). At E5 an aperture was created in the blunt side of the egg and the outer membrane removed, as described in Section 2.4. Forceps were used to manipulate the embryo into a position such that the lower half of the embryo was accessible. Using fine forceps, one of the embryo's lower limb buds was severed by pinching off the limb close to the pelvic region. The aperture was sealed with tape (Scotch Magic Tape). The eggs were returned to the incubator and incubated at 39.5°C until harvesting. Where experiments examined the effects of factor supplementation on target ablation, cells containing the pcDNA3 vector, the pcDNA3/BDNF construct, the pcDNA3/NT-3 construct or a mixed population of the two latter cell lines were pressure injected into the optic tectum of the embryo immediately following limb bud removal, as described in Section 2.4.

### 2.6 Dissection and Processing of Spinal Tissue

Spinal tissue was harvested from the chick embryo at daily intervals between E6 and E15 for analysis of the motoneuron population counts during NOCD and at E10 or E15 for factor supplementation analysis. The aperture in the egg was widened using surgical scissors and the embryo removed using hooked forceps. Following rapid decapitation the embryos were immersed in 1X PBS to avoid desiccation of the tissue. The embryos

were dissected with the aid of a dissecting microscope. Using fine, curved forceps the internal organs and tissue of the body cavity were removed. The aorta and *vena cava* were peeled back so as to expose the spinal cord. The spinal tissue was transversely dissected above and below the lumbar segments 1 and 8 respectively using spring scissors and the dorsal and ventral roots of the lumbar spinal nerves were severed. The lumbar spinal tissue was immersion fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 1 hour.

The fixed tissue was dehydrated on a rotator at room temperature in 70% ethanol overnight. The 70% ethanol was replaced and incubated for a further hour followed by three 2 hour incubations in 100% ethanol. The tissue was placed in 100% butanol for 2 hours. The 100% butanol was replaced and the sample was further incubated overnight. The tissue was infiltrated and embedded in glycolmethacylate. This involved infiltrating the tissue overnight in Technivit 7100 infiltration solution (1g Technovit 7100 Hardener I/ 100ml Technovit 7100 liquid) (Heraeus Kulzer GmbH). The infiltrated tissue was embedded in glycolmethacylate embedding solution (1ml Technivit 7100 Hardener II/ 15ml Technivit 7100 infiltration sclution) (Heraeus Kulzer GmbH). The glycomethacrylate blocks were allowed to set completely before adhering a backing block. Backing blocks were made by combining a 2:1 ratio of Technovit 3040 powder to liquid (Heraeus Kulzer GmbH). The backing block resin was poured into an embedding mould and allowed to set. Liquid Technovit 3040 resin was used to adhere the back of the methacrylate blocks to the backing block.



## 2.7 Sectioning

Methacrylate blocks were sectioned on a microtome (Leica RM 2165) to a thickness of 20µm. Sections were sampled from the 4$^{th}$ section following the first appearance of tissue in the sections and every 30$^{th}$ section thereafter until the tissue was exhausted. Two consecutive sections were sampled each time for the purpose of allowing for faults in the former section. Sections were immersed in water before placing on the slide. Slides were then incubated at 60°C for 30 minutes.

## 2.8 Staining

Slides were dried in an oven at 90°C for 3 hours. Slides were then stained with Harris haematoxylin (Amber Scientific) for 45 minutes, washed in water and immersed in Scott's tapwater (8mM KHCO3, 200mM MgSO4(7H20)) for 90 seconds. If necessary, sections were placed in acid alcohol (5% concentrated HCl, 70% ethanol) for 30 seconds to remove excess stain and again treated with Scott's water. Slides were counterstained with 0.5%w/v aqueous eosin (Amber Scientific) for 15 minutes, dipped in 100% ethanol, placed in 70% ethanol for 30 seconds and washed in water. The slides were dried at 60°C for 3 hours and placed in xylene for a few minutes prior to cover slipping. Cover slips were adhered to slides using polystyrene mounting medium.

## 2.9 Stereology

The optical disector/fractionator technique was used to determine the number of motoneurons present in the LMC of the spinal cord. The LMC was discernible as a population of primarily large deeply staining cells clearly demarcated from the ventrolateral grey matter. Within the LMC, motoneurons were differentiated from the surrounding cells as being large deeply staining cells with large nuclei and deeply stained nucleoli. Motor nuclei were counted using a X100 oil immersion lens (NA 1.4, Olympus) on an Olympus BX50 compound microscope. The image was projected on to a cumputer monitor via a JAI Protec video camera. The Castgrid 1.10 (Olympus) software package was used to generate a random sample field and an unbiased, two-dimensional counting frame over the projected image. A counting frame with an area of 1067μm was used within a sampling grid consisting of 2500μm sampling frames (X = 50μm, Y = 50μm). The counting frame and sampling frame areas were reduced to 1067μm and 1600μm (X = 40μm, Y = 40μm) respectively when analysing E6 and E7 samples owing to the denser cell populations. A microcator (Microcator 2000, Heidenhain) monitored the depth of the section being analysed.

The upper surface of each section was brought into focus. The first 3μm of the section was traversed to allow for aberrations on the section surface and the microcator set to zero. The section was traversed a further 10μm through the z-axis. Nuclei were counted as they came into focus within



this intermediate zone of the section provided they did not intersect the forbidden boundaries of the counting frame or their extensions.

The estimated number (N) of motoneurons in the LMC was calculated using the equation

$$N = \Sigma Q^{\cdot} \cdot \frac{A_{X,Y}}{A_f} \cdot \frac{h}{t} \cdot R$$

where $\Sigma Q^{\cdot}$ is equal to the sum of the motoneuron counts from each section, $A_{X,Y}/A_f$ is equal to the proportion of the sample field area ($A_{X,Y}$) analysed in the counting frame area ($A_f$), h/t is equal to the the section thickness (h) divided by the amount of the z-axis analysed (t) and R is equal to the sampling frequency. Thus, where the sections were sampled every 50μm in the X and Y axis and 10μm of each section was sampled using a counting frame with an area of 1067μm,

$$N = \Sigma Q^{\cdot} \cdot \frac{(50 \cdot 50)}{1067} \cdot \frac{20}{10} \cdot 30$$

Motoneuron counts were expressed as mean counts with standard errors of the means.

## 2.10 Statistical Analysis

Statistical analysis was carried out using Minitab 13 (Minitab Inc). Student's t-test and Tukey's pos-hoc test for significance were used to compare sample groups. Two way ANOVA was used to analyse the interaction between embryonic ages under each regime *(Appendix 1)*.

## 2.11 Oligonucleotide Primers

The following oligonucleotide primers were employed in this study to amplify cDNA fragments of TrkB, TrkC and β-actin respectively from chick derived cDNA. The resulting product fragment lengths for each primer pair are given.

TrkB – 798 nucleotide pair cDNA fragment

| | |
|---|---|
| chTrkBF | $^{5'}$TCCTCTCCATCACATCTC$^{3'}$ |
| chTrkBR | $^{5'}$TCCCATAATACAACTCCC$^{3'}$ |

TrkC - 740 nucleotide pair cDNA fragment

| | |
|---|---|
| chTrkCF | $^{5'}$TCTTCAACAAACCTACTCAC$^{3'}$ |
| chTrkCR | $^{5'}$ACCACACACACCATAGAAC$^{3'}$ |

β-actin – 785 nucleotide pair cDNA fragment

| | |
|---|---|
| chactinF | $^{5'}$AAACTACCTTCAACTCCATC$^{3'}$ |
| chactinR | $^{5'}$CCTTCATTCACATCTATCAC$^{3'}$ |



## 2.12 Dissection of Spinal Cords and RNA Extraction

At E5, embryos were implanted with the modified 2.3D cell line containing either the pcDNA3/BDNF construct or the pcDNA3/NT-3 construct, as per Section 2.4. Embryos that had not undergone implantation were used as controls.

Total RNA was extracted from chick spinal cord tissue at E8, E10 and E15. The embryo was dissected as per Section 2.5 to expose the spinal tissue. Transverse incisions were made immediately above and below the lumbar region of the spine using spring scissors. The dorsal and ventral roots were severed and the spinal tissue was lifted from the body cavity and immersed in 1X PBS. The tissue surrounding the spinal cord was cut and peeled away using fine forceps. Any ventral and dorsal root tissue attached to the spinal cord was removed. The spinal cords were placed in separate microfuge tubes with 500μl RNA-Bee™ (Tel-Test) and fragmented by homogenisation. A further 500μl of RNA-Bee™ was added to the homogenate and mixed. Chloroform (100μl/ml of homogenate) was added to the homogenate and mixed vigorously for 15 seconds before placing the tubes on ice for 5 minutes. The tubes were centrifuged in a microcentrifuge at 8,000xg for 15 minutes and the upper aqueous phase was transferred to a fresh microfuge tube. An equal volume of isopropanol was added to the upper aqueous phase and incubated at 4°C for 15 minutes. The tubes were centrifuged at 8,000xg for 15 minutes, the supernatant was discarded and the

pellet washed with 70% ethanol. The solution was re-centrifuged at 8,000xg for 5 minutes and the pellet was resuspended in 50µl of millipore purified water. The concentration and purity of the RNA was measured spectrophotometrically at 260nm and 280nm. The samples were stored at -20$^{O}$C.

### 2.13 Reverse Transcription

Chick mRNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase. 1µg total RNA and 250ng oligo dT was incubated at 65$^{O}$C for 10 minutes to denature the RNA followed by immediately cooling the reaction tubes on ice to prevent re-annealing of the RNA. 1X AMV RT buffer (Promega), 10mM DTT (Promega), 0.5mM of each dNTP (Promega), 20U RNasin (Promega), and 10U of AMV reverse transcriptase (Promega) were added to the reaction tube and the volume was made up to 20µl using millipore purified water. The reaction was incubated at 42$^{O}$C for 1 hour followed by an 80$^{O}$C incubation for 5 minutes to denature the enzyme. The product was stored at -20$^{O}$C until required.

### 2.14 Polymerase Chain Reaction (PCR) Cycle Optimisation

cDNA derived from P2 chick cerebellum was used to optimise the PCR cycle number, such that the period of exponential amplification could be determined. 1µl of the cDNA product was added to a 25µl reaction containing 0.8µM of each of the chTrkBF and chTrkBR primers, 1X thermophilic DNA



polymerase buffer (Sigma), 0.2mM of each dNTP (Promega), 2.5mM $MgCl_2$ (Promega), and 1U DNA Taq polymerase (Promega) per reaction. Reaction samples were first produced as a master mix prior to aliquotting into reaction tubes, in order to ensure uniformity between reactions. Negative controls, consisting of reactions in which the cDNA templates were not added and reactions containing the original RNA samples in place of the reverse transcription products, were performed in parallel. Reactions were carried out in 200µl domed cap tubes in a thermal cycler possessing a heated lid (GeneAmp PCR System 2400, Perkin Elmer). The tubes were heated to 94$^{O}$C for 5 minutes to fully denature the cDNA. The PCR reaction was incubated for a 45 second denaturing step at 94$^{O}$C, a 45 second annealing step at 60$^{O}$C and a 1 minute extension step at 72$^{O}$C. The number of amplification cycles used was determined by optimising the reaction for exponential amplification (Refer to Section 2.23).

### 2.15 Electrophoresis of Samples

5µl of loading buffer was added to the PCR samples. The solution was mixed and the tubes pulsed in a microcentrifuge (Microcentaur, MSE). The samples were then electrophoretically analysed by running the samples on a 1.2% agarose gel (Progen) containing 300ng/ml ethidium bromide (BioRad), at 80V for 40 minutes in 1X TBE buffer (2mM EDTA, 100mM Tris.HCl, 100mM, Boric acid, pH 8). A 1Kb ladder (GIBCOBRL) was also run on the gel as a size

marker. The gel was viewed under ultra violet light to ensure amplification had taken place.

### 2.16 Southern Hybridisation

To determine the relative amount of amplified cDNA present in the gel, the cDNA was transferred via capillary action to a nylon membrane and probed using radioactive DNA probes.

### 2.17 Capillary Transfer of DNA from Agarose Gels to Nylon Membranes

Following the agarose gel electrophoresis of the PCR products, the corner of the gel was cut to orientate the gel. The gel was rinsed with distilled water and the DNA denatured by immersing the entire gel in denaturation solution (0.4M NaOH, 1M NaCl) for 20 minutes with constant, gentle agitation. The gel was then rinsed with distilled water and immersed twice in neutralising solution (1.5M NaCl, 0.5M Tris.HCl pH7.5, 0.001M EDTA) for 20 minutes. While the gel was being treated, a nylon membrane (Hybond™ N+ Amersham) was cut slightly larger than the gel and the corner of the membrane cut to match the gel. The DNA in the gel was then transferred to the membrane by capillary action. This involved placing the gel onto three sheets of 3MM Wattman's paper and placing the paper between two reservoirs containing 20X SSC (3M NaCl, 0.3M Na citrate) to form a wick. The level of 20X SSC was kept approximately 1cm below the base of the gel. Any air bubbles between the gel and the wick were removed



and the nylon membrane was laid over the gel with the cut corner of the membrane orientated over the corresponding corner of the gel. Any air bubbles were removed from between the gel and the membrane and two pieces of 3MM Wattman's paper, cut to the same size as the membrane and soaked in 20X SSC, were placed over the membrane. Additional pieces of 3MM Wattman's paper and paper towels were stacked upon the gel. A weight was then placed upon the towels and the transfer was allowed to take place overnight.

After the transfer was completed, the towels and paper were removed and the well positions of the gel were marked on the membrane with pencil. The membrane was removed from the gel and the gel was viewed under UV light to ensure that the transfer was successful. The membrane was placed DNA side up on to three sheets of 3MM Wattman's paper, soaked in 0.4M NaOH for 15 minutes to fix the DNA and rinsed with 5X SSC. The membrane was then probed via hybridisation.

### 2.18 Amplification of cDNA for Probes

The full length TrkB, TrkC and β-actin cDNA used to generate the probes was amplified by PCR from P2 cerebellum total RNA (obtained from Karen Bailey) reverse transcribed as per Section 2.12. The oligonucleotide primers chTrkBF and the reverse complement, chTrkBR were used to amplify the TrkB cDNA. Similarly, the oligonucleotide primers chTrkCF and the reverse

complement, chTrkCR were used to amplify the TrkC cDNA and the oligonucleotide primers chactinF and the reverse complement, chactinR were used to amplify the β-actin cDNA (Refer to Section 2.10). Approximately 500pg of cDNA was added to a 25 µl reaction containing 0.8µM of each primer (chTrkBF and chTrkBR, chTrkCF and chTrkCR or chactinF and chactinR), 1X PCR reaction buffer (Sigma), 0.2mM of each dNTP (Boehringer Mannheim), 2.5mM $MgCl_2$ (Sigma), and 1U Hot Start Taq polymerase (Sigma). Reactions were carried out in 200µl domed cap tubes in a thermal cycler possessing a heated lid (GeneAmp PCR System 2400, Perkin Elmer). The tubes were heated to 94$^{O}$C for 5 minutes to fully denature the cDNA. The PCR reaction was incubated for a 45 second denaturing step at 94$^{O}$C, a 45 second annealing step at 60$^{O}$C and a 1 minute extension step at 72$^{O}$C for 35 cycles. Negative controls, consisting of reactions in which the cDNA templates were not added, were used.

### 2.19 Electrophoresis and Prep-a-gene Purification of cDNA for Probes

The samples were electrophoretically analysed by running the samples on an ethidium bromide stained 1.2% agarose gel. A 1Kb ladder (GIBCOBRL) was also run on the gel as a size marker. Solutions containing 500ng and 1µg of pcDNA3 vector DNA were also loaded on to the gel to facilitate the estimation of the mass of the amplified fragments. The gel was viewed under ultra violet light. An estimate of the PCR product mass was made by comparing the band intensity of the amplified bands to those of the known



masses of vector. The bands corresponding to TrkB, TrkC and β-actin were excised separately from the gel and purified using a Prep-a-Gene kit (Biorad).

Purification involved placing the excised gel slice into a microfuge tube containing 3 gel volumes of binding buffer and incubating the tube at 55$^{O}$C until the gel had melted. 5µl of binding matrix was added to the tube and incubated at room temperature for 10 minutes with agitation. The matrix was pelleted by spinning the tubes for 30 seconds in a microcentrifuge. The supernatant was discarded and the pellet was washed twice by resuspending the pellet in 25 pellet volumes of wash buffer, pelleting the matrix by centrifuging for 30 seconds and discarding the supernatant for each wash. Following the final wash the DNA was eluted by resuspending the pellet in 5µl of elution buffer and incubating the tube at 55$^{O}$C for 5 minutes. The matrix was pelleted by centrifugation for 30 seconds and the supernatant was transferred to a separate tube. The pellet was resuspended in 5µl of elution buffer and the elution step was repeated. The second volume of supernatant was added to the first and the pellet was discarded. The eluted samples were then used to produce the TrkB, TrkC and β-actin radioactively labelled probes.

### 2.20 Preparation of Radioactive Probes

Radioactively labelled probes were generated using a Gigaprime DNA labelling kit (Bresatec). Approximately 70ng of DNA in a volume of 6µl was denatured by heating the sample to 95$^{O}$C for 5 minutes. After

denaturation the samples were immediately placed on ice. To the denatured DNA sample was added 6µl of decanucleotide solution, 6µl of nucleotide/buffer cocktail (tube 2A), 5µl (50 µCi) $^{32}$P-dATP and 1U Klenow enzyme. The reaction was incubated for 37$^{O}$C for 15 minutes and the reaction was stopped by the addition of EDTA (pH 8) to a final concentration of 20mM. Sephadex® G-50 (nick) columns (Pharmacia Biotech) were used to separate incorporated radioactive nucleotides from unincorporated nucleotides. The preservative buffer was removed from the column and 400µl of TE buffer was added to the column to wash out any remaining preservative buffer. The column was then equilibrated via the addition of 3ml TE buffer. The products of the labelling reaction were then added to the column and allowed to enter the sephadex matrix. Two volumes of 400µl of TE buffer were sequentially placed into the column, allowing the first volume to enter the matrix before adding the second. The solution containing the radioactive probe, which was eluted upon adding the second volume, was collected and checked for radioactivity to ensure the probe had been successfully labelled. The probe was stored at -70$^{O}$C until required.

### 2.21 Hybridisation of the Nylon Membrane with a Radioactive Probe

The membrane was placed into a hybridisation tube together with 20ml of hybridisation solution (5X SSC, 0.1% bovine serum albumen, 0.1% Ficoll™, 0.1% polyvinylpyrrolidone, 0.5% SDS) and rotated for an hour at 65$^{O}$C. 500mg of salmon sperm DNA was denatured by heating to 95$^{O}$C for 5 minutes and then placed on ice for 1 minute. Likewise, 400µl of the



radioactive probe was denatured at 95$^{O}$C for 5 minutes and then placed on ice for one minute. Both the salmon sperm DNA and the probe were then added to fresh hybridisation solution and the membrane was incubated at 65$^{O}$C for a further 12 hours with rotation. Following hybridisation, the membrane was washed twice with 2X SSC, 0.5% (w/v) SDS for 10 minutes at 65$^{O}$C, once with 1X SSC, 0.1% (w/v) SDS for 15 minutes at 65$^{O}$C and finally once with 0.1X SSC, 0.1% (w/v) SDS for 15 minutes at 65$^{O}$C. The membrane was then sealed in plastic and developed by phosphoimaging.

### 2.22 Phosphor imaging

Following hybridisation, the membrane was placed in a phosphor imager cassette (Molecular Dynamics) and exposed for several days. The phosphor imager screen was then scanned using Storm (Molecular Dynamics) and analysed using ImageQuant (Molecular Dynamics) and Excel 2002 (Microsoft) software.

### 2.23 Optimisation of RT-PCR Amplification

The PCR reaction conditions were optimised to determine the range of cycles during which exponential amplification was occurring. Total RNA extracted from the chicks was reverse transcribed as per Section 2.13. The reverse transcription product was then used to optimise the PCR cycle number to be used when analysing Trk receptor expression levels Cycle optimisation was performed using TrkB. PCR was carried out as per Section 2.14. The

oligonucleotide primers chTrkBF and the reverse complement, chTrkBR were used to amplify the TrkB cDNA. A series of reactions were performed varying by 5 cycle increments between 0 and 35 cycles of amplification.

The PCR products were analysed by gel electrophoresis as per Section 2.15. The electrophoresed products were then transferred to a nylon membrane via capillary action and probed using radioactive cDNA probes (Refer to Sections 2.16 to 2.21). The probed membrane was analysed via phosphor imaging. The relative signal intensities of the probed bands were determined and examined to determine at which cycles amplification was occurring exponentially. A cycle number was chosen from within this range and used for further reactions.

### 2.24 Optimisation of RNA Concentration for RT-PCR

As β-actin exhibits higher levels of expression compared to Trk receptors, it was necessary to determine whether the initial concentration of total RNA in the RT-PCR needed to be varied in order to produce comparable template concentrations. Tenfold serial dilutions of the P2 cerebellum-derived total RNA were produced. The serially diluted RNA was reverse transcribed. Using TrkB as a standard, TrkC and β-actin were amplified from the diluted reverse transcription products using the optimised cycle number determined as per Section 2.23. The PCR products were electrophoresed on a DNA agarose gel. The products were transferred to a nylon membrane and simultaneously



probed using equal amounts of each probe. The membrane was analysed via phosphor imaging (Refer to Sections 2.13 to 2.22). The dilutions at which the signal intensity of the amplified TrkC and β-actin cDNA corresponded to the signal intensity of TrkB were used when examining the relative expression levels of the receptors (Refer to Section 2.25).

### 2.25 Analysis of Receptor Expression Levels

Spinal cord total RNA, extracted in Section 2.12, was used to determine the relative levels of TrkB and TrkC expression in relation to that of β-actin, via RT-PCR. For each total RNA sample, separate amplification reactions were carried out for TrkB, TrkC and β-actin. Total RNA samples were diluted to the required concentration for each sequence as determined in Section 2.24. The RNA samples were separately reverse transcribed and amplified by PCR (Refer to Sections 2.13 and 2.14) using primers specific to the sequence being amplified. Reactions were amplified using the optimised cycle number determined as per Section 2.23. PCR products were grouped according to the amplified sequence and each group was electrophoresed on a separate DNA agarose gel. The PCR products were transferred to separate nylon membranes via capillary action and probed separately using sequence specific probes. The probed membranes were exposed simultaneously on the same phosphor imaging screen. The screen was then scanned (Refer to Sections 2.15 to 2.22). The resulting signal intensities of TrkB and TrkC were compared to that of β-actin for each sample group.

**2.26 Harvesting of Cerebrospinal Fluid (CSF) from Graft Implanted Embryos (Appendix 2)**

2.3D cells were transfected with pcDNA3 expression vectors, containing either the BDNF or NT-3 constructs, using the same method as outlined in Section 2.2. At E5, chick embryos were implanted with either the NT-3 expressing cell line or a combination of the BDNF and the NT-3 expressing cell lines, as per Section 2.3. Implanted embryos were incubated at 39.5°C until harvesting.

At E8, embryos were removed from the egg and decapitated. Using a 1ml syringe fitted with a 26 gauge needle, CSF was gently extracted from the optic tectum and placed in a microcentrifuge tube. Samples were briefly centrifuged at 8,000xg in a microcentrifuge to remove protease and erythrocyte contamination, which are known to degrade peptides. The CSF, present as supernatant, was removed and stored at -70 °C until use.

**2.27 DRG Neuronal Bioassay (Appendix 2)**

The DRG bioassay was a modification of the procedure described by Barde *et al* (1980). White Leghorn chicken eggs were incubated at 39°C with the blunt end facing up until embryonic day 8 (E8). Embryos were removed from the egg, killed by decapitation and the body transferred to a petri dish containing phosphate buffered saline. The heart, liver and intestines were discarded, exposing the spinal cord and DRG. The DRG



were gently dissected from surrounding tissue and transferred to a sterile 15ml tube. Following dissection, the isolated DRG were centrifuged at 160xg for 5 minutes and the supernatant discarded. The DRG were resuspended in 3ml of 1X PBS containing 0.1% Trypsin/EDTA and incubated at 37°C for 15 minutes. The trypsin was inhibited by the addition of 0.3ml fetal calf serum and the cells centrifuged as described above. The cells were washed twice in 5ml PBS and then resuspended in 3ml of DMEM/10% Horse serum (HS). The cells were dissociated by rapid pippetting using a siliconised pasteur pipette that had previously had its end rounded off by gentle flaming. The cell suspension was made up to 6ml with an additional 3ml of DMEM/10%HS and the cells pre-plated on a Nunc petridish for 3.5 hours in a 37°C humidified incubator with 10%$CO_2$. During the pre-plating step the non-neuronal cells adhere to the petridish allowing the purification of neuronal cells by gently removing the media containing the non-adherent cells. After 3.5 hours the neuronal cells and media were removed and a small sample used to obtain a cell count. Cells were then plated at a concentration of 5000 cells per well (in a final volume of 500µl) in a 24 well Limbro plate. These plates had previously been treated for 2 hours with poly-ornithine followed by a 2 hour incubation with laminin. This treatment is required for cell survival. Factors (including purified neurotrophic factors, transfected cell media and CSF fluid) were added to the cells and the cells incubated at 37°C in a humidified incubator for 48 hours. Surviving neurons were then counted by randomly counting 10 fields and comparing the counts to control samples containing either no factor or 1ng/ml BDNF or NT-3.

# CHAPTER 3

# THE EFFECTS OF SUPPLYING SPINAL MOTONEURONS WITH A CONSTANT SOURCE OF NEUROTROPHINS

# CHAPTER 3

## 3.1 INTRODUCTION

The neurotrophins BDNF and NT-3 are known to be potent trophic factors for cultured spinal motoneurons derived from chick embryos during NOCD (Becker *et al*, 1998). Such responsiveness is supported by the expression of the corresponding receptors TrkB and TrkC in embryonic spinal motoneurons. However, the same trophic response has not been observed in developing chick motoneurons *in vivo*. While BDNF has been found to rescue one third of those motoneurons that normally succumb during NOCD (Oppenheim *et al*, 1992; Calderó *et al*, 1998), NT-3 has not been shown to significantly rescue spinal motoneurons during this period (Calderó *et al*, 1998). Furthermore, no single neurotrophic factor has been observed to rescue the entire spinal motoneuron population from NOCD. Whether this phenomenon results from specific subsets of motoneurons within the spinal cord being unresponsive to the supplied factors or whether it is simply an artefact of long term factor withdrawal, is largely undetermined.

Previous studies that have examined the trophic effects of neurotrophins on developing spinal motoneurons have administered factor to the embryos at intermittent stages during the experimental period (Oppenheim *et al*, 1992; Calderó *et al*, 1998). However, the duration between administrations in these studies has usually exceeded both the known half-



life of the model neurotrophin NGF and the observed time frame during which neurons succumb to NGF deprivation (Tria *et al*, 1994; Deckwerth & Johnson, 1993). Therefore, it remains ambiguous as to whether the lack of survival is a true indication of the trophic responsiveness of motoneurons or whether it is the cumulative result of factor deprivation. Furthermore, there is increasing evidence that neurotrophic factors work in combination on gross cell populations to elicit a full complete response (Kato & Lindsay, 1994; Mitsumoto *et al*, 1994). Within the PNS neurotrophic factors may act sequentially, with cells switching their trophic requirements. When isolated from stages prior to target innervation, cultured murine trigeminal ganglion neurons subsequently die unless BDNF or NT-3 are present in the culture medium. When isolated at later periods, trophic responsiveness to BDNF and NT-3 is lost in favour of NGF (Paul & Davies, 1995). Such apparent shifts in trophic response are supported by a corresponding change in Trk receptor expression from TrkB and TrkC to TrkA in these populations *in vivo* (Pinon *et al*, 1996). Similarly TrkC is also expressed early in the target innervation of superior cervical sympathetic ganglia. However, at later stages TrkC expression decreases as the neurons begin to express TrkA (Birren *et al*, 1993; Wyatt & Davies, 1995). *In vitro* studies have shown that dorsal root ganglia are responsive to BDNF, NT-3 and NGF early in development but subsequently narrow their trophic requirements to NGF (Memberg & Hall, 1996; Lefcort *et al*, 1996). Early in post-natal development a substantial proportion of the dorsal root ganglia population undergo a further shift in trophic responsiveness towards GDNF dependence (Molliver *et al*, 1997).

Not all neuronal populations appear to undergo temporal changes in their trophic requirements. Murine knockout studies have shown *trkA-/-* mice do not show any reduction in the number of spinal ganglion neurons. While *trkB-/-* and *trkC-/-* mice do display a reduction in the number of spinal ganglion neurons, the neuronal loss observed in *trkB-/-;trkC-/-* mice is equivalent to the combined loss observed in the single Trk knockouts. Such observations suggest neuronal subsets of these populations are responsive to either BDNF or NT-3 but do not require both factors (Minichiello *et al*, 1995). Within the CNS, it is unclear as to whether any neuronal populations require the sequential action of different neurotrophic factors for the maintenance of survival. Evidence from the PNS suggests it is probable that such molecular fine-tuning occurs in at least some cell populations of the CNS.

In this chapter we employed a neuroepithelial cell line genetically modified to produce either BDNF or NT-3 to deliver a continuous supply of these neurotrophins to the developing spinal cord. We aim to determine the extent to which constant supplementation of BDNF and NT-3 individually can rescue motoneurons from NOCD *in vivo*. BDNF and NT-3 will also be supplied in combination to determine whether the addition of both factors can elicit a greater survival response. The effect of the neurotrophins on motoneuron survival will be examined both after the main phase of neuronal degeneration and a stage several days later. We aim to examine whether



single factors can maintain their trophic support or whether the trophic requirements undergo temporal changes.

The optical disector/fractionator combination will be used to examine the changes in neuronal survival resulting from factor administration. The advent of the optical disector has facilitated a wide array of unbiased cell counting techniques. Nurcombe *et al* (1991) used the optical disector/Calvalieri combination to quantitatively profile motoneuron death during NOCD and examine the ability of various growth factors to rescue LMC motoneurons during this period. The optical disector/fractionator combination represents a less complex counting regime compared to the optical disector/Calvalieri combination. Estimation of cell number is independent of tissue volume thus minimising bias. The optical disector/fractionator combination has been successfully used to examine spinal motoneuron survival in postnatal mice (Lowry *et al*, 2000) and represents an effective stereological tool for examining changes in motoneuron population density.

## 3.2 RESULTS

### 3.21 Establishing a Profile of Cell Loss During NOCD

Prior to examining the trophic effects of supplementing motoneurons with neurotrophic factors it was necessary to establish a profile of the number of motoneurons in the LMC spanning the period of NOCD. From this profile we were able to define the developmental stages between which NOCD was occurring and determine the optimal developmental stage to analyse the change in motoneuron populations. Chick embryos were sacrificed at daily intervals between embryonic day 6 and embryonic day 15 (E6 to E15). The lumbar spinal cords were removed from the embryos, fixed and embedded in glycolmethacrylate. It was necessary to increase the manufacturer recommended processing times for the spinal tissue in order to prevent shattering of the tissue due to inadequate infiltration. The tissue was sectioned and the motoneurons were counted using the optical disector/fractionator combination *(Figure 3.1)*. The LMC were discernible from the grey matter of the spinal cord as populations of large, deeply stained cells projecting bilaterally from the ventrolateral grey matter *(Figure 3.2)*. Within the LMC, motoneurons were evident as large cells with deeply stained cytoplasm and nucleoli.



While the ventrolateral protrusions of the LMC were evident at E5, the motoneurons were largely indistinguishable from the surrounding cells, including those of the grey matter. By E6 motoneurons were sufficiently distinguishable to be reliably counted. Thus, a profile of the motoneuron counts between E6 and E15 was produced (Figure 3.3). Published data suggests that LMC motoneuron cell loss is minimal prior to this period (Chu-Wang & Oppenheim, 1978).

A dramatic loss of motoneurons in the embryonic LMC was observed between E6 and E9. During this period 38% of motoneurons present at E6 were lost. Following E10, cell loss continued at a reduced rate. Between E10 and E15 a further 8% of motoneurons were lost. These observations are consistent with previously published studies (Chu-Wang & Oppenheim, 1978, Oppenheim *et al*, 1992). Motoneuron counts across the period examined were also similar to those previously published (Nurcombe *et al*, 1991; Oppenheim *et al*, 1992; Calderó *et al*, 1998). Such studies have also shown that following E15 cell numbers plateau to a level comparable to that of post-natal animals.

Figure 3.1: The process of counting particles using the optical disector/fractionator combination. The figure shows a series of sequential optical sections through a 20μm section of an E10 chick LMC superimposed with a counting frame (Gunderson, 1977). Z refers to the depth within the focal plane, referenced from the depth at which sampling commenced, from which the image was sampled. Arrows indicate nuclei that may be included in particle counts. Using the optical disector, the LMC population was visualised under a X100 (NA 1.4) oil immersion lens. A sampling region containing the entire LMC motoneuron population was manually selected. A random sample field, consisting of 2500μm$^2$ sampling frames (X = 50μm, Y = 50μm), was laid over the selected sample region. A 1067μm$^2$ counting frame was superimposed over the image at a fixed point within the sample frame. Particles within each counting frame in the sample field were counted. Within each sample frame, the lens was focused at the surface of the section. The lens was then focused 3μm through the surface of the section, to allow for surface aberrations, where the microcator measuring the depth of the focal plane was set to zero. The lens was focused through the focal plane. Nuclei were counted as they came into focus within the counting frame. Those nuclei whose outer edges lay entirely within the counting frame were counted. Those nuclei whose outer edges crossed those borders of the counting frame marked as thin lines were also counted. The forbidden regions of the counting frame are marked as bold lines. Any nuclei whose outer edges crossed the forbidden zones of the frame, including their extensions upwards from the upper left hand corner and downwards from the lower right hand corner to infinity, were not counted. Those nuclei whose outer edges lay entirely outside the counting frame were not counted. Nuclei that were in focus at Z = 0 were omitted from the counts. Any allowably countable nuclei in focus at Z=10 were included in the counts. Bar = 10μm

Z = 0.0

Z = 3.0

Z = 7.5

Z = 10.0

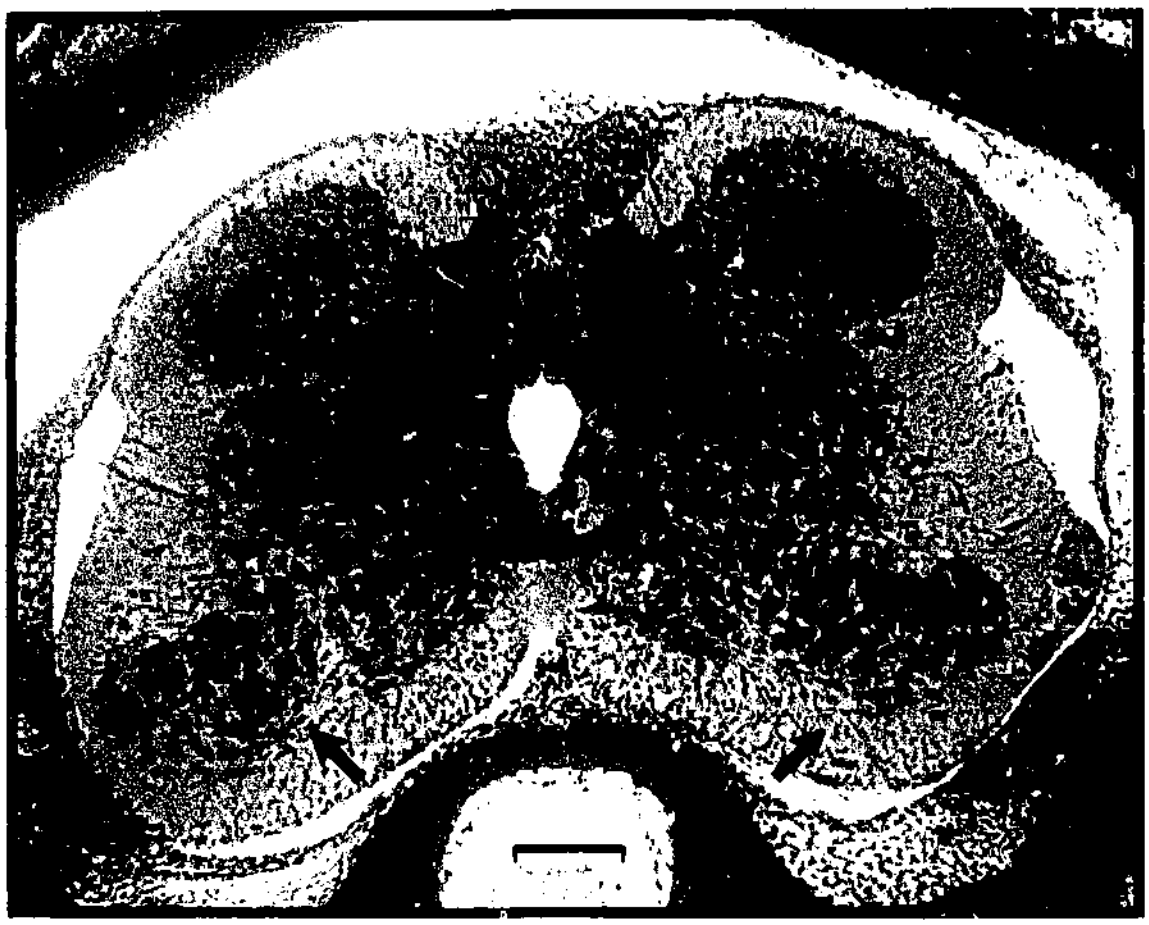


Figure 3.2: A cross section of the E10 chick spinal cord. The LMC (marked with arrows) are present as discrete populations of large, deeply stained cells projecting from either side of the ventrolateral grey matter. Within the LMC motoneurons were evident as large cells with deeply stained cytoplasm and nucleoli. Bar = 100μm

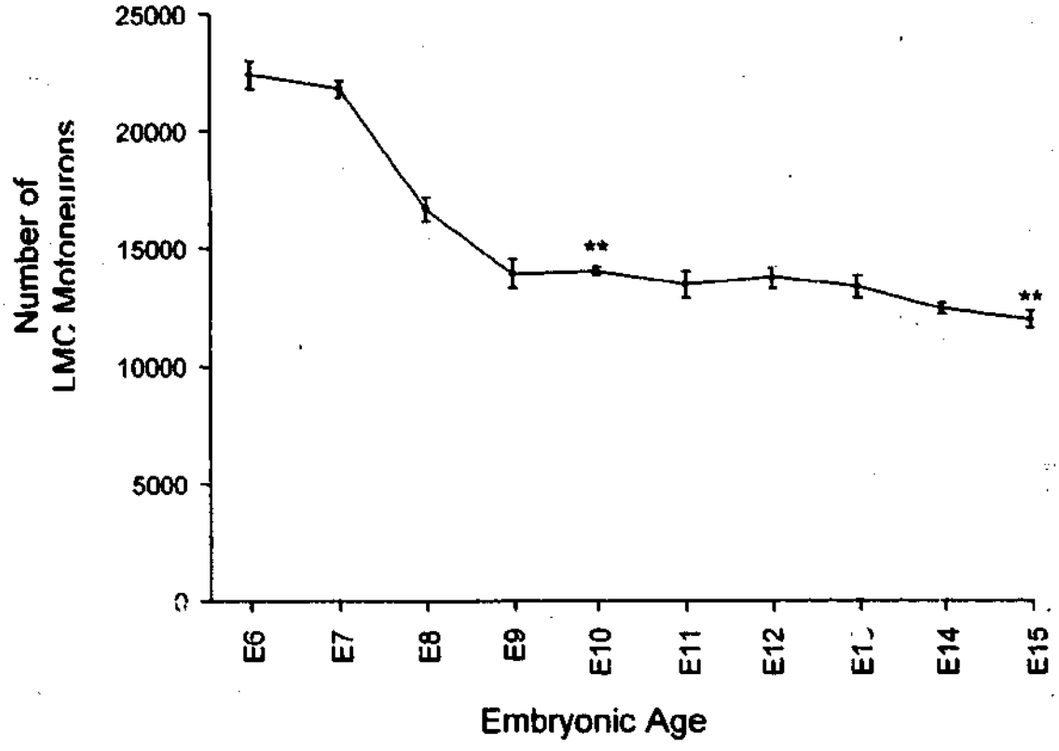



Figure 3.3: Temporal profile of the loss of motoneurons during NOCD. Following an initial rapid reduction in LMC motoneuron density cell loss is seen to continue at a reduced rate. Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord. Motoneuron nuclei counts were recorded daily between E6 and E15. Statistical significance indicated at E10 refers to the difference in cell counts between E6 and E10. Statistical significance indicated at E15 refers to the difference in cell counts between E10 and E15. ** = $P<0.01$

### 3.22 Preparation of the Genetically Modified 2.3D Cell Line

Preparation of the modified 2.3D cell line was carried out by Karen Bailey (School of Biological Sciences, Monash University). The murine expression vector, pcDNA3 (Invitrogen), was employed as a means to continuously deliver exogenous neurotrophins to the developing spinal motoneurons of the chick. Two recombinant pcDNA3 constructs, containing either a pre-pro-BDNF or pre-pro-NT-3 cDNA insert, had previously been generated (Bailey) and transformed into DH5α *E. coli* cells. The recombinant vectors were purified using a standard large scale plasmid DNA preparation followed by PEG precipitation.

The purified pcDNA3 constructs were transiently transfected into a 2.3D neuroepithelial cell line via calcium phosphate precipitation. Transfected cells containing the pcDNA3/BDNF construct, the pcDNA3/NT-3 construct or the pcDNA3 parent vector were generated. Cells were cultured in a selective medium prior to preparation for transplantation. While such transient transfections may produce cells expressing varying levels of recombinant protein, the number of cells used in the embryo transplantation was at a quantity previously determined to secrete factor in excess of the optimum levels known to promote survival. Unpublished work performed by a past honours student within our research group suggests that, when transfected with the pcDNA3/NT-3 construct, the 2.3D cell line is capable of sustaining neurotrophic activity until at least E8 *(Appendix 2)*. Thus, implantation of the



2.3D graft does not appear to result in significant post implantation elimination in response to tissue damage, as has been previously observed for other graft implantation paradigms (Suhr & Gage, 1993). Such data also suggests that cross species immunoreactivity does not present a problem at these early stages in development.

### 3.23 The Effects of Supplying Neurotrophins to Developing Embryos at E10

Analysis of the profile of the motoneuron counts between E6 and E15 showed that the greatest period of NOCD occurs between E6 and E9. Therefore, E5 was chosen as the most appropriate stage to begin factor supplementation as it was just prior to the onset of NOCD. At earlier developmental stages the embryos were too immature to facilitate adequate implantation of the transfected neuroepithelial cell line used to deliver neurotrophins to the developing motoneurons. Analysis of the motoneuron counts following factor supplementation was performed at E10. Thus the period of administration spans that of the predominant phase of NOCD.

At E5 embryonated eggs were manipulated and the factor secreting 2.3D cell lines were pressure injected into the ventricle of the optic tectum. Embryos were implanted with either the pcDNA3/BDNF construct transfected 2.3D cells or the pcDNA3/NT-3 construct transfected 2.3D cells. Embryos were also injected with a combination of the two cell lines. Negative

controls were produced by implanting embryos with 2.3D cells transfected with the parent pcDNA3 vector. Lumbar spinal tissue was harvested at E10 and embedded in glycolmethacrylate. The tissue was sectioned and the motoneurons were counted using the optical disector/fractionator combination.

Constant supplementation with either BDNF ($P<0.01$) or NT-3 ($P<0.01$) was able to produce an increase in survival over that of the E10 control *(Figure 3.4)*. Supplementation with BDNF rescued 40% of those LMC motoneurons normally lost between E6 and E10. Supplementation with NT-3 resulted in the rescue of 36% of the LMC motoneurons normally lost during that period. The administration of a combination of BDNF and NT-3 resulted in an increase in motoneuronal survival over that achieved by the single factors alone ($P<0.05$). However, the increase observed was not additive suggesting a high level of redundancy in the factor responsiveness of the motoneurons. The failure to achieve a level of survival comparable to that of the E6 motoneuron population suggests that, while the two neurotrophins employed in this study are potent trophic factors for the LMC motoneuron population, subpopulations of LMC motoneurons may require the trophic support of other factors.



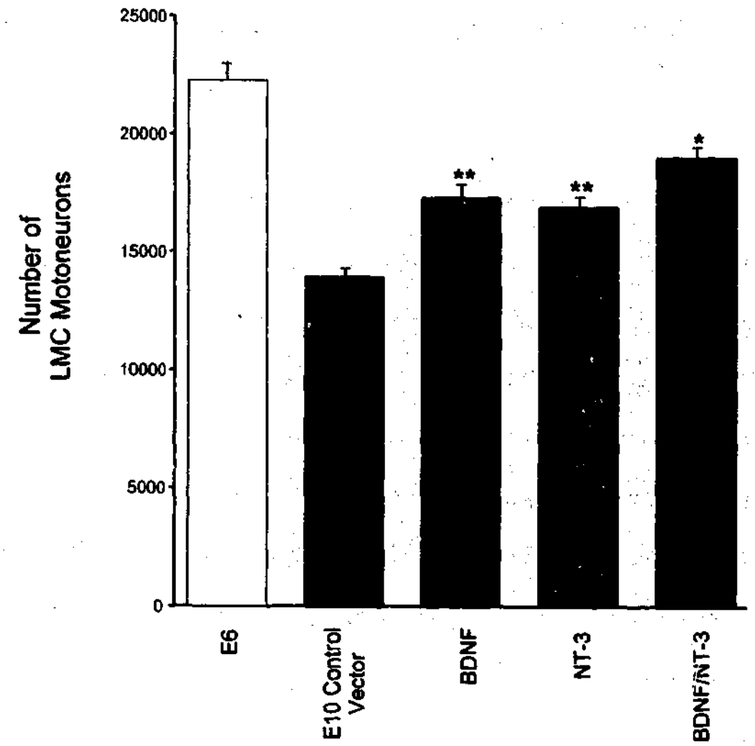



Figure 3.4: The effects of continuous supplementation of neurotrophins, alone and in combination, on LMC motoneuron survival at E10. Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord. At E5 embryos were implanted with 2.3D cells transfected with either the pcDNA3/BDNF construct or the pcDNA3/NT-3 construct. The graph indicates the number of LMC motoneurons present at E10 following implantation of BDNF secreting cells, the NT-3 secreting cells or a mixed population of the two recombinant cell types. The control group consists of embryos implanted with 2.3D cells containing the unmodified parental vector. Data for E6 is provided for comparison. Asterisks above the single factor groups refer to the significant difference between the survival achieved by the vector control and the single factor trials. Asterisks above the factor combination group refer to the significant difference between the highest level of survival observed in the single factor trials and the survival achieved by both factors in combination. * = $P<0.05$ ** = $P<0.01$

### 3.24 The Effects of Supplying Neurotrophins to Developing Embryos at E15

Studies employing intermittent administration to deliver neurotrophins to the developing nervous system have not been able to sustain the level of neuron survival observed at E10 over the entire period of NOCD. Whether these observations reflect changes in factor responsiveness or the cumulative effects of factor deprivation have been somewhat ambiguous. Thus, embryos implanted with the neurotrophin secreting graft were also analysed at E15. This allowed us to examine whether constant factor supplementation could sustain the degree of neuron survival observed at E10 over the extended developmental period during which cell death occurs.

Between E10 and E15 the rates of survival had decreased in all three supplementation regimes *(Figure 3.5)*. Only BDNF alone ($P<0.01$) and in combination with NT-3 ($P<0.05$) produced survival levels significantly greater than that of the pcDNA3 control. At E15 supplying a combination of BDNF and NT-3 did not produce a significant increase in survival over that achieved by supplementation with BDNF alone. The supplementation regimes at E10 and E15 showed a significant level of interaction ($P<0.05$) (see Appendix 1: A1.15). The rate at which motoneurons are lost between E10 and E15 differs across the different factor supplementation



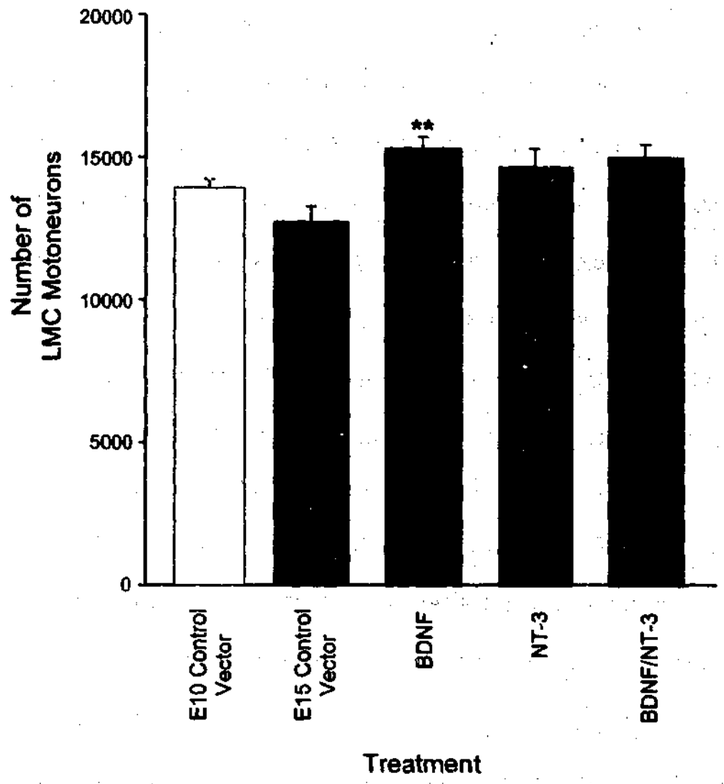



Figure 3.5: The effects of continuous supplementation of neurotrophins, alone and in combination, on LMC motoneuron survival at E15. The graph indicates the number of LMC motoneurons present at E15 following implantation of BDNF secreting cells, the NT-3 secreting cells or a mixed population of the two recombinant cell types at E5. The control group consists of embryos implanted with 2.3D cells containing the unmodified parental vector. Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord. Asterisks above the single factor groups refer to the significant difference between the survival achieved by the E15 vector control and the E15 single factor trials. No significant difference was seen between the single factor trials and the factor combination trials.

regimes. Therefore, the reduction in cell survival that is observed between E10 and E15 in the factor supplemented embryos does not solely result from the normal cell loss that occurs between E10 and E15 in untreated embryos. Nor can this observation be explained entirely by a loss of integrity in cell graft expression. As the parent vector and transfection methodology used to generate each factor secreting cell graft were identical, any cell loss resulting from a reduction in graft mediated support should be uniform across the treatment regimes. Thus, it appears that the factor responsiveness of LMC motoneurons do change throughout the period of NOCD.

Control counts using the parental vector were not significantly different from the counts obtained from embryos that did not undergo *in ovo* manipulation for E10 ($P>0.05$) or E15 ($P>0.05$). Thus, it appears that the presence of the 2.3D cell line and the pcDNA3 expression vector does not affect the LMC motoneuron populations. It is also evident that trauma from embryo manipulation or pressure injection does not effect these cell populations.



## 3.3 DISCUSSION

### 3.31 Motoneuron Survival Throughout NOCD

An understanding of the temporal changes in neuronal number during NOCD is essential if we are to assess the ability of exogenous trophic factors to support cell populations during this period. A profile of the normal LMC motoneuron counts between E6 and E15 was generated to establish when cell death was occurring and at what rate. Not only did this allow us to determine the optimal period in embryonic development for the trials to take place, the generation of such a profile also ensured that the pattern of population death observed corresponded to the levels previously recorded by other studies. Such a correlation of the observed cell numbers is essential if we are to compare the observations from these experiments to similar published data.

An initial, rapid loss of LMC motoneurons was observed to occur between E6 and E9. During this period approximately 39% of the LMC motoneurons present at E6 were lost. These observations support studies carried out by Hamburger (1975), Arakawa *et al* (1990), Nurcombe *et al* (1991), Oppenheim *et al* (1992) and Calderó *et al* (1998) that observed similar degrees of cell loss occurring during this period of chick embryonic development. Cell counts observed in our study were also similar to those of the aforementioned studies affirming the suitability of the optical

disector/fractionator combination for reliably estimating motoneuron population density. Following this initial period of NOCD, cell loss was then observed to continue, albeit at a much reduced rate. Similarly observations of cell death following the initial period of NOCD have previously been noted by Chu-Wang & Oppenheim (1978). While this study did not attempt to examine cell death beyond E15, Chu-Wang & Oppenheim (1978) found that motoneuron loss in the LMC plateaus following E15. The same study found cell loss prior to the period analysed in this study to be minimal. Thus, we are confident that the majority of LMC motoneuron loss during NOCD in the chick is confined to the developmental periods examined in the cell count profile.

The actual developmental stage of an embryonic chick can vary by up to 12 hours from the estimated embryonic age depending on the time of fertilisation. From the profile, cell loss was observed to occur at a rate of up to 23% between daily samples. Examining the survival effects of exogenous neurotrophic factors on LMC motoneurons during such a high rate of cell loss has the potential for evidence supporting factor-mediated survival to be masked by dramatic fluctuations in population density caused by sampling error. Based on the temporal examination of cell survival, continuous factor administration was initiated at E5. This ensured that factor supplementation occurred prior to the onset of NOCD so that LMC motoneuron populations would have access to exogenous trophic support prior to becoming factor dependant. Factor-mediated motoneuron survival was analysed at E10 and at E15 providing us the opportunity to examine survival potentiation during



the major phase of cell death and determine whether factor-mediated survival can be sustained over the entire period of NOCD.

### 3.32 Constant Expression Systems

The employment of intermittent administration to study the *in vivo* response to exogenous neurotrophins is limited by the possible detrimental effects caused by the suggested short half-life of neurotrophins and the rapid onset of neurodegeneration observed following factor deprivation (Deckwerth & Johnson, 1993; Tria *et al*, 1994). Several studies have sought to rectify this by employing various methods of continuous factor supplementation. Among these the use of grafted cell lines, genetically modified to secrete factor, have proven effective at providing constant supplementation for prolonged periods. Using modified fibroblast cells, Grill *et al* (1997b) showed that continuous supplementation of NGF could substantially augment axonal growth in the chronically lesioned rat spinal cord for up to three months. In subsequent experiments using the same method of cellular delivery, NT-3 produced a greater response in corticospinal axon growth following spinal lesion when compared to previously published data for single dose treatment (Grill *et al*, 1997a).

This study employed the 2.3D cell line, a murine cell line derived from E10 neuroepithelial cells (Bartlett *et al*, 1988), transfected with the murine expression vector pcDNA3. Within the postnatal murine system,

neuroepithelial cell grafts are capable of surviving *in situ* for substantially longer periods than were utilised in this study (Kilpatrick *et al*, 1994). However, the use of a murine xenograft in a chicken system does present some possible immunological problems. However, analysis of the integrity of the 2.3D/pcDNA3 expression system by previous members of our research group showed that 2.3D cells implanted at E5 did not appear to succumb by E8 *(Appendix 2)* suggesting the chicken's immune system at the early developmental stages examined in this study is insufficiently developed to initiate graft rejection.

### 3.33 Continuous Supplementation of BDNF or NT-3 to the Developing Spinal Cord Motoneurons during NOCD

When supplied alone, BDNF and NT-3 were both found to rescue a substantial proportion of the LMC motoneurons that normally succumb to NOCD between E5 and E10. Both factors appeared to be equally as potent with neither factor producing a significant increase in survival over the other. This study employed a continuous expression system to supply a constant source of neurotrophins to developing spinal motoneurons. To the best of our knowledge, previous studies analysing the effects of neurotrophic factors on spinal motoneurons during NOCD have relied on intermittent administration of the factors. Continuous supplementation of BDNF to the embryonic chick between E5 and E10 resulted in the survival of 40% of those LMC motoneurons that are normally lost. This is slightly higher than the previously



published data for intermittent administration, which produced 33% survival in response to BDNF (Oppenheim *et al*, 1992; Calderó *et al*, 1998), however, the level of survival we observed is still within the range of the published data. Without a comparative study it cannot be determined whether there is any actual increase with constant supplementation. In contrast to the observations by Calderó *et al* (1998), who found intermittent administration of NT-3 ineffective at rescuing LMC motoneurons, we observed that continuous supplementation of NT-3 rescued 36% of those neurons that ordinarily succumb to NOCD.

Methods of effective factor administration are limited by the half-life of the factor in question. As a model neurotrophin, NGF is known to have an elimination half-life of up to 4.5 hours when subcutaneously injected (Tria *et al*, 1994). Furthermore, NGF deprivation leads to a rapid loss of neurons with 50% of neuron soma displaying detectable atrophy 19 hours after factor withdrawal (Deckwerth & Johnson, 1993). Therefore, administration of neurotrophins at daily intervals, as is a commonly practiced regime of factor supplementation, may exceed the period for which the factor remains biologically active. Even for studies that have used shorter periods between administration, it is not clear how long after the factor withdrawal the processes leading to factor deprivation induced death become irreversible. However, Grill *et al* (1997a) noted that, when supplied via a recombinant cell graft to adult rats following spinal cord injury, NT-3 had a much greater effect on corticospinal axon growth when compared to similar experiments using

intermittent administration. Such observations suggest that the cumulative effect of neurotrophin withdrawal may have an effect on the maintenance of neuronal survival.

The effect of cumulative factor deprivation is a likely explanation for the differences in motoneuron survival observed in this study when compared to the observations of other studies. While an increase in BDNF-promoted survival over that of the observations in studies by Oppenheim *et al* (1992) and Calderó *et al* (1998) cannot be conclusively established, it does appear that the constant supplementation of NT-3 dramatically increases the level of LMC motoneuron survival over that of observations based on intermittent administration (Calderó *et al*, 1998). Why the difference in the levels of motoneuron survival between constant and intermittent supplementation was apparently greater for NT-3 compared to BDNF is unknown. There is the possibility that the half-life for the NT-3 molecule is much shorter than that of BDNF.

While it appears that for NT-3, at least, continuous supply of the factors does produce a greater survival response when compared to intermittent administration, neither factor was capable of inducing total survival of motoneurons during NOCD. It is unlikely that the lack of survival is due to an inability to obtain secreted factor. Neurotrophins are highly diffusible proteins and administration of factor on to the chorioallantoic membrane results in motoneuron survival (Oppenheim *et al*, 1992). Thus,



factor secretion directly into the ventricular space of the embryonic CNS should provide adequate factor supply to the spinal cord. It appears that while continuous trophic support can increase the survival response of some neurotrophic factors, it is likely that sub-populations of motoneurons possess distinct neurotrophic requirements.

### 3.34 A Combination BDNF and NT-3 Slightly Increases Motoneuron Survival during NOCD Over That of Single Factors

The lack of total support by any one factor in this and other studies suggests the presence of motoneuron sub-populations, which differ in their trophic requirements. Calderó *et al* (1998) found that BDNF, NT-3 and NGF rescued a greater proportion of DRG during NOCD when administered in combination compared with the administration of single factors. When supplied in combination, FGF-II and CNTF rescue 100% of spinal motoneurons *in vitro* (Arakawa *et al*, 1990). Mitsumoto *et al* (1994) found that co-treatment of BDNF and CNTF arrested disease progression in *wobbler* mice, a murine model of motoneuron disease. A combination of these two factors has also been shown to increase motoneuron survival of axotomised neurons over that of the single factors (Vejsada *et al*, 1995). While the spatial and temporal expression patterns of CNTF and FGF-II do not suggest these factors play a primary role in motoneuron support during NOCD (Oppenheim *et al*, 1991) such studies support the concept that different factors are required by different motoneurons during NOCD.

Our results also support the requirement of the motoneuron population for multiple factors. When supplied in combination, BDNF and NT-3 promoted a significant increase in motoneuron survival compared to single factor supplementation. However, the combination of factors was unable to support the entire LMC motoneuron population, suggesting neurotrophic factors outside of the known neurotrophin family play a role in supporting cells during NOCD. The co-treatment of embryos with BDNF and NT-3 did not produce an additive increase in survival. Thus, there appears to be a degree of redundancy in the factor requirements of motoneurons, where individual cells may be responsive to either factor. Whether this redundancy is caused by promiscuity of Trk molecules or the expression of multiple Trk receptors on the cell surface is not known. However, such trophic redundancy may suggest a reason for the apparent lack of spinal motoneuron loss observed in BDNF, NT-3 and NT-4/5 knockout mice (Jones *et al*, 1994; Klein *et al*, 1994; Conover *et al*, 1995).

### 3.35 Survival Effects of BDNF and NT-3 Are Not Maintained Over a Longer Duration

Numerous neurotrophic factors have been found to promote increased survival during NOCD. However, those studies that have examined neuron survival over an extended period have often encountered problems with maintaining the level of survival. Oppenheim *et al* (1992) found that intermittent administration of BDNF was capable of rescuing 33% of



motoneurons that would otherwise succumb by E10. While increased motoneuron survival in response to BDNF was still observable by E16, the level of survival had fallen to a level similar to that of normal E9 embryos. Prolonged treatment usually fails to maintain levels of cell survival over that of controls. However, constant expression systems have been shown to provide trophic support to neurons *in vivo* for several months (Zhu *et al*, 1992; Grill *et al*, 1997a). Whether the reduction in trophic support is due to the cumulative effects of factor deprivation or whether it reflects a temporal change in factor responsiveness has not previously been resolved. Within the peripheral nervous system it appears that trophic requirements of neurons do change and factors act sequentially to promote their survival (Davies, 1997).

We examined whether the constant expression of exogenous BDNF and NT-3, alone and in combination, could maintain the level of survival observed at E10 over the prolonged period of NOCD observed in Section 3.21. Compared to E10, neurotrophin mediated survival at E15 was observed to decrease under all three factor supplementation regimes. E15 motoneuron populations only appeared significantly responsive to BDNF. Supplementation with NT-3 did appear to slightly increase survival over normal E15 levels, however, the increase was not statistically significant. It may be necessary to increase the sample size used in this study to determine whether NT-3 is actually capable of promoting survival at E15. Co-treatment of embryos with BDNF and NT-3 did not produce survival levels

greater than that promoted by BDNF alone. As with the peripheral nervous system, it appears that neuronal populations within the central nervous system may also undergo constant regulation of the neuronal population mediated by temporal changes in trophic support. It also appears that factors outside the neurotrophin family are necessary to support different neuronal populations. The neurotrophin family has been a major focus in neuronal survival during NOCD, due primarily to the correlation between their expression and the duration of cell death. The importance of neurotrophic factors that have been shown to support motoneurons during NOCD yet display ubiquitous or atypical expression patterns has been questioned (Oppenheim *et al*, 1991). However, such pleiotrophic factors may play a role in the control of cell survival during this period. The comparatively low trophic effect of BDNF and NT-3 observed at E15 does not necessarily imply these factors are not involved in the trophic support of spinal motoneurons at this stage. These factors may still act as potential sequential support for those motoneurons that succumbed to factor deprivation by E10.

We do however note that the use of a murine expression system in a chicken model does possess some limitations to the interpretation of the results. While the 2.3D xenograft does not appear to be rejected by the host at E8 (Pankhurst, 1996) the effectiveness of the embryo's immune response at later stages has not been determined. As transient transfection was used to incorporate the pcDNA3 expression construct into the 2.3D cell line, the stability of the expression system may become an issue as the duration of



trophic support increases. This expression system has not previously been used to examine trophic support over such an extended period. Even in stable recombinant cell implants, transgene expression levels have been found to decrease over time following implantation (Palmer *et al*, 1991). Thus, while the cell line may still be viable, the integrity of the expression system in the latter stages of NOCD may be compromised. It should also be noted that by E15 the comparative size of the ventricular space is greatly reduced. It is possible that the observed reduction in survival may be caused by a restriction of the access of the factor to the motoneurons. Thus, the lack of support observed at E15 may still result from cumulative factor deprivation.

It is clear that the increased trophic response resulting from a combination of BDNF and NT-3 observed at E10 is not maintained through to E15. Such a reduction can be explained by the lower responsiveness of motoneurons to NT-3 compared with BDNF. It does, however, seem unlikely that the integrity of the NT-3 expression system should differ significantly from the BDNF expression system. As such, the changed levels of factor responsiveness would not appear to result solely from the limitations of our constant expression system. Thus, the change in factor responsiveness should be seen, at least in part, to suggest an actual shift in the factor requirements of LMC motoneurons.

Our results do suggest that factor deprivation is an important consideration when determining the effectiveness of neurotrophic support.

Stage III trials for BDNF as a therapeutic agent against ALS have had limited success. (The BDNF Study Group [Phase III], 1999). The trials relied on daily administration of BDNF to patients over a three month period after which no significant difference between the gross sample groups were observed. If the cumulative effects of factor deprivation do have serious implications for the effectiveness of a factor's trophic support, it may be necessary to reconsider the methods of drug delivery before deeming a therapeutic agent ineffective.

# CHAPTER 4

# THE EFFECTS OF SUPPLYING SPINAL MOTONEURONS WITH A CONSTANT SOURCE OF NEUROTROPHINS FOLLOWING TARGET ABLATION

# CHAPTER 4

## 4.1 INTRODUCTION

Neurons are known to become dependant on their target fields following innervation. Target fields secrete specific neurotrophic factors and it is the ability to respond to these trophic signals that determines whether the innervating neuron survives or succumbs to NOCD. Classic experiments have shown that the removal of the target field prior to NOCD results in a reduction of those neurons that ordinarily innervate that tissue (Hamburger & Levi-Montalcini, 1949). A potent method of studying this phenomenon employs the amputation of the limb bud prior to neuronal innervation. The limb bud constitutes a major innervation target of LMC motoneurons and the removal of the limb bud prior to innervation results in the loss of a large proportion of these motoneurons. Furthermore, connections made by the motoneurons of each motor column appear to be bilaterally distinct (Oppenheim *et al*, 1978). Therefore, an intact limb bud contralateral to the amputated limb bud does not provide target-derived support to the motor column ipsilateral to the amputated tissue. Thus, one need only examine a single LMC to determine the total effect of target ablation.

In Chapter 3 we showed that constant supplementation of BDNF and of NT-3 was capable of promoting an increase in the levels of LMC motoneuron survival during NOCD. This observed responsiveness of chick



spinal motoneurons to BDNF and NT-3 correlates to observations in the rat, which show BDNF and NT-3 expression to occur in the hindlimb target tissue while the TrkB and TrkC receptors are expressed by the spinal motoneurons (Henderson *et al*, 1994). Despite the evidence suggesting BDNF and NT-3 are potent survival factors for spinal motoneurons during NOCD, intermittent administration of either of these factors to embryos that are amputated prior to target innervation fails to prevent the increased neurodegeneration resulting from target ablation (Calderó *et al*, 1998). It remains unclear as to whether the inability to promote cell survival in the absence of a target is an accurate reflection of neuronal responsiveness to neurotrophins or whether it is the result of accrued factor deprivation between administrations exacerbated by the absence of target derived support.

In this chapter we examined the effects of continuously supplying neurotrophins to LMC spinal motoneurons following the removal of the embryonic limb bud. We have employed the same expression system utilised in Chapter 3 to continuously deliver BDNF and NT-3 to the embryonic spinal cord. We aim to determine the extent to which constant supplementation of these two factors individually can reduce the extent of motoneuron loss that results from the removal of the target field. BDNF and NT-3 will also be supplied in combination to determine whether the addition of both factors can elicit a greater survival response. The effect of the neurotrophins on motoneuron survival following target ablation will be examined both after the main phase of normal neurodegeneration and at the

later stages of NOCD. We aim to examine whether these factors can maintain neuron survival or whether the supported motoneurons undergo temporal changes in their trophic requirements in the absence of a target field.



## 4.2 RESULTS

### 4.21 Removal of the Developing Limb Buds

Motoneuron loss during NOCD is known to be exacerbated by the removal of the limb bud prior to this period due to the reduction in the target field. Limb bud removal was performed on developing chick embryos to ensure this increased cell loss did occur within the developmental time period being examined in this study. Embryonated white Leghorn eggs were incubated at 39$^{O}$C without turning until E5. At E5 an aperture was created in the blunt end of the egg. The chorioallantoic membrane was removed and the embryo was manoeuvred to expose one of the lower limb buds. Using fine forceps the lower limb bud was severed close to the pelvic region. Care was taken to ensure the blood vessels surrounding the embryo and the embryo's internal circulatory system did not haemorrhage during surgery as this inevitably resulted in the death of the embryo. However, localised bleeding resulting directly from the removal of the limb bud did not appear to affect embryo survival. Following limb bud removal the egg was sealed and further incubated until E10.

At E10 the embryos were removed from the egg and decapitated. Only embryos from which the lower limb had been completely removed were used for analysis. The side from which the limb had been removed was recorded. Embryos were dissected as for Chapter 3 and the lumbar region of

the spinal cord was excised including a portion of the sacral spinal cord in the excised tissue to facilitate orientation whilst embedding. The tissue was fixed in Carnoy's fixative and processed and embedded into glycolmethacrylate. Before the glycolmethacrylate resin was allowed to set in the cutting moulds, the tissue was orientated within the mould such that the rostral region of the lumbar was positioned closest to the cutting face. This allowed for the determination of which motor column was ipsilateral to the amputated limb. As for Chapter 3, the embedded tissue was sectioned to a thickness of 20μm, sampling every thirtieth section from the fourth section containing tissue. The tissue was then stained with haematoxylin and eosin. The motoneurons of the LMC were counted using the optical disector/fractionator combination with the same sampling paradigm outlined in Chapter 3. Based on the orientation of the tissue in the block, the motoneurons of the motor column ipsilateral to the amputated limb were counted. The motoneurons of the motor column contralateral to the amputated limb were also counted to determine the extent to which unilateral limb bud removal affects the two LMC populations.

In accordance with previously published studies, limb amputation at E5 resulted in a substantial loss of LMC motoneurons *(Figure 4.1)*. Approximately 55% of those motoneurons present at E10 were lost in the motor columns ipsilateral to the amputated limbs (*Figure 4.2)*. Cell loss was evident in all sections sampled from each embryo. LMC contralateral to the

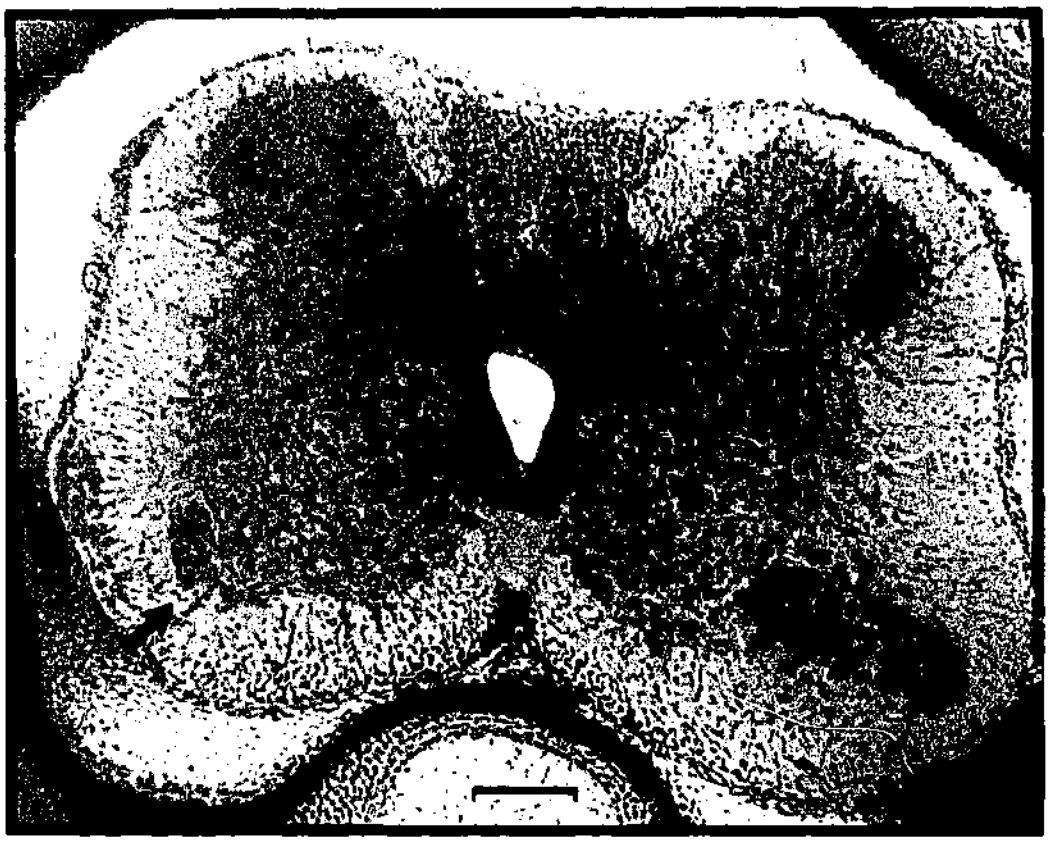


Figure 4.1: A cross section of a chick lumbar spinal cord at E10 showing a reduction in LMC motoneuron density resulting from target ablation. At E5 a lower limb bud was removed from the embryo. Lumbar spinal cords were harvested at E10 and examined microscopically. The area of the LMC ipsilateral to the amputated limb (marked with an arrow) is visibly reduced at E10 in comparison to the contralateral LMC The reduction in LMC area corresponds to a reduction in the number of motoneurons within the LMC. Bar = 100μm

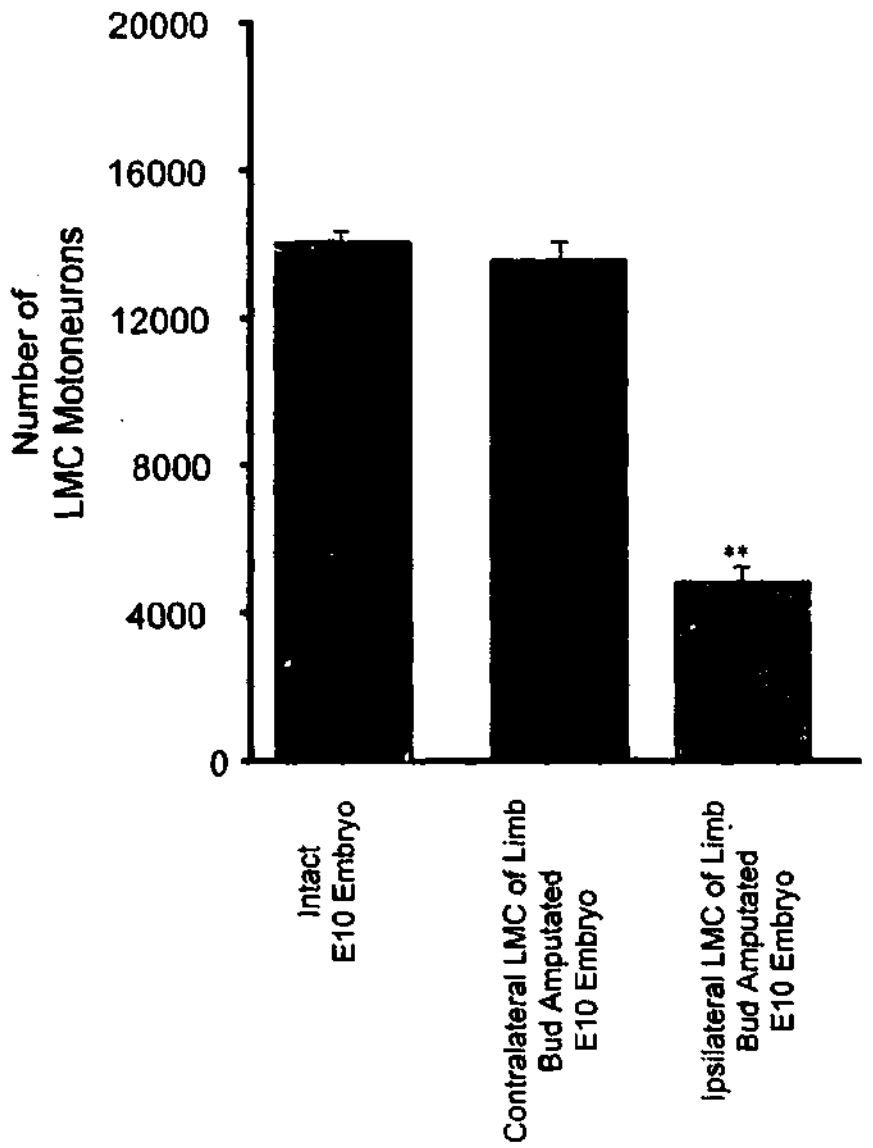



Figure 4.2: The reduction of motoneuron survival following the removal of the lower limb bud. At E5 a lower limb bud was surgically removed from the chick embryo. The graph indicates the number of LMC motoneurons present at E10 in non-amputated embryos compared to amputated. Within amputated embryos, the LMC contralateral to the site of amputation did not show a significant difference in motoneuron density when compared with non-amputated embryos. Motoneuron density in the LMC ipsilateral to the site of amputation was significantly different to that of the contalateral LMC and those of non-amputated embryos. ** = $P<0.01$ Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord.

site of amputation did not display any significant reduction in motoneuron density in comparison to intact embryos. It appears that the targets innervated by the motoneurons within each LMC are bilaterally discrete. Thus, motoneuron loss in the LMC ipsilateral to the site of amputation should be indicative of the complete extent of LMC motoneuron loss resulting from target ablation.

## 4.22 The Effects of Supplying Neurotrophins to Developing Embryos Following Target Ablation

The expression model used in the previous chapter was employed to determine the extent to which BDNF and NT-3, alone and in combination, could support motoneuron survival following a significant reduction of the target field. 2.3D cells modified to contain the pcDNA3, pcDNA3/BDNF or pcDNA3/NT-3 constructs were prepared as for the previous chapter. The factor-secreting, modified 2.3D cells were implanted into the optic tectum of E5 chicks. At the time of cell graft implantation a lower limb bud was removed from the embryo as for Section 4.21. To analyse the effects of supplying the factors in combination, amputated embryos were also implanted with a mixed population of the two neurotrophin-secreting cell types. Control trials were conducted by implanting 2.3D cells transfected with the parental pcDNA3 vector into embryos following limb bud removal. Lumbar spinal tissue was harvested at E10 and E15. The tissue was embedded, sectioned and counted as per Section 4.21.

Following limb bud removal, implantation of the control modified 2.3D cells containing the parent vector resulted in a reduction in the LMC motoneuron population ipsilateral to the amputated limb. There was no significant difference in the numbers of LMC motoneurons in the target ablated embryos implanted with the control cell line when compared with those target ablated embryos that had not received a graft. Thus, it appears that the implantation of the vector transfected 2.3D cells and the cranial trauma resulting from implantation does not have an impact on the survival of the LMC motoneurons. Any impact on motoneuron survival in these trials can thus be attributed to the secretion of factor by the modified grafts and not the presence of the graft itself.

Constant supplementation of BDNF ($P<0.01$) and of NT-3 ($P<0.01$) to the limb bud amputated embryos resulted in a statistically significant increase in survival at E10 over that of the amputated controls *(Figure 4.3)*. BDNF was capable of restoring approximately 73% of the LMC motoneuron density normally seen at E10 in intact embryos. Similarly, NT-3 was capable of rescuing approximately 57% of LMC motoneurons that would otherwise succumb to target ablation exacerbated cell death during NOCD.

By E15, BDNF and NT-3 were still capable of providing trophic support to the LMC motoneurons of the amputated embryos *(Figure 4.4)*. In contrast to the observations for embryos which did not undergo limb bud removal, the number of motoneurons supported by BDNF and NT-3 in the



mputated embryos at E15 did not decrease in comparison to those at E10. As a result both BDNF and NT-3 maintained LMC motoneuron survival to a level comparable to normal E15 embryos

A combination of BDNF and NT-3 did not produce an increase in motoneuron survival over that of the single factors alone at E10 or E15 ($P>0.05$). Thus, it also appears that those cells that are responsive to BDNF are also responsive to NT-3. It should be noted that the motoneuron counts evident at E15 may not be reflective of the total number of potentially responsive cells at this stage in non-amputated embryos as it does not include those initially unresponsive motoneurons which may change their trophic requirements in favour of the neurotrophins. In contrast to the results obtained in Chapter 3 for intact embryos, no loss of LMC motoneurons occurred between E10 and E15 in amputated embryos supplied with either factor alone or in combination.

Neither BDNF nor NT-3, alone or in combination, were capable of rescuing motoneurons to a level comparable to intact factor supplemented embryos. The difference in motoneuron survival between intact and amputated embryos decreased between E10 and E15 under all factor supplementation regimes. This was most likely caused by the reduction in motoneuron survival in intact embryos between E10 and E15 compared with the maintenance of motoneuron numbers in amputated embryos during this period.

Figure 4.3: The effects of continuous supplementation of neurotrophins, alone and in combination, on LMC motoneuron survival in target ablated embryos at E10. At E5 embryos were implanted with 2.3D cells transfected with either the pcDNA3/BDNF construct or the pcDNA3/NT-3 construct. At this time a lower limb bud was surgically removed. The graph indicates the number of LMC motoneurons present at E10 following implantation of BDNF secreting cells, the NT-3 secreting cells or a mixed population of the two recombinant cell types. The control vector group consists of amputated embryos implanted with 2.3D cells containing the unmodified parental vector. The E10 intact control group, implanted with the parental vector cell graft, did not undergo limb bud amputation. Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord. Lines and error bars above columns show motoneuron numbers in non-amputated E10 embryos as determined in Chapter 3. Significance values for intact embryos refers to the difference between intact and amputated embryos under the given supplementation regime. Constant supplementation of BDNF ($P<0.01$) and of NT-3 ($P<0.01$) significantly increased motoneuron survival over that of the amputated control. Supplying BDNF and NT-3 in combination did not significantly increase survival over that of single factors ($P>0.05$). Neither factor, alone nor in combination, rescued motoneurons to a level comparable with non-amputated controls ($P<0.01$).



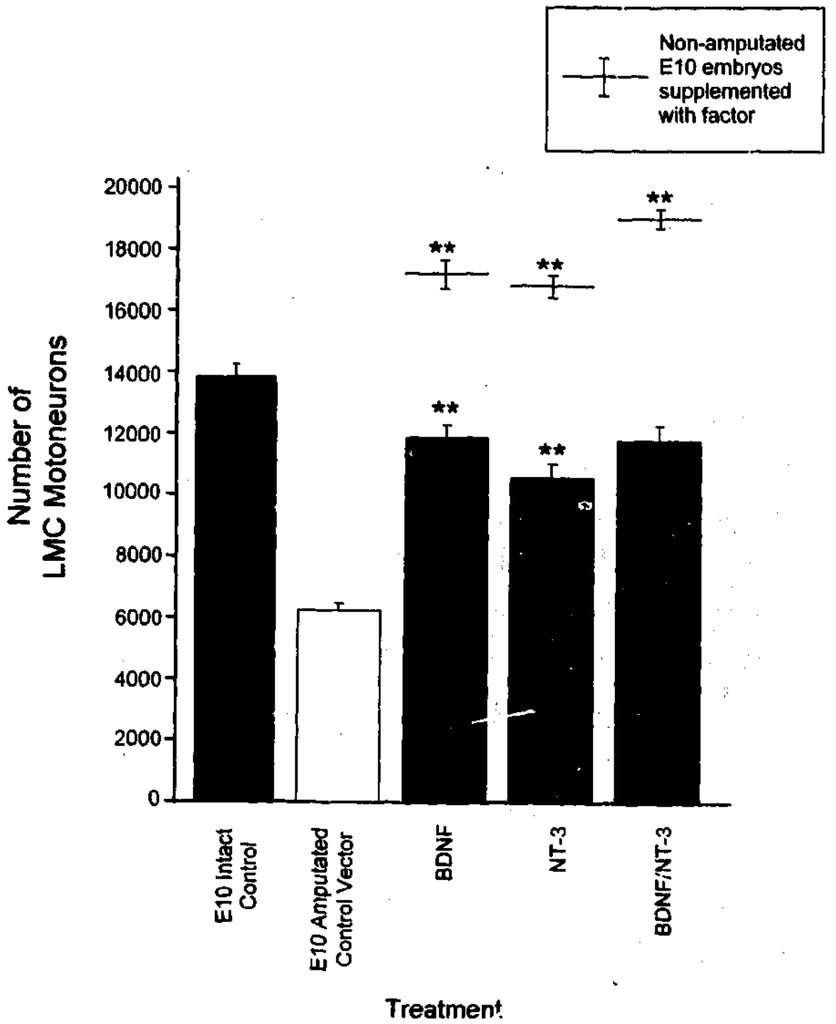
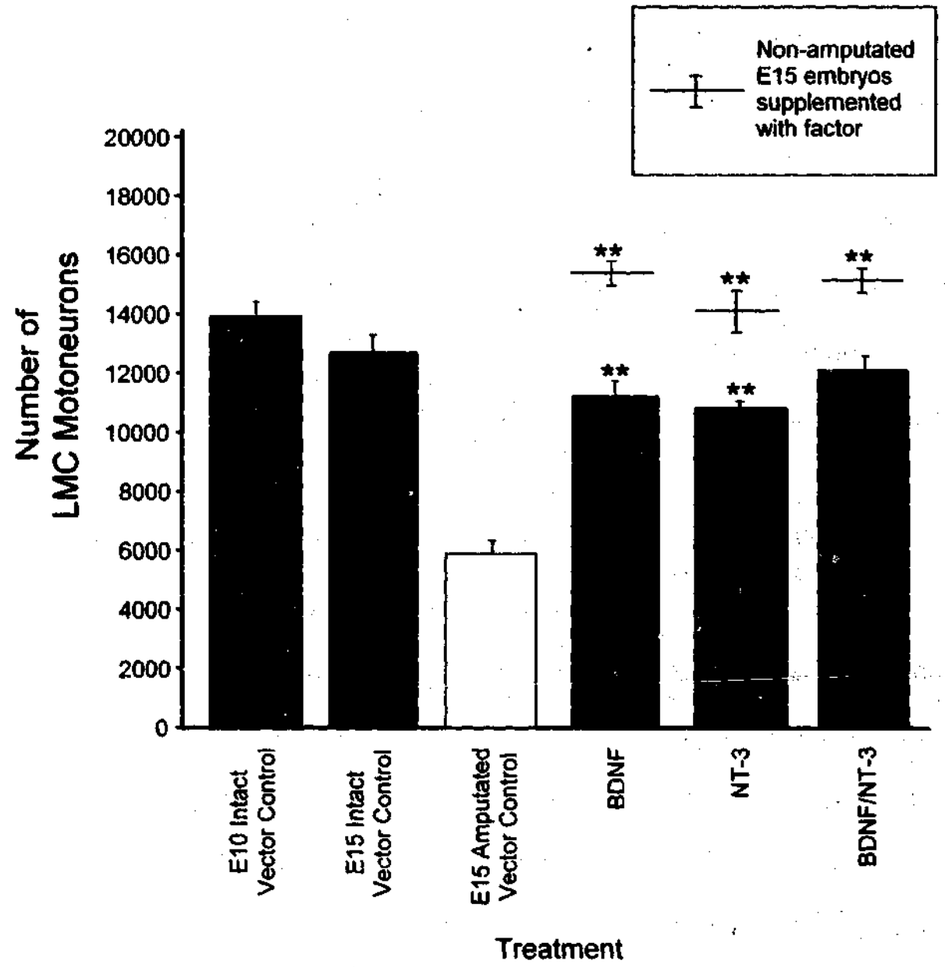


Figure 4.4: The effects of continuous supplementation of neurotrophins, alone and in combination, on LMC motoneuron survival in target ablated embryos at E15. The graph indicates the number of LMC motoneurons present at E15 following implantation of BDNF secreting cells, the NT-3 secreting cells or a mixed population of the two recombinant cell types. The control vector group consists of amputated embryos implanted with 2.3D cells containing the unmodified parental vector. The E10 and E15 intact control groups, implanted with the parental vector cell graft, did not undergo limb bud amputation. Lines and error bars above columns show motoneuron numbers in non-amputated E10 embryos as determined in Chapter 3. Significance values for intact embryos refers to the difference between intact and amputated embryos under the given supplementation regime. Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord. Constant supplementation of BDNF ($P<0.01$) and of NT-3 ($P<0.01$) significantly increased motoneuron survival over that of the amputated control. Supplying BDNF and NT-3 in combination did not significantly increase survival over that of single factors ($P>0.05$). BDNF, both alone and in combination with NT-3, rescued motoneurons to a level that was not significantly different from E15 intact control embryos (BDNF: $P>0.05$: BDNF + NT-3 $P>0.05$).





In the vector control embryos there was no significant reduction in LMC motoneuron numbers between E10 and E15 following limb bud removal. (P>0.05) (*figure 4.5*). This observation is in contrast to the observations in Chapter 3, where motoneuron death was found to continue between E10 and E15 in normal embryos (P<0.05). These findings suggest that the target tissue continues to influence the survival of motoneuron populations throughout the extended period of NOCD. It appears that under normal conditions the embryonic LMC undergoes a gross elimination of motoneurons. Following this the target tissue continues to remove support to those inappropriately connected motoneurons.

The removal of the limb bud prior to motoneuron innervation would remove all trophic support to those motoneurons that ordinarily innervate the leg resulting in the exacerbated cell death that was observed in E10 populations (*figure 4.2* and *figure 4.5*). However, in the absence of target tissue no further changes in target mediated support can occur. Therefore, the LMC motoneurons in the amputated chick remain at a constant level of survival between E10 and E15 (*figure 4.5*).



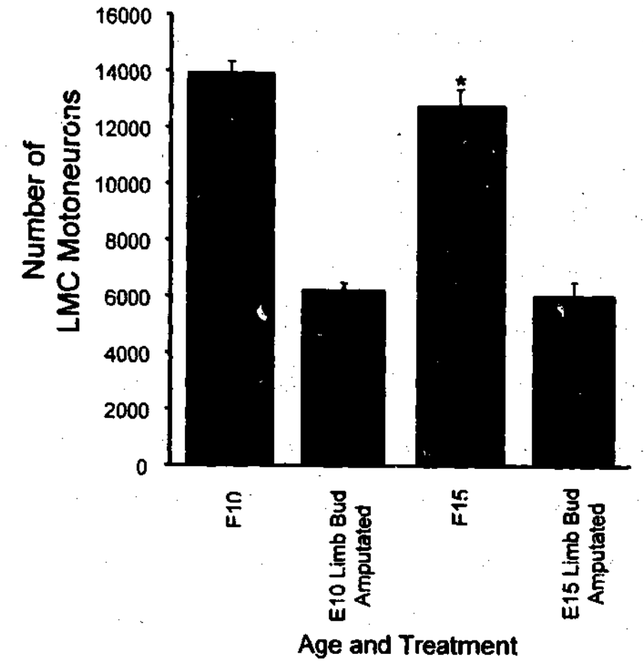



Figure 4.5: The effects of target ablation on LMC motoneuron density at E10 and E15. All embryos were implanted with the 2.3D cell line transfected with the parental vector at E5. Intact and limb bud amputated embryos were examined at E10 or E15. Values are expressed as mean ± SE numbers of motoneurons per LMC in the embryonic chick spinal cord. Asterisks indicate the level of significant difference between different developmental periods under the same experimental regime. * = P<0.05

## 4.3 DISCUSSION

### 4.31 Motoneuron Loss in Response to Target Ablation

Studies dating back nearly a century have shown that neuronal target fields are essential for the maintenance of neuronal populations ordinarily innervating that tissue (Shorey, 1909; Hamburger & Levi-Montalcini, 1949). Hamburger and Levi-Montalcini (1949) showed that the target field itself is responsible for supporting neurons. Enlargement of the peripheral fields, by grafting supernumerary limb buds on to the developing chick prior to axonal migration, was shown to increase the number of neurons in spinal populations ipsilateral to the graft. Similarly, the reduction of target tissue, via the removal of the limb bud, resulted in a reduction of the neuronal population normally innervating the limbs.

It is now clear that the changes in neuronal survival that occur when the size of the target field is altered are merely an exaggeration of the processes that occur during NOCD. The expansion or retraction of the target field results in a corresponding increase or decrease in the spatial levels of trophic support. Target tissues have been found to support the innervating neurons via the secretion of various neurotrophic factors, a phenomenon which has formed the basis of the neurotrophic theory proposed by Oppenheim (Reviewed by Oppenheim, 1989). During NOCD, mRNA for both BDNF and NT-3 is expressed in the limb buds and mRNA for the TrkB and



TrkC receptors is expressed in spinal motoneurons (Henderson *et al*, 1994), suggesting BDNF and NT-3 provide such target derived support. Such findings are somewhat contradicted by the inability of these factors to elicit a significant trophic response in spinal motoneurons during early NOCD following limb bud removal (Calderò *et al*, 1998).

The LMC motoneurons of the lumbar spinal cord are a useful population for examining target mediated trophic support as a large proportion of these motoneurons innervate the lower limbs, a significant yet experimentally removable target field. In this study, we have utilised the well established technique of unilateral limb bud amputation prior to the onset of target innervation to examine the ability of neurotrophins to support motoneurons in the absence of target tissue support.

Following the removal of the hind limb bud, cell loss in the chick hind limb innervating motoneuron populations is known to begin between E6 and E7. Between E5 and E6, embryos that have undergone limb bud removal still possess a normal complement of cells (Oppenheim *et al*, 1978; Lanser & Falley, 1984). In this study limb bud amputation was carried out at E5, prior to the known period of target innervation. Removal of the limb bud prior to target innervation resulted in the loss of over 70% of LMC spinal motoneurons present prior to NOCD. These observations support published studies, which found similar reductions in population density in response to limb bud amputation (Hamburger & Levi-Montalcini, 1949; Oppenheim *et al*,

1978; Calderò *et al*, 1998). Complete cell loss within the effected LMC was not observed. Those LMC motoneurons that did not succumb most likely support the lumbar region of the embryo and possibly any tissue from the upper pelvic region of the limb that may not have been totally removed during surgery.

Amputation of the lower limb bud was shown to only affect the survival of those motoneurons of the LMC ipsilateral to the lesion site. Similar observations have been made by Oppenheim *et al* (1978). The motor columns appear to innervate bilaterally discrete target fields. Thus, trophic support of LMC motoneurons appears to emanate from tissue ipsilateral to the LMC with little or no support derived from tissue on the contralateral side of the body. This unilateral nature of target innervation by the LMC motoneurons allows us to determine the complete effect of neurotrophic factor supplementation on LMC motoneuron populations following unilateral limb bud amputation by counting a single LMC. Thus there is no need to consider possible trophic interference from contralateral target tissue when analysing such data.



### 4.32 BDNF and NT-3 Decrease Motoneuron Loss in Response to Target Ablation

Constant supplementation of BDNF and NT-3 individually resulted in a significant decrease in the level of cell death normally occurring by E10. Oppenheim *et al* (1992) observed that BDNF was capable of eliminating LMC motoneuron loss between E9 and E16 resulting from deafferentation in the developing chick. By E16, BDNF was shown to be capable of promoting motoneuron survival in deafferented embryos to a level comparable to that of embryos that had been supplied with BDNF but had not undergone deafferentation. In this study neither BDNF nor NT-3 were capable of supporting LMC motoneurons in amputated subjects to a level comparable to normal E10 levels. Only BDNF, alone and in combination with NT-3, could rescue E10 and E15 motoneurons to levels comparable with normal E15 embryos. This would suggest that target derived trophic support plays a greater role in the survival of LMC motoneurons during NOCD than the anterogradely transported trophic factors derived from the spinal and supraspinal afferents.

### 4.33 Constant Factor Supplementation is Important for Rescuing Motoneurons Following Target Ablation

Studies that have examined the effects of intermittent administration of neurotrophic factors to target ablated motoneurons during NOCD have failed to show a trophic response to BDNF or NT-3 (Calderò *et al*, 1998). Such findings contradict the expression patterns of BDNF, NT-3 and their Trk receptors, which do support a role for these factors in target derived support (Henderson *et al*, 1993, Kahane *et al*, 1996). Calderò *et al* (1998) examined limb bud removal induced motoneuron death prior to E7.5 noting that factors that potentially support these neuronal populations may elicit a response following this period. We have observed higher survival rates in this study at E10 by using constant administration than have previously been achieved with the seemingly ineffective factors at E7.5. The observation that NT-3 is expressed in the innervating leg segments from as early as E5 and that BDNF expression is first seen at E6 (Kahane *et al*, 1996) leads to some confusion in the interpretation of the published data. Our results suggest constant exogenous support may become even more important when analysing the effects of supplying exogenous factor in the absence of trophic redundancy resulting from target ablation. If, as temporal expression patterns suggest, distinct motoneuron populations are responsive to BDNF and NT-3 early in NOCD, the removal of the target field prior to target innervation would eliminate any other target-derived factor support. With only one trophic factor to support the neuronal populations following



target ablation, the potential for factor withdrawal resulting from intermittent factor administration may have a greater impact, as secondary support from other factors is not available.

The ability of constant factor supplementation to improve neuronal survival in the absence of trophic support from the innervated target has been documented in studies employing induced neuronal trauma in adult rats. Neurotrophins have been shown to prevent neurodegeneration following axotomy in rats (Schnell *et al*, 1994; Vesjada *et al*, 1994). However, such effects are usually transient when factors are supplied at intermittent periods (Vesjada *et al*, 1994). By supplying neurotrophic factors via the implantation of a genetically modified fibroblast cell line, Grill *et al* (1997a; 1997b) found that the augmentation of neuronal support via NGF and NT-3 following axotomy of the mid-thoracic spinal cord continued for over three months. Strömberg *et al* (1990) found that 3T3 fibroblast cells modified to secrete NGF rescued axotomised cholinergic neurons for up to six weeks post lesion. Such observations provide further evidence for the increased effect on cell degeneration that factor deprivation can have on neurons deprived of trophic support from their innervated tissue.

**4.34 Not All Target Ablated LMC Motoneurons are Responsive to Neurotrophins.**

While BDNF and NT-3 were able to support cell survival in amputated embryos to levels approaching those of normal E10 embryos, those motoneurons that survived may not necessarily represent the populations that would normally be supported by the target field. When comparing motoneuron survival in factor supplemented, target ablated embryos with motoneuron survival in factor supplemented, intact embryos, as presented in Chapter 3, a reduction in motoneuron number is seen across supplementation regime. Therefore, a proportion of LMC motoneurons that are normally supported by the limb bud, succumbed to target ablation in the presence of neurotrophins. It is evident that other neurotrophic factors are also supplied by the target tissue during NOCD.

Numerous neurotrophic factors have been shown to support neuronal populations during NOCD (Nurcombe *et al*, 1991; Oppenheim, 1991; Calderò *et al*, 1998), however, by only supplying neurotrophins we may be selecting specifically for neurotrophin responsive motoneurons. Whether the selection of motoneurons with specific trophic requirements, instead of the normal complement of cells, would have any impact of the functionality and connectivity of the motoneurons in a normal subject is unclear. However, if these factors are to serve a therapeutic purpose,



understanding the impact of preferentially supporting certain neural populations may be necessary.

It is probable that target-derived support for motoneurons is far more complex than single factor supplementation experiments may allow for. The complexity of target derived support may account for the discrepancies in the factor responsiveness of motoneurons *in vivo* and *in vitro*. While cultured rat motoneurons derived from populations undergoing NOCD do respond to BDNF and NT-3 in rats (Henderson *et al*, 1993; Hughes *et al*, 1993), many studies have reported that chick motoneurons are unresponsive when these factors are supplied alone to the culture medium (Arakawa *et al*, 1990; Bloch-Gallego *et al*, 1991). However, in the presence of muscle extract BDNF and NT-3 become potent survival factors for cultured chick motoneurons (Becker *et al*, 1998). It would appear that in addition to the secretion of target specific trophic factors, the target fields in the chick also supply innervating motoneurons with an apparently less population specific array of factors required for neuronal support. The decrease in availability of this additional target derived support caused by the reduction of the target field may account for the inability of either BDNF or NT-3 to increase motoneuron survival in amputated embryos to the levels seen in intact factor administered embryos. The retention of target-derived support following deafferentation may explain why Oppenheim *et al* (1992) found BDNF to be so potent in preventing deafferentation induced cell death.

### 4.35 Supplying BDNF and NT-3 in Combination Does Not Increase LMC Motoneurons Survival Over That of Single Factors

As was the case in Chapter 3, constant supplementation of BDNF and NT-3 in combination to the amputated embryos was unable to promote a level of survival significantly greater than that achieved by single factors alone. It appears that motoneurons display some degree of neurotrophic redundancy, being responsive to multiple factors. Within the spinal cord expression of BDNF and NT-3 occurs largely in distinct populations of motoneurons (Kahane *et al*, 1996). Conversely, TrkB and TrkC display a considerable amount of overlap in their expression patterns (Merlio *et al*, 1992; Henderson *et al*, 1993). In light of the potential trophic redundancy observed in this study, it would appear that motoneurons may be responsive to a number of trophic factors and have the potential to innervate a number of target fields. The specificity of support for innervating motoneurons from specific target fields may result from a complex requirement of neurons for specific temporal combinations of neurotrophic factors. The regulation of such changing support may be at least in part controlled by the target field itself. Coupled with a degree of trophic redundancy, as observed in our study, such a system would allow for the required specificity necessary for correct synaptic connectivity yet provide sufficient latitude to allow the innervation of neurons of varying trophic requirements.



It is evident from this study that, although BDNF and NT-3 are potent survival factors for motoneurons, they are not the only neurotrophic factors capable of eliciting a trophic response in LMC motoneurons during NOCD. Neurotrophins most likely play a part in a more complex system of multi-factoral support and regulation of neuronal cell population size. The potential remains for other target derived factors outside the neurotrophin family to be required to elicit a complete trophic response. Such factor support would be absent in the target ablation model we have employed. As such, any potential interaction of these factors with the factors employed in this study would not be evident.

### 4.36 Motoneuron Survival Levels Plateau Following Initial Target Ablation Induced Neurodegeneration

Between E10 and E15, no significant change in the level of factor promoted survival was seen in embryos treated with BDNF and NT-3, alone or in combination. This is in contrast to the evidence for temporal changes in the trophic requirements of motoneurons documented in Chapter 3. Such results would imply that the trophic requirements for these surviving motoneuronal populations do not appear to change. This observation was unexpected, given the apparent reduction in neurotrophin responsiveness seen in intact embryos. The neuronal populations sustained by neurotrophin supplementation following target ablation do represent a considerably smaller population than those present in the intact factor supplemented

embryos. While those populations present in the target ablation experiments are reflective of the cells that are sustainable on BDNF or NT-3 alone, they do not represent possible neurotrophin responsive populations which may also require other neurotrophic factors. The potential remains for changes in the trophic requirements of these populations to explain the loss of motoneuron populations seen between E10 and E15 in the neurotrophin supplemented embryos not subjected to limb bud removal. Thus, those populations that are solely dependent on neurotrophins may not undergo the temporal changes in their neurotrophic factor requirements known to occur throughout NOCD in the PNS (Reviewed by Davies *et al*, 1997).

Amputated control subjects, supplied with the parent vector, showed no extra loss of LMC motoneurons between E10 and E15. This also is in contrast to the observations from intact embryos, where the continued loss of up to 8% of the motoneuron population is observed over this period. The lack of additional motoneuron loss after E10 in amputated embryos suggests motoneurons in the LMC do not innervate the target field at later periods during NOCD in large enough numbers to fully explain the cell loss normally observed between E10 and E15. The lack of cell loss after E10 in amputated embryos would suggest most neurons in this area have innervated their targets and have become factor dependent. It appears likely that following innervation of the target by the motoneurons, the target-derived trophic support changes in an effort to further reduce support to inappropriately connected neurons. Such changes in trophic requirements



most likely allow the removal of cells from the neuronal population that, though responsive to the initially secreted factor, may still have formed improper or superfluous synaptic connections. While the importance of sequential changes to neuronal factor requirements in the CNS remains unclear, our observations for untreated embryos suggest that such a mechanism for fine tuning neuronal connections may be present within the developing CNS. Target ablation results in a rapid decrease in the potentially factor responsive motoneuron population due to the absence of target-derived support. In the absence of a target field, the mechanisms by which neuronal population density is controlled are removed. Therefore, the population density remains at a basal level.

While the lack of cell loss seen in both neurotrophin supplemented and unsupplemented amputated embryos during late NOCD may suggest that the trophic responses of some populations at least do not change their trophic requirements, the lack of change may also reflect a stagnation of natural processes of trophic requirement change resulting from the removal of the target field. In the absence of a more complex combination of target secreted factors, cells may respond by remaining responsive to those factors present in order to survive. There is also the potential that the target tissue itself plays a role in promoting or signalling the change in factor responsiveness of the innervating neurons. If the target tissue does play a direct role in the temporal changes in factor responsiveness of motoneurons, target ablation may eliminate cues for

trophic change resulting in a lack of requirement change over the period of NOCD.

# CHAPTER 5

## THE CHANGES IN TRKB AND TRKC EXPRESSION IN THE SPINAL CORD IN RESPONSE TO SUPPLEMENTATION WITH A CONSTANT SOURCE OF NEUROTROPHINS

# CHAPTER 5

## 5.1 INTRODUCTION

The ability of chick spinal motoneurons to respond to BDNF and NT-3 (Becker *et al*, 1998) is supported by the expression of their specific Trk receptors, TrkB and TrkC, within the spinal cord (Ip *et al*, 2001). However, the mechanisms by which Trk transcription is regulated in response to neurotrophin activity are poorly understood. Intermittent administration of NGF has been shown to increase TrkA mRNA in the sympathetic and sensory neurons of neonatal rats. NGF has also been shown to up-regulate the transcription of TrkA in PC12 cells (Miller *et al*, 1991; Zhou *et al*, 1995). Similar responses to BDNF have been suggested for TrkB transcriptional regulation (Sommerfeld *et al*, 2000). While prolonged exposure to BDNF does decrease the surface expression of TrkB *in vitro*, the reduction in surface protein does not reflect a reduction in transcript level. Evidence suggests that transcript levels may subsequently increase in response to the elimination of surface expressed TrkB (Sommerfeld *et al*, 2000).

The expression system employed in our study produces a constant supply of neurotrophins to the CNS at concentrations previously determined to be in excess of optimal concentrations. While several studies have examined the short term effects of neurotrophin supplementation on receptor expression, the long term effects of supplying neurotrophins at saturating



concentrations have not been well documented. Miller *et al* (1991) found that supplying NGF to PC12 cells resulted in an increase in TrkA mRNA transcription within 6 hours of administration. Although this increase was sustained for two days, by the third day neurotrophin responsiveness returned to normal levels. In the same study, daily injections of NGF to neonatal rats resulted in an increase in TrkA mRNA in sympathetic and sensory neurons over the 10 day period examined.

Outside of the nervous system, saturating concentrations of epidermal growth factor (EGF) has been shown to reduce EGF receptor expression. Gilligan *et al* (1992) found that administering saturating concentrations of EGF to a hepatoma-derived cell line that overexpressed EGF receptor resulted in an initial accumulation of EGF receptor mRNA within 4 to 8 hours. However, after 18 hours receptor mRNA biosynthesis was reduced below normal levels. Therefore, while published studies suggest neurotrophin supplementation can increase Trk receptor transcription (Miller *et al*, 1991; Zhou *et al*, 1995; Sommerfeld et al, 2000), prolonged exposure to high concentrations of neurotrophins may inhibit the biosynthesis of Trk mRNA.

The absence of high levels of neurotrophic factors in later stages of life is believed to be a factor in the inability of the body to arrest the progression of neurodegenerative diseases, such as motoneuron disease. The potential use of neurotrophins as therapeutic agents for such diseases is

therefore an area of intense study. Aged rats display reduced expression levels of TrkB and TrkC compared to younger animals (Johnson *et al*, 1996). It is therefore necessary to understand the potential of neurotrophins to increase receptor levels in order to assess their therapeutic value.

In Chapter 3, we observed an increase in LMC motoneuron survival in response to the constant supplementation of BDNF and NT-3, alone and in combination. It is of interest to determine whether this increase in motoneuron survival corresponds to an increase in the expression of the appropriate Trk receptors. In this chapter we examined the temporal changes in TrkB and TrkC expression in the spinal cords of embryos supplemented with BDNF or NT-3 via the constant expression system described in Chapter 3. The relative levels of TrkB and TrkC mRNA in the spinal cords were analysed by comparison with the expression levels of the housekeeping gene β-actin using RT-PCR. β-actin is a cytoskeletal protein that displays constant levels of expression, thus allowing its use as an endogenous standard. We aim to determine whether levels of TrkB and TrkC mRNA expression change in response to the constant supplementation of BDNF and NT-3.



## 5.2 RESULTS

### 5.21 Optimisation of the RT-PCR

Relative quantitative RT-PCR was employed to compare the expression levels of the TrkB and TrkC receptors in the embryonic spinal cord following constant supplementation with either BDNF or NT-3. The housekeeping gene β-actin was used as an endogenous standard from which the levels of Trk expression relative to that of the actin gene could be estimated. This allowed us to generate a profile of the temporal changes in TrkB and TrkC expression levels in embryos under different supplementation regimes.

Comparative analysis of RNA expression levels using RT-PCR requires sampling of the RT-PCR product whilst in the exponential phase of DNA amplification. During this phase the reaction is not limited by reactant concentration and the concentration of PCR product is proportional to the concentration of original mRNA species of interest (reviewed in Freeman *et al*, 1999). It was therefore necessary to determine during which period exponential amplification of the cDNA was occurring in order to assess the optimal number of amplification cycles to use when analysing the receptor expression levels.

Total RNA extracted from P2 chick cerebellum was used, in preference to embryonic spinal tissue, to optimise the amplification cycle number. Owing to the small size of the tissue and the low expression levels of Trk receptors, large quantities of embryonic spinal tissue would have been required to optimise the RT-PCR. The P2 chick cerebellum is known to express TrkB and represents a more abundant source of Trk receptor mRNA. The total RNA was reverse transcribed. The reverse transcription product was added to a master mix containing the PCR reagents before aliquoting into separate reaction volumes. This ensured consistency amongst reagent concentrations between individual reactions. Primers directed towards TrkB were used in the reactions.

The samples were amplified under identical reaction conditions. The reactions were sampled at 5 cycle intervals between 0 and 35 cycles of amplification. The resulting RT-PCR products were electrophoresed and radioactively probed via Southern hybridisation. The resulting signal intensities for each sample were analysed via phosphor imaging. The intensity of the radioactive signal at each cycle period was assessed to determine when exponential amplification was occurring.

*Figure 5.1* shows a profile of the rate of product amplification over the PCR reaction period. Following the start of the reaction an initial lag phase of cDNA amplification was observed to occur until cycle 10 after which amplification continued at an exponential rate. After cycle 20 the rate of



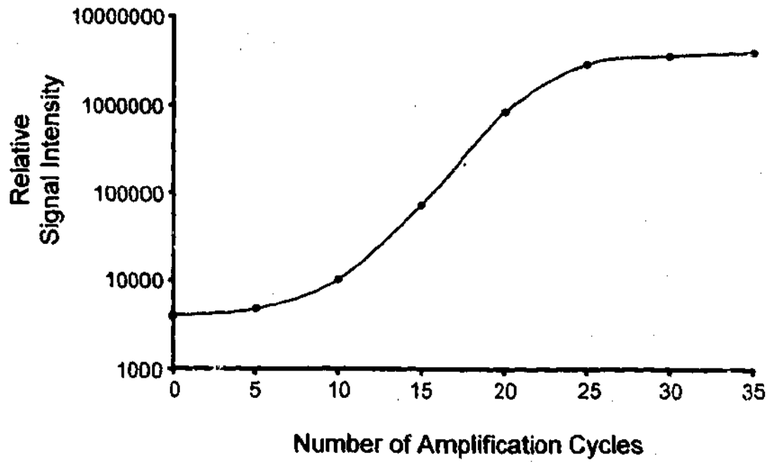



Figure 5.1: Optimisation of PCR cycle number. Effective analysis of expression levels using RT-PCR necessitates the exponential amplification of the cDNA products of reverse transcription. The graph shows the relative amounts of RT-PCR product sampled at 5 cycle intervals between 0 and 35 cycles of amplification. The amount of RT-PCR product is expressed as the intensity of radioactive signal as determined by phosphor imaging and is presented on a logarithmic scale. An initial lag phase of amplification is seen to occur between the start of the PCR reaction and the $10^{th}$ cycle of amplification. Exponential amplification is seen to occur between 10 and 20 cycles of amplification. After the $20^{th}$ amplification cycle the production of PCR product begins to plateau.

amplification began to plateau. The optimal RT-PCR cycle number needed to assess relative expression levels was defined as being within the cycle range within which amplification was exponential and reactants were in excess. From the data an amplification period of 15 cycles was chosen to analyse the relative expression levels of the Trk receptors.

### 5.22 Optimisation of Relative RNA and cDNA Levels Prior to Expression Analysis

The change in expression levels of TrkB and TrkC in response to supplying their respective ligands, BDNF and NT-3, was examined by estimating their levels of expression in relation to the expression levels of the cytoskeletal protein, β-actin. The expression levels of β-actin are constant within the spinal cord and thus can be used as a standard to determine whether the expression of TrkB or TrkC varies under different experimental regimes.

Chick embryos were implanted at E5 with cell lines containing either the pcDNA3/BDNF construct or the pcDNA3/NT-3 construct, as described in Chapter 2. Embryos that had not undergone any manipulation were used as controls. Graft implanted and control embryos were sacrificed at E8, E10 and E15 and the lumbar spinal cords were harvested. 3 to 5 embryos from each age/supplementation group were sacrificed to produce the RNA samples.



Total RNA was extracted from the spinal cord samples in each experimental group. Approximately 1μg of each RNA sample was reverse transcribed. The optimal number of PCR amplification cycles that will allow sampling during the phase of exponential amplification is dependant upon the initial concentration of cDNA in the reaction. As the genes normally display similar, low levels of expression, the variation in TrkB and TrkC mRNA concentrations normally observed throughout embryonic development should not affect the integrity of the expression level estimations derived from RT-PCR. However, β-actin is expressed in much higher quantities than TrkB or TrkC and, thus, the optimal cycle number determined for TrkB may not be appropriate for assessing the expression levels of undiluted β-actin. It was therefore deemed appropriate to dilute the cDNA sample used for β-actin amplification to a level comparable to that of the Trk receptors. Thus, the reverse transcription products from the control groups were amplified in separate reactions by PCR using primers directed towards TrkB, TrkC and β-actin to assess the relative gene concentrations. As it was anticipated that β-actin would be present at much higher levels, cDNA was serially diluted tenfold and added to separate reactions containing the β-actin specific primers.

The PCR samples were amplified for 15 cycles, electrophoresed on the same gel and analysed using Southern hybridisation. The membrane was probed simultaneously with equal concentrations of the TrkB, TrkC and β-actin probes. The probed membrane was then exposed onto

autoradiographic film. The relative concentrations of the PCR products were estimated from the intensity of the exposed radioactive signal *(Figure 5.2)*. From the autoradiograph TrkB and TrkC were found to be amplified in similar quantities. As was expected, β-actin showed greater levels of amplification owing to the higher quantities of β-actin cDNA in the original samples. When the cDNA samples were diluted one hundred-fold, the level of β-actin amplification was reduced to a level comparable to that of TrkB and TrkC. Thus, all reverse transcribed cDNA samples were diluted one hundred-fold and these samples were used for amplifying the β-actin internal standard.

### 5.23 Estimation of Relative RNA Levels Using RT-PCR

Reverse transcribed samples derived from BDNF-supplemented, NT-3-supplemented and control, E8, E10 and E15 embryos were amplified by PCR using primers directed towards TrkB, TrkC or β-actin. To ensure uniformity of the reaction mixes, master mixes were prepared for each gene-specific series of reactions and the cDNA samples were added to aliquots of the master mixes. All reactions were amplified for 15 cycles. The PCR products were electrophoresed on an agarose gel and transferred to a nylon membrane via capillary action. Each gene-specific series of PCR products was electrophoresed on a separate gel to better facilitate gene specific probing. Following hybridisation, the resulting signal intensities were quantified. For each RNA sample group, the signal intensities of TrkB and of TrkC were expressed as a proportion of the signal intensity of β-actin.



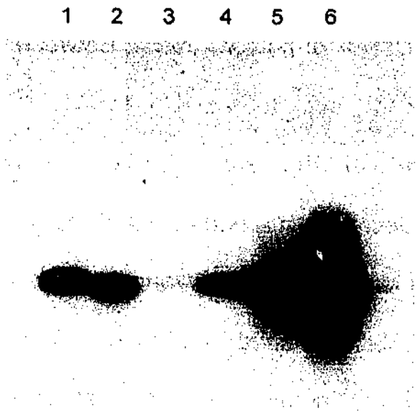



Figure 5.2: An autoradiograph of the relative levels of amplified PCR product analysed by Southern hybridisation. Lane 1 contains the TrkB PCR product. Lane 2 contains the TrkC PCR product. Lanes 3 to 6 contain the amplified products of serially diluted β-actin, such that lane 3 contains $10^{-3}$ diluted cDNA template, lane 4 contains $10^{-2}$ diluted cDNA template, lane 5 contains $10^{-1}$ diluted cDNA template and lane 6 contains undiluted cDNA template. TrkB and TrkC template were shown to be present at similar concentrations. The levels of β-actin template were shown to be comparable to TrkB and TrkC when diluted one-hundredfold.

At E8 both TrkB and TrkC were expressed at levels approximately 0.003% of that of β-actin in control embryos. Expression levels of both TrkB and TrkC were seen to increase between E8 and E10 to a maximal level of 0.006% and 0.007% of β-actin levels. Between E10 and E15 expression of both receptors was seen to decrease to levels similar to those at E8 *(Figure 5.3 and Figure 5.4)*. The expression levels of TrkB and TrkC are known to increase and decrease in a pattern reflective of the onset and subsidence of NOCD in various tissues (Chu-Wang & Oppenheim, 1978; Ip *et al*, 2001). Based on the data obtained in Chapter 3 from the temporal profile of cell death, the levels of Trk receptor expression in the non-supplemented controls do appear to be elevated during the period when NOCD is occurring. By E15, when NOCD in the spinal cord appears largely completed, Trk expression levels are seen to decrease.

When supplied with BDNF the relative expression levels of TrkB were seen to decrease below that of normal levels at E10 *(Figure 5.3)*. Small yet not significant decreases in expression were also seen at E8 and E15. While the level of TrkB expression in BDNF supplemented embryos does vary between ages, the fluctuation in expression levels appears to parallel the fluctuation in expression that is observed in normal embryonic development.

TrkB was also seen to decrease below that of normal levels, at all ages examined, when embryos were supplied with NT-3. Furthermore,



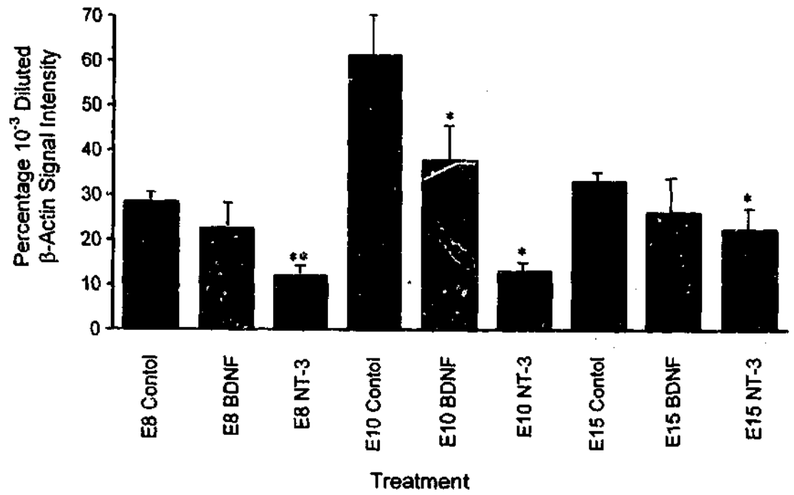



Figure 5.3: The effect of neurotrophin supplementation on TrkB expression in the spinal cord. The graph shows the relative expression levels of TrkB in BDNF supplemented and NT-3 supplemented embryos and in non-supplemented control embryos. Y-values refer to the intensity of the TrkB PCR product's radioactive signal expressed as a percentage of the signal intensity of the $10^{-3}$ diluted β-actin PCR product.

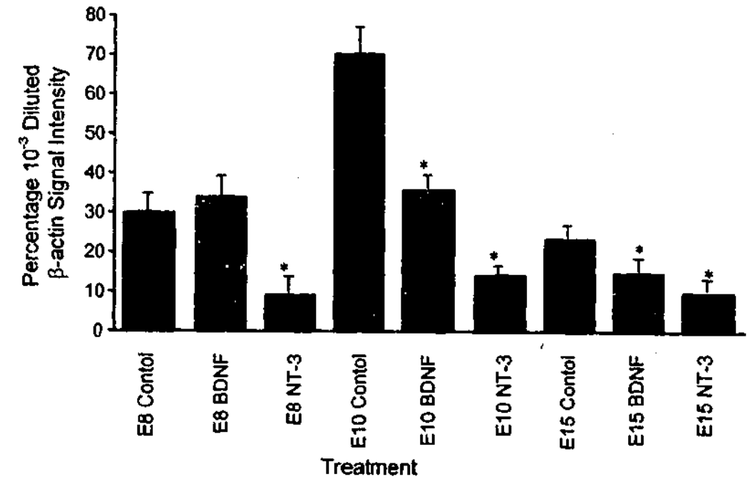



Figure 5.4: The effect of neurotrophin supplementation on TrkC expression in the spinal cord. The graph shows the relative expression levels of TrkC in BDNF supplemented and NT-3 supplemented embryos and in non-supplemented control embryos. Y-values refer to the intensity of the TrkC PCR product's radioactive signal expressed as a percentage of the signal intensity of the $10^{-3}$ diluted β-actin PCR product

administration of NT-3 is also shown to decrease the level of TrkB expression below that observed when embryos are supplied with BDNF. In contrast to the expression patterns seen in embryos supplemented with BDNF, TrkB expression in embryos supplemented with NT-3 does not appear to fluctuate in accordance with normal TrkB levels. An initial reduction in TrkB expression is seen at E8, however, expression levels are seen to increase at subsequent ages such that the highest level of expression is seen at E15.

When embryos were supplemented with BDNF or NT-3, TrkC expression was also found to be suppressed *(Figure 5.4)*. When NT-3 was supplied to the embryo, TrkC expression levels were reduced at all ages. A slight yet not significant increase in expression was observable at E10. While this increase may be due to experimental error it may also be reflective of the normal increase in expression levels seen in the control embryos. When embryos were supplied with BDNF, the TrkC expression levels appeared to drop below that of the control embryos at E10 and E15. As was seen in the expression data for TrkB, supplementation of NT-3 resulted in a decrease in TrkC expression below those levels seen in both control and BDNF supplemented embryos.



## 5.3 DISCUSSION

### 5.31 Optimisation of the Cycle Number and cDNA Concentrations

PCR amplification of cDNA under conditions of reactant excess is necessary if a reliable estimate of the level of original template is to be made. When reactants become limited within the reaction, the integrity of amplification efficiency is compromised due to spatial variations in reactant concentration within the reaction volume. Under optimum conditions the amplification of DNA occurs exponentially. Under our reaction conditions, amplification was seen to be exponential between 10 and 20 cycles. Based on the profile of PCR amplification it was determined that the samples to be analysed should be amplified for 15 cycles. As this stage was in the middle of the phase of exponential amplification, slight variations in the template concentrations should be able to occur without detriment to the integrity of the reaction.

In analysing Trk expression levels, β-actin was chosen as an endogenous standard. β-actin is a cytoskeletal protein that exhibits stable levels of transcripts throughout the body. However, β-actin transcripts are present in significantly higher amounts in comparison to TrkB and TrkC. Such a variation in transcript concentration would most likely affect the integrity of the optimised RT-PCR conditions. It was therefore necessary to dilute the

template one-hundredfold to a concentration comparable to that of the TrkB and TrkC transcripts.

### 5.32 TrkB and TrkC Expression in Non-Factor Supplemented Embryos

The expression levels of both TrkB and TrkC were shown to fluctuate over the developmental period examined. Expression levels of both receptors were found to increase between E8 and E10. Expression was found to subsequently decrease at E15. These patterns of expression are consistent with published observations of Trk expression within the embryonic chick (Ip *et al*, 2001). The rise in TrkB and TrkC transcription in the spinal cord appears to occur during the major period of NOCD in the LMC motoneuron populations, as observed in Chapter 3. The expression of high levels of TrkB and TrkC mRNA in neural cells in the spinal cord is consistent with the responsiveness of LMC motoneurons to BDNF and NT-3 supplementation, as was found in Chapter 3 and Chapter 4.

Expression of the two receptors was found to be highest towards the end of the major phase of cell loss, suggesting that the responsiveness to BDNF and NT-3 is greatest during this period. This may partly explain the discrepancy between the results obtained in this study for factor responsiveness following target ablation, as presented in Chapter 4, and studies by Calderò *et al* (1998), which found LMC motoneurons to be unresponsive to BDNF and NT-3 following target ablation. Calderò *et al*



(1998) examined LMC motoneuron loss in E7.5 target ablated embryos. In this study embryonic LMC motoneurons were examined at E10 and were found to be responsive to both BDNF and NT-3. As TrkB and TrkC expression is maximal after the embryonic period examined by Calderò *et al* (1998), the responsiveness of motoneurons to BDNF and NT-3 that was observed in this study may result from a shift in factor requirement throughout NOCD.

The lowered level of Trk expression seen at E15 also corresponds to the reduction in cell death observed during NOCD at this period. It would appear that at this stage the pressures on cell survival are not as great in these latter stages of cell death. In the case of BDNF, expression levels were found to be higher at E15 than E8, while E15 levels of NT-3 expression fell below that seen at E8. Ip *et al*, (2001) showed that while NT-3 expression in the chick spinal cord decreased after E12, TrkB remained at detectable levels into post-natal development. Thus, the comparably higher expression of TrkB at E15 may reflect an apparent longer duration of TrkB activity.

### 5.33 TrkB and TrkC Expression in Factor Supplemented Embryos

The expression of both TrkB and TrkC was seen to decrease below normal levels when embryos were supplemented with either BDNF or NT-3. It appears that both factors down regulate the transcription of TrkB and TrkC. In contrast, Miller *et al* (1991) and Zhou *et al* (1995) showed that NGF up

regulated the expression of TrkA transcripts in PC12 cells. This increase in expression was observable after 7 days of factor administration. While exposure to BDNF for several hours does decrease the surface expression of TrkB *in vitro* (Sommerfeld *et al*, 2000; Haapasolo *et al*, 2002), the reduction in surface protein does not reflect a reduction in transcript level. Evidence suggests that transcript levels may subsequently increase in response to the elimination of surface expressed TrkB (Sommerfeld *et al*, 2000).

The constant expression system used in this study was implanted into the embryo at a density previously determined to produce factor in excess of the concentrations known to optimally support cell survival. It is possible that the constant supplementation of BDNF and NT-3 in excess results in the down regulation of Trk receptor expression. A reduction of receptor transcripts would appear to be an appropriate regulatory response in order to control the population density in the presence of abnormally high trophic support. Thus, it is probable that the constant supplementation of neurotrophins to the spinal cord resulted in sustained down regulation of Trk receptor expression throughout the period examined.

While the increase in factor-mediated TrkA upregulation observed by Miller *et al* (1991) and Zhou *et al* (1995) appears to contradict our observations of TrkB and TrkC downregulation, an explanation for such a discrepancy may be found in the method of factor administration. Miller *et al* (1991) and Zhou *et al* (1995) employed daily administration of NGF in optimal



but most likely not excessive concentrations. Neuronal degradation can be observed between 12 and 19 hours following NGF deprivation (Deckwerth & Johnson, 1993). Coupled with the short half-life of NGF (Tria *et al*, 1994), it is possible that NGF concentrations in daily supplemented neuronal cells do not remain at levels high enough to efficiently promote the negative regulation of receptor expression.

The effect of providing excessive concentrations of neurotrophic factors to neurons on receptor expression has been poorly studied. Within the hepatic system, saturated levels of EGF have been shown to decrease EGF receptor expression in the NPLC/PRF/5 cells, a hepatoma-derived cell line that overexpresses EGF receptor (Gilligan *et al*, 1992). Within these cells, saturated levels of EGF result in an initial increase of EGF receptor mRNA accumulation within 4 to 8 hours followed by a decrease in receptor transcription by 18 hours. Subsaturated concentrations of EGF resulted in an initial decrease in surface EGF receptor levels followed by a 67% increase in receptor levels by within 20 hours. Parallel patterns of receptor expression have been noted for NGF and BDNF (Miller *et al*, 1991; Sommerfeld *et al*, 2000; Haapasolo *et al*, 2002). Thus, it is possible that the Trk receptor regulatory mechanisms may respond in a similar manner to the EGF system by down regulating Trk transcription in response to excessive neurotrophin signalling.

When embryos were supplemented with NT-3, the expression of TrkB decreased. Similarly when embryos were supplemented with BDNF the expression of TrkC decreased. The latter observation was particularly unexpected because, while NT-3 is capable of mediating activity through both TrkB and TrkC, the activity of BDNF is primarily mediated through TrkB. The explanation for this observation is not entirely clear.

It is possible that the constant supplementation of each neurotrophin is selecting against the expression of other neurotrophic factor receptors. The results from Chapter 3 suggest that supplementation of BDNF and NT-3 selects for neurons that are normally responsive to those factors. By supplementing factor at a period prior to target innervation, we may be inadvertently inducing neurons to express receptors specific to the supplied factor. The maintenance of factor responsiveness in the absence of a target field, as observed in Chapter 4, may suggest that while the target tissue selects neurons based on their factor responsiveness, neurons can also change their trophic requirements to suit the target field.

It is also possible that the downregulation of Trk receptors by non-specific ligands results indirectly via the downregulation of the factor specific receptor. Trk signal transduction is primarily mediated via the receptor's tyrosine kinase domain. Members of the Trk family share high levels of sequence homology in this region. As a consequence, common pathways are involved in the signal transduction of TrkB and TrkC (Yuen & Mobley,



1999). It is, therefore, likely that a number of the pathways that regulate transcription in response to neurotrophin activity are also shared between Trk molecules. Thus, if cells expressing both receptors were exposed to high levels of BDNF or NT-3, the regulatory mechanisms that stimulate the suppression of the specific Trk receptor's expression would most likely suppress the expression of the other Trk receptor.

Regulation of non-specific Trk receptor expression via ligand-specific Trk signal transduction pathways would require neurons to express both receptors. In Chapter 3, the levels of cell survival in embryos supplemented with a combination of BDNF and NT-3 suggested that a large proportion of motoneurons were responsive to both BDNF and NT-3. Such observations support the concept that, within the spinal cord, Trk receptors may be regulated in the presence of neurotrophins with low binding affinity via the regulatory mechanisms of the neurotrophin's specific Trk receptor.

The apparent downregulation of Trk receptors by non-specific ligands may also result indirectly from the action of adjacent factor responsive glial cells. The data obtained in Chapters 3 and 4 relate only to the factor responsiveness of LMC motoneurons, however, the LMC contains a variety of neuronal and non neuronal cells and neurotrophin activity is not limited to motoneurons (Leibrock *et al*, 1989; Merlio *et al*, 1992). It is not clear whether the administered neurotrophins are acting directly on receptive motoneuron populations or whether the motoneurons are being supported indirectly via

the aid of other cells. Therefore, the potential remains, for example, for BDNF responsive glia to supply adjacent cells with NT-3 resulting in the regulation of TrkC by the glia-supported cells.

The primers used in this study were directed towards the extracellular regions of TrkB and TrkC. Therefore, the observed expression levels for TrkB and TrkC represent both the full length receptors and their truncated isoforms. The function of the truncated Trk receptor isoforms remains unclear, however, they are capable of mediating signal transduction (Baxter *et al*, 1997; Palko *et al*, 1999). The full length and truncated Trk receptors exhibit different temporal patterns of expression and are often localised to distinct cell types. Expression of the TrkB.T1 truncated isoform is predominantly associated with glial cells while TrkB.T2 and full length TrkB expression is higher in neurons (Biffo *et al*,1995; Alderson *et al*, 2000; Rose *et al*, 2003). Full length TrkC is expressed by both neurons and glia, however, expression of the truncated TrkC isoforms is localised to glial cells (Valenzuela *et al*, 1993).

From our results, we cannot determine whether there is any bias in the type of receptor isoforms being downregulated in response to neurotrophin supplementation. TrkB.T1 expression is increased during the period of target innervation and NOCD (Baxter *et al*, 1997). Thus, truncated isoforms appear to be important in the mediation of cell responsiveness to neurotrophins during NOCD. A reduction of the glial associated isoform



expression may account for the observed decrease in TrkB and TrkC. If this was the case, the abundant levels of graft-supplied neurotrophins may make glial support redundant. In such a situation, the glia may reduce paracrine support to the motoneurons by decreasing their own responsiveness to the neurotrophins. It would, therefore, be of interest to further analyse the expression of the Trk receptors in response to factor supplementation using primers directed towards the specific TrkB and TrkC isoforms. This would allow us to determine whether the expression levels of any isoforms are preferentially decreased in response to factor supplementation and from this infer which cell types are being affected.

Supplementation of NT-3 resulted in a greater decrease in TrkC and TrkB mRNA levels when compared with BDNF. The decrease in Trk expression also occurred at E8 in NT-3 supplemented embryos while the decrease wasn't observed in BDNF supplemented embryos until E10. The regulation of NT-3 activity would, therefore, appear more tightly controlled and more responsive to abnormal levels of trophic support. NT-3 is generally expressed at lower levels than BDNF. The binding affinity of NT-3 to TrkC is also higher than the binding affinity of BDNF to TrkB (Yuen & Mobley, 1999). Therefore, the NT-3-TrkC ligand-receptor system would appear much more susceptible to fluctuations in the ligand levels in comparison to the BDNF-TrkB system and as a result the over-expression of NT-3 may cause greater disruption to the normal trophic balance compared with BDNF. The

requirement for more stringent regulation of NT-3 activity would explain the greater levels of Trk transcriptional suppression in response to NT-3.

It should be noted that the Trk receptors are expressed in numerous cell populations throughout the spinal cord (Tessarollo *et al*, 1993). The expression patterns of the Trk receptors within the spinal cord observed in this chapter should not be directly related to the trophic responsiveness of LMC motoneurons observed in Chapters 3 and 4 without first considering the potential for interference via other Trk expressing cells in the spinal cord. Whether the various neural populations within the spinal cord exhibit similar patterns of trophic responsiveness to LMC motoneurons is unclear. The degree to which changes in LMC motoneuron Trk expression influence the gross levels of Trk in the spinal cord is also unclear. However, the observations made in this chapter regarding the regulation of TrkB and TrkC by BDNF and NT-3 do correspond to the apparent responsiveness of LMC motoneurons to BDNF and NT-3 supplementation.

The regulation of Trk receptors in relation to ligand concentration is an issue that may need addressing when considering the effectiveness of neurotrophins in promoting motoneuron survival. Supplying neurotrophins in too low concentrations would likely not promote survival to the full potential of the factor. Despite the apparent down regulation of TrkB and TrkC within the spinal cord, constant supplementation of BDNF and/or NT-3 did result in an increase in LMC motoneuron survival during NOCD. It is unclear whether the



reduction in Trk transcription resulting from factor supplementation had any detrimental effect on the survival of the motoneurons. While the reduction in Trk expression would decrease the ability of the factors to elicit a trophic effect, the high levels of graft secreted neurotrophins may have promoted sufficient neurotrophic activity to make the effect of decreased Trk expression negligible.

# CHAPTER 6

## GENERAL DISCUSSION

# CHAPTER 6

## GENERAL DISCUSSION

The experiments detailed in this thesis have utilised a modified neuroepithelial cell line to examine the effects of supplying a constant source of neurotrophins to chick LMC motoneurons during NOCD. Several studies have shown that intermittent administration of BDNF and NT-3 during chick embryonic development does not fully prevent LMC motoneuron death resulting from NOCD (Oppenheim *et al*, 1992; Calderó *et al*, 1998). Therefore, it was of interest to determine whether the survival could be increased by supplying a constant source of factor and whether supplying factors in combination could increase motoneuron survival over that of single factors.

Examination of the LMC motoneuron population at daily intervals during NOCD has shown that the majority of cell loss occurs between E6 and E9. During this period 38% of those motoneurons present at E6 are lost. Between E9 and E15 cell loss continues but at a much lower rate. These observations support other studies that have shown similar changes in LMC motoneuron population density during this period (Hamburger, 1975; Chu-Wang & Oppenheim, 1978; Nurcombe *et al*, 1991; Oppenheim *et al*, 1992; Calderó *et al*, 1998). Motoneuron loss both prior and following the period examined in this



thesis have previously been shown to be minimal (Chu-Wang & Oppenheim, 1978; Oppenheim *et al*, 1992). Examination of the TrkB and TrkC mRNA expression levels in the spinal cord over the developmental period examined in this thesis showed that expression of both receptors increases between E8 and E10. This increase corresponds to the main period of motoneuron loss during NOCD, where innervating neurons become dependant on target derived trophic support. The increase in the amount of neurotrophin receptor seen in this study supports the evidence suggesting neurotrophins play a major role in mediating cell survival during NOCD (Henderson *et al*, 1993; Becker *et al*, 1998). By E15 TrkB and TrkC expression decreases to levels comparable to E8. By E15 NOCD was seen to be largely complete. Therefore, the selection pressure against the motoneuron population is not as strong and the requirement for neurotrophin support has reduced.

When embryos were supplied with either BDNF or NT-3, the level of motoneuron survival at E10 increased in comparison to normal embryos. It is interesting to note that in this study NT-3 promoted motoneuron survival to levels comparable to BDNF. While NT-3 has been shown to support LMC motoneurons *in vitro* (Becker *et al*, 1998), previous studies, which have supplied NT-3 via intermittent administration, have found NT-3 to be ineffective at promoting motoneuron survival (Calderó *et al*, 1998). Thus, it appears that the method of factor supplementation does impact on the ability of neurotrophins to promote cell survival. We have relied upon the published results of other

research groups to compare the effectiveness of constant versus intermittent factor supplementation. It is unclear whether the slightly higher levels of BDNF promoted motoneuron survival observed in this study compared with previously published data (Calderó *et al*, 1998), results from the method of factor supplementation or whether it reflects variation in the sampling and counting methodologies. A direct comparison of constant and intermittent factor administration would be useful in resolving this matter.

When embryos were supplied with a combination of BDNF and NT-3, the level of E10 motoneuron survival was greater than that achieved by either factor alone. However, the combination of factors did not produce an additive effect on cell survival suggesting that many motoneurons within the LMC are responsive to both factors. While BDNF and NT-3 did increase LMC motoneuron survival during NOCD, neither factor, alone nor in combination, was capable of totally preventing cell loss during this period. Therefore, it appears that other neurotrophic factors are involved in supporting the LMC motoneuron population during NOCD. This study has focused on neurotrophins as important survival factors for neurons undergoing NOCD because neurotrophin and Trk expression appears to correlate with the onset and subsidence of NOCD (Chu-Wang & Oppenheim, 1978; Ip *et al*, 2001). However, several other neurotrophic factors that are expressed when NOCD is not occurring have been shown to promote neuronal survival during NOCD (Arakawa *et al*, 1990; Nurcombe *et al*, 1991; Oppenheim *et al*, 1991; Calderó *et al*, 1998). Therefore, while neurotrophins



tend to elicit NOCD specific trophic support, more broadly expressed factors are likely to play important roles in the maintenance of neuronal populations during NOCD. From our experiments we cannot draw any conclusions regarding which factors, other than BDNF and NT-3, are involved in supporting motoneurons during NOCD. It would be of interest to repeat the factor supplementation experiments using other neurotrophic factors known to elicit an effect on spinal motoneurons to determine what combinations of factors are required to completely rescue the LMC motoneuron population.

LMC motoneuron population density was found to decrease between E10 and E15. By E15 NT-3 did not promote a significant increase in motoneuron survival over that of normal E15 embryos. BDNF did promote motoneuron survival at E15, however, a decrease in motoneuron number was still observed between E10 and E15. In comparison to the increased motoneuron survival resulting from supplying neurotrophins in combination, the level of motoneuron survival observed at E15 in embryos supplied with both factors did not differ from that of BDNF alone. The lack of response to multiple factors is probably a reflection of the reduced trophic ability of NT-3. The normal decrease in motoneuron number between E10 and E15 cannot totally explain the reduction in cell survival between E10 and E15 in factor supplemented embryos. The rate of cell loss during this period varied disproportionately between factor supplementation regimes. Therefore, it appears that the factor requirements of the motoneurons change between E10 and E15. Evidence

suggests that temporal switching of neurotrophic factor requirements occurs over the duration of NOCD in the PNS (Davies, 1997). Factor requirement switching in the CNS has been insufficiently documented. However, the results presented in this thesis suggest that it is likely to occur in some CNS populations.

The exacerbated motoneuron loss resulting from limb bud removal is a classical model for studying the role of the target tissue in NOCD (Hamburger & Levi-Montalcini, 1949). Removal of the chick lower limb bud prior to NOCD resulted in the loss of 55% of LMC motoneurons normally present in the E10 embryo. By E15, no further motoneuron loss occurred. This is in contrast to the continued cell loss between E10 and E15 normally seen in non-amputated embryos. Therefore, the signaling events controlling the apparent fine tuning of neural connections at the latter phase of NOCD must act via the target tissue.

When limb bud amputated embryos were supplied with either BDNF or NT-3, the motoneuron loss associated with target ablation was reduced. A combination of BDNF and NT-3 was unable to increase survival over that of individual factors at the two embryonic ages examined. This suggests a high degree of redundancy exists in the factor requirements of the neurotrophin responsive motoneurons. BDNF has previously been found to support deafferented motoneurons (Oppenheim *et al*, 1992). However, Calderó *et al* (1998) found that intermittent administration of BDNF or NT-3 could not rescue



motoneurons following limb bud removal. Our results provide further support for the importance of constant factor supplementation when assessing the trophic capacity of neurotrophic factors. Limb bud removal results in the elimination of target derived support. Factor deprivation resulting from lengthy periods between administrations may be more detrimental when compared with non-amputated embryos because support cannot be supplemented by the target tissue.

Neither factor was capable of rescuing E10 target ablated LMC motoneuron populations to a level comparable to normal E10 embryos. However, by E15 the LMC motoneuron population density in amputated embryos supplied with either factor was not significantly different to normal E15 levels. This is most likely explained by the maintenance of motoneuron numbers in factor supplied, target ablated embryos between E10 and E15, in contrast to normal embryos where neuron loss continues throughout this period.

The continued cell loss between E10 and E15 observed in normal embryos coupled with the changes in the factor response in non-target ablated, factor supplied embryos during this period suggests some LMC motoneurons undergo changes in their factor requirements throughout NOCD. This is most likely a means to fine tune neuronal connections to remove inappropriate or superfluous connections. In factor supplied, target ablated embryos the trophic

support is static and initially responsive motoneurons are not subsequently selected against. Therefore, the population density can be maintained.

Examination of TrkB and TrkC mRNA expression levels in response to factor supplementation found that, in the presence of excess factor, Trk expression was suppressed below that of normal levels. As far as we are aware this is the first study to provide evidence for negative regulation of Trk expression in response to neurotrophins. However, within the hepatic system, downregulation of receptor expression in response to high concentrations of EGF has been documented (Gilligan *et al*, 1992). Previously published data has shown that the addition of NGF to PC12 cells results in an increase in TrkA production (Miller *et al*, 1991; Zhou *et al*, 1995). Zhou *et al* (1995) found NGF could sustain this increase in trkA expression for up to 14 days. Taken together with our data, it appears that while neurotrophins can induce Trk expression, when neurotrophins are present in high concentrations the motoneuron population reduces its ability to respond to the trophic support by decreasing receptor production. Suppressing the ability of the neurons to respond to the neurotrophin stimulus effectively acts to regulate cell survival in order to maintain normal population densities. Interestingly, the supplementation of BDNF and of NT-3 led to a decrease in the expression of non-specific Trk receptors. It's possible that the supplementation of neurotrophins prior to NOCD conditioned the motoneurons to be responsive to the supplemented factor resulting in fewer cells expressing mRNA of other neurotrophic factor receptors.



A more thorough examination of neurotrophin receptor expression is necessary to understand exactly why TrkB and TrkC expression is decreasing in response to neurotrophin supplementation. Oligonucleotide primers directed towards the extracellular domain of the TrkB and TrkC sequence were employed in this study. This allowed us to examine the effects of factor supplementation on total TrkB and TrkC mRNA expression. However, we were unable to differentiate between the expression levels of the full length receptors and their truncated isoforms. Trk receptor isoforms are preferentially expressed in different cell types (Valenzuela *et al*, 1993; Biffo *et al*,1995; Alderson *et al*, 2000; Rose *et al*, 2003). Future studies, using primers specific to the various TrkB and TrkC isoforms, would allow us to determine the ratios of full length and truncated receptor expression. From this we may be able to infer which cells are downregulating TrkB or TrkC expression and examine whether the known functions of the isoforms have an affect on which isoforms are preferentially expressed. Analysing TrkB and TrkC mRNA expression via *in situ* hybridisation or RT-PCR *in situ* hybridisation would also allow us to determine whether the neurotrophins are acting directly on the motoneurons or indirectly via the surrounding glia. Examining the expression pattern of the p75 receptor in response to neurotrophin supplementated embryos would also help in understanding how and why TrkB and TrkC expression decreases in response to BDNF and NT-3 supplementation. It is possible that the BDNF and NT-3 are mediating activity via p75 and the decrease in TrkB and TrkC mRNA expression

reflects a preference towards p75 signal transduction. P75 may even be directly downregulating TrkB and TrkC expression.

It is unclear whether the down regulation of Trk receptors in response to excess neurotrophins had an effect on neurotrophin mediated cell survival. While Trk receptor expression did decrease with the constant expression system employed in this study, the high quantity of factor may have promoted sufficient neurotrophic activity to rescue the majority of responsive motoneurons. The level of survival promoted by BDNF in non-amputated embryos in this study was comparable to that of previous studies (Oppenheim *et al*, 1992; Calderó *et al*, 1998), so it would appear that the decrease in receptor mRNA levels had little impact on promoting motoneuron survival.

The experiments presented in this thesis have shown the importance of constant supplementation when determining the effects of supplying degenerating neurons with neurotrophic factors. BDNF has been shown to promote LMC motoneuron survival during NOCD (Oppenheim *et al*, 1992; Calderó *et al*, 1998). We have also shown that NT-3 can increase *in vivo* LMC motoneuron survival during NOCD when it is supplied throughout the developmental period. Previous studies have found NT-3 ineffective at rescuing these motoneurons *in vivo* (Calderó *et al*, 1998), although NT-3 is capable of promoting motoneuron survival *in vitro* (Becker *et al*, 1998). In contrast to previous studies, we have also shown that BDNF and NT-3 are capable of



rescuing LMC motoneurons following target ablation. In light of the therapeutic potential of neurotrophins in the treatment of neurodegenerative diseases, there is a need to reassess the most appropriate method of factor administration when examining factor's trophic potential. Despite experimental data suggesting the potential value of BDNF in the treatment of spinal neurodegenerative diseases, clinical trials for the treatment of motoneuron disease have not been successful (The BDNF Study Group (Phase III), 1999). Before dismissing the therapeutic value of BDNF in the treatment of motoneuron disease, it is necessary to assess the impact of the periodic drug administrations that have been employed in such clinical trials. If the constant supplementation can improve the effectiveness of neurotrophins, as our study suggests, we may need to change the mode of drug delivery before we can conclusively discount neurotrophins as effective therapeutic agent for motoneuron disease.

# APPENDIX 1

## A1.1 Statistical Analysis of the Sample Groups From Chapter 3

**A1.11** Refer to *Figure 3.3*. A comparison of the number of LMC motoneurons present at E6, E10 and E15 in untreated embryos. P-values derived from the Tukey test for multiple comparisons

| | E6 | E10 |
|---|---|---|
| E10 | P <0.0001 | |
| E15 | P <0.0001 | P <0.0001 |

**A1.12** A comparison between the number of LMC motoneurons present in untreated embryos and embryos implanted with the vector control. P-values derived from the two-sample Student t-test.

E10 P = 0.7281

E15 P = 0.3227

**A1.13** Refer to *Figure 3.4.* A comparison of the number of LMC motoneurons present at E10 following factor administration. P-values derived from the Tukey test for multiple comparisons.

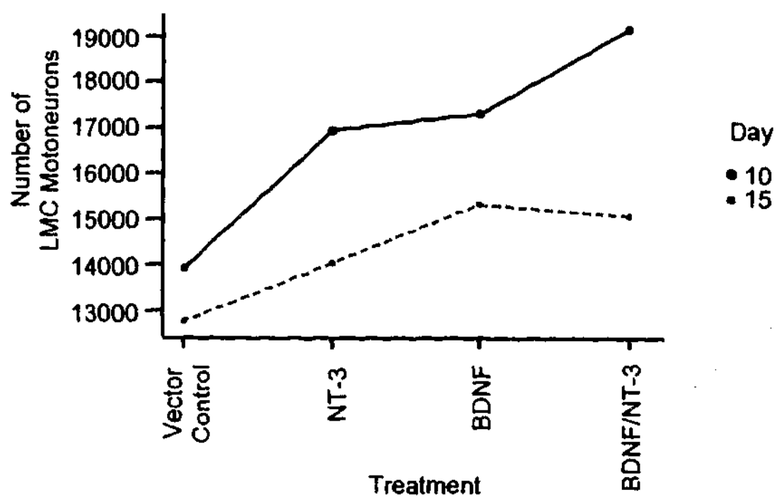
| | Vector Control | BDNF | NT-3 |
|---|---|---|---|
| BDNF | P <0.0001 | | |
| NT-3 | P = 0.0001 | P = 0.9418 | |
| BDNF + NT-3 | P <0.0001 | P = 0. 0284 | P = 0.0063 |

**A1.14** Refer to *Figure 3.5.* A comparison of the number of LMC motoneurons present at E15 following factor administration. P-values derived from the Tukey test for multiple comparisons.

| | Vector Control | BDNF | NT-3 |
|---|---|---|---|
| BDNF | P = 0.0068 | | |
| NT-3 | P = 0.3264 | P = 0.3246 | |
| BDNF + NT-3 | P = 0.0173 | P = 0. 9856 | P = 0.5204 |



**A1.15** An interaction plot showing the interaction between the mean number of LMC motoneurons present in embryos at E10 and E15 under the various factor administration regimes. The interaction between the two ages was found to be significant; P <0.001. Motoneuron loss between E10 and E15 occurs at different rates across factor administration regime groups. Therefore, variation between E10 and E15 in the trial groups cannot be attributed solely to the normal motoneuron loss seen in the control group. P-values derived by ANOVA.



## A1.2 Statistical Analysis of the Sample Groups From Chapter 4

**A1.21** Refer to *Figure 4.2* A comparison between the number of motoneurons present at E10 in the LMC in normal (control) embryos and in the LMC's, both contralateral and ipsilateral to the removed limb bud, in target ablated embryos. P-values derived from the Tukey test for multiple comparisons.

| | Contralateral LMC | Ipsilateral LMC |
|---|---|---|
| Control | P = 0.482762 | P <0.0001 |
| Ipsilateral LMC | P = 0.0004 | |

**A1.22** Refer to *Figure 4.3* A comparison between the number of LMC motoneurons present at E10 and E15 in normal and target ablated embryos. P-values derived from the two-sample Student t-test.

E10/E15 normal embryos P <0.0001

E10/E15 target ablated embryos P = 0.6568

**A1.23** Refer to *Figure 4.4*. A comparison of the number of motoneurons per LMC present at E10 following target ablation and factor administration. P-values derived from the Tukey test for multiple comparisons.

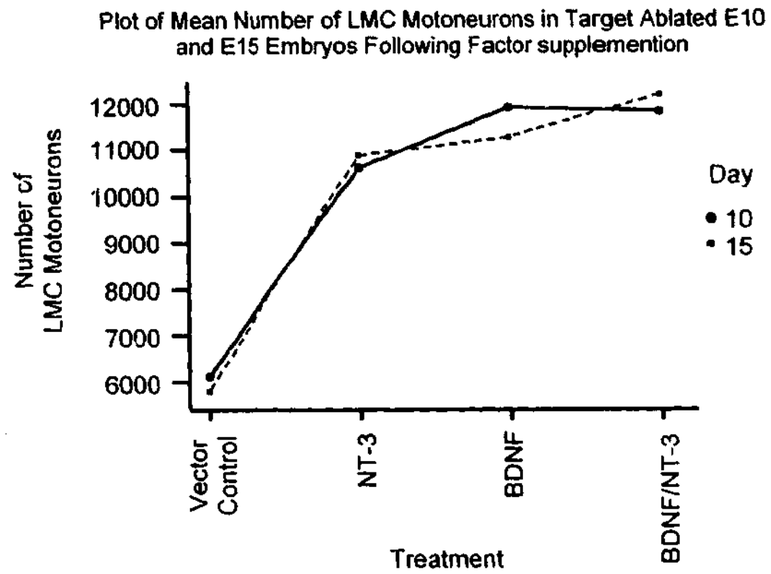


| | Vector Control | BDNF | NT-3 |
|---|---|---|---|
| BDNF | P <0.0001 | | |
| NT-3 | P <0.0001 | P = 0.5030 | |
| BDNF + NT-3 | P <0.0001 | P = 0. 9084 | P = 0.1740 |

**A1.24** Refer to *Figure 4.5*. A comparison of the number of motoneurons per LMC present at E15 following target ablation and factor administration. P-values derived from the Tukey test for multiple comparisons.

| | Vector Control | BDNF | NT-3 |
|---|---|---|---|
| BDNF | P <0.0001 | | |
| NT-3 | P <0.0001 | P = 0.4025 | |
| BDNF + NT-3 | P <0.0001 | P = 0. 3584 | P = 0.0093 |

**A1.25** An interaction plot showing the interaction between the mean number of LMC motoneurons present in target ablated embryos at E10 and E15 under the various factor administration regimes. The interaction between the two ages was not found to be significant. There is no significant variation in the rate of cell loss between E10 and E15 across factor administration regime groups. P- values derived by ANOVA.






## A1.3 Statistical Analysis of the Sample Groups From Chapter 5

**A1.31** Refer to *Figure 5.2*. The level of TrkB mRNA expression in E8, E10 and E15, factor supplemented embryos compared to control non-supplemented embryos at similar ages. P-values derived from the Tukey test for multiple comparisons.

| | BDNF | NT-3 |
|---|---|---|
| E8 | P = 0.0001 | P = 0.1931 |
| E10 | P = 0.0290 | P = 0.0117 |
| E15 | P = 0.2795 | P = 0.0377 |

**A1.31** Refer to *Figure 5.3*. The level of TrkC mRNA expression in E8, E10 and E15, factor supplemented embryos compared to control non-supplemented embryos at similar ages. P-values derived from the Tukey test for multiple comparisons.

| | BDNF | NT-3 |
|---|---|---|
| E8 | P = 0.3448 | P = 0.0087 |
| E10 | P = 0.0029 | P = 0.0005 |
| E15 | P = 0.0444 | P = 0.0107 |

# APPENDIX 2

## A2.1 INTRODUCTION

The following is a summary of work carried out by Jacinta Pankhurst, an honours student under the supervision of Dr Karen Bailey (Pankhurst, 1996). A DRG neuronal bioassay was used to assess the ability of the constant expression system employed in our study to successfully implant into the chick CNS and maintain the expression of neurotrophins *in vivo*.

## A2.2 RESULTS

*Figure A2.1* summarises the results of the DRG neuronal bioassay. CSF extracted from embryos implanted with recombinant 2.3D cells expressing NT-3 and a combination of BDNF and NT-3 show a level neurotrophic activity comparable to that obtained by the purified factor. The data suggests that the recombinant grafts do express active neurotrophins *in vivo*. Expression is maintained for the 96 hour period examined in the study. Thus, the 2.3D graft does not appear to result in significant post implantation elimination in response to tissue damage as is known to occur for other graft implantation paradigms (Suhr & Gage, 1993). The maintenance of survival of a murine-derived graft within the chick implies that cross-species immunoreactivity does not noticeably affect the integrity of the graft at this early stage in embryonic development.

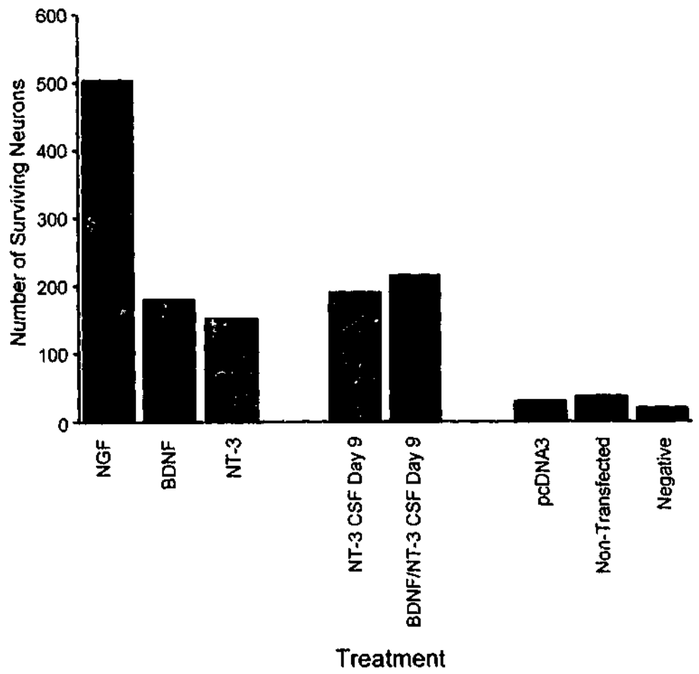



Figure A2.1: A DRG neuronal bioassay of the neurotrophic activity of CSF derived from embryonic chicks implanted with the pcDNA3/neurotrophin constant expression system. The first group of columns show the level of neuronal survival promoted by the addition of purified NGF, BDNF and NT-3 to the culture media. The unusually high levels of surviving neurons in response to NGF may indicate the presence of sympathetic chain neurons. The second group of columns show the level of neuronal survival promoted by the addition of CSF derived from embryos transplanted with the NT-3 and BDNF/NT-3 combination expressing cell grafts 96 hours after implantation. The third column shows the negative controls. Controls were produced by extracting CSF from embryos injected with 2.3D cells transfected with the parent vector, with non-transfected 2.3D cells and with 1XPBS.



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**Addendum - Appendix 3**
**Raw Data for the Results Presented Throughout this Thesis.**

Table 1: LMC motoneuron counts between E6 and E15. Each value represents the estimated number of motoneurons in a single LMC. Both LMC's were examined per embryo. Refer to Figure 3.3.

| E6 | E7 | E8 | E9 | E10 | E11 | E12 | E13 | E14 | E15 |
|---|---|---|---|---|---|---|---|---|---|
| 20805 | 20946 | 15463 | 10262 | 13636 | 12652 | 12652 | 15323 | 12230 | 11808 |
| 21087 | 21227 | 14761 | 11808 | 14198 | 13917 | 12230 | 14479 | 12230 | 11668 |
| 24742 | 19681 | 14901 | 11527 | 14620 | 12230 | 14761 | 16166 | 11949 | 11527 |
| 22071 | 21789 | 16166 | 14058 | 14339 | 9138 | 11808 | 13776 | 12792 | 12089 |
| 27272 | 21368 | 15745 | 14760 | 14620 | 13355 | 14761 | 12652 | 10864 | 12652 |
| 23336 | 23617 | 18837 | 16447 | 13636 | 10684 | 15042 | 12230 | 12792 | 9981 |
| 21368 | 23607 | 18978 | 16307 | 13214 | 14339 | 12089 | 10684 | 12230 | 11387 |
| 22492 | 20665 | 18837 | 14620 | 14339 | 13497 | 13355 | 10829 | 13495 | 11528 |
| 20665 | 21508 | 15182 | 12511 | 13355 | 9700 | 16166 | 13074 | 11387 | 13636 |
| 23055 | 21930 | 18837 | 12933 | 14901 | 10543 | 15604 | 13214 | 13214 | 14058 |
| 21368 | 23617 | 15463 | 16307 | 13917 | 14479 | 12933 | 13636 | 12652 | 13355 |
| 20524 | 21508 | 17010 | 15604 | 14058 | 14058 | 14198 | 14761 | 13776 | 10965 |
| 22398.75 | 21788.58 | 16681.67 | 13928.67 | 14069.42 | 13500 | 13799.92 | 13402 | 12467.53 | 12054.5 |
| ±574.53 | ±359.68 | ±496.50 | ±605.27 | ±153.27 | ±548.14 | ±426.65 | ±481.62 | ±242.25 | ±340.03 |

Table 2: LMC motoneuron counts in E10 and E15 embryos under different factor supplementation regimes. Each value represents the estimated number of motoneuron in a single LMC. Both LMC's were examined per embryo. Refer to Figures 3.4 and 3.5.

| Treatment regime in E10 embryos | | | | Treatment regime in E15 embryos | | | |
|---|---|---|---|---|---|---|---|
| Vector control | BDNF | NT-3 | BDNF+NT-3 | Vector control | BDNF | NT-3 | BDNF+NT-3 |
| 14339 | 19681 | 16166 | 20665 | 9278 | 14339 | 13355 | 16167 |
| 13495 | 19119 | 18556 | 19962 | 10684 | 16166 | 17291 | 16162 |
| 13355 | 15885 | 15323 | 19259 | 12793 | 13776 | 15745 | 13495 |
| 14198 | 16869 | 16729 | 19962 | 13355 | 14339 | 14429 | 12230 |
| 13355 | 15464 | 18556 | 15604 | 13636 | 13777 | 11668 | 16447 |
| 14198 | 14901 | 17853 | 17291 | 15042 | 15182 | 13496 | 13214 |
| 16588 | 17241 | 14339 | 19962 | 8997 | 12933 | 14339 | 17432 |
| 11246 | 20243 | 16166 | 19540 | 12511 | 16167 | 14339 | 19119 |
| 14198 | 15604 | 16869 | 19259 | 14479 | 17291 | 12371 | 12652 |
| 15604 | 16026 | 17713 | 17713 | 13073 | 15744 | 14198 | 14058 |
| 14198 | 19540 | 18416 | 19211 | 14058 | 16026 | 13214 | 15886 |
| 12230 | 17150 | 16711 | 21509 | 15182 | 18134 | 14020 | 13918 |
| 13917 | 17310.25 | 16949.75 | 19161.42 | 12757.33 | 15322.83 | 14038.75 | 15065 |
| ±401.36 | ±540.03 | ±384.47 | ±461.48 | ±600.44 | ±446.54 | ±423.65 | ±610.02 |

Table 3: LMC motoneuron counts in E10 and E15 target ablated embryos under different factor supplementation regimes. Each value represents the estimated number of motoneuron in a single LMC. Only the LMC ipsilateral to the removed limb was used to produce the factor supplementation data. Refer to Figures 4.2, 4.3 and 4.4.

| Ungrafted E10 controls | | Treatment regime in E10 embryos | | | | Treatment regime in E15 embryos | | | |
|---|---|---|---|---|---|---|---|---|---|
| LMC contralateral to removed limb | LMC ipsilateral to removed limb | Vector control | BDNF | NT-3 | BDNF+NT-3 | Vector control | BDNF | NT-3 | BDNF+NT-3 |
| 14198 | 6607 | 5482 | 10965 | 11246 | 11808 | 7310 | 13355 | 10402 | 9138 |
| 14198 | 5904 | 5623 | 10824 | 9840 | 10402 | 3655 | 11386 | 11246 | 13355 |
| 11668 | 2952 | 8013 | 14339 | 7872 | 11808 | 4920 | 8294 | 10262 | 12090 |
| 13355 | 3374 | 6747 | 12511 | 9981 | 10684 | 7310 | 10121 | 12094 | 11387 |
| 14902 | 4076 | 5763 | 10402 | 11527 | 15182 | 6888 | 12933 | 9981 | 14199 |
| 13777 | 5904 | 6185 | 12090 | 11246 | 10684 | 5342 | 11246 | 11106 | 12512 |
| | | 5623 | | 12371 | | | | | |
| | | 6326 | | | | | | | |
| 13580 | 4802.833 | 6220.25 | 11855.17 | 10583.29 | 11761.33 | 5904.17 | 11222.5 | 10848.5 | 12113.5 |
| ±494.755 | ±623.848 | ±297.95 | ±595.04 | ±561.28 | ±727.43 | ±612.754 | ±759.17 | ±319.97 | ±717.21 |

Table 4: The relative radioactive signal intensity of amplified TrkB cDNA at various cycles of amplification during PCR, as measured via phosphor imaging. The intensity values are relative to the background signals. Refer to Figure 5.1

| Amplification Cycle Number | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 |
| 4005.57 | 4807.24 | 10367.72 | 74139.84 | 862711.3 | 2988182 | 3714953 | 4071534 |

Table 5: The total number of spinal cord tissue samples that were used for the extraction of total RNA for each factor supplementation regime.

| | | Factor Supplied | | |
|---|---|---|---|---|
| | | Vector Control | BDNF | NT-3 |
| Age at time of harvest | E8 | 4 | 4 | 4 |
| | E10 | 5 | 5 | 5 |
| | E15 | 3 | 3 | 3 |

Table 6: The expression levels of CSF-derived TrkB mRNA at various ages following factor supplementation. TrkB mRNA concentration is expressed as a percentage of the β-actin mRNA concentration. Refer to Figure 5.2

| E8 Treated Embryos | | | E10 Treated Embryos | | | E15 Treated Embryos | | |
|---|---|---|---|---|---|---|---|---|
| Vector Control | BDNF | NT-3 | Vector Control | BDNF | NT-3 | Vector Control | BDNF | NT-3 |
| 37.741 | 40.368 | 19.963 | 93.868 | 67.275 | 17.244 | 39.198 | 42.453 | 34.478 |
| 23.281 | 5.912 | 5.912 | 41.474 | 18.603 | 5.395 | 27.410 | 5.048 | 6.007 |
| 24.281 | 21.671 | 10.766 | 48.520 | 27.778 | 16.578 | 32.983 | 31.184 | 27.118 |
| 28.434 | 22.65 | 11.986 | 61.287 | 37.885 | 13.072 | 33.197 | 26.228 | 22.534 |
| ±2.331 | ±4.979 | ±2.149 | ±8.208 | ±7.466 | ±1.922 | ±1.702 | ±5.539 | ±4.266 |

Table 7: The expression levels of CSF-derived TrkC mRNA at various ages following factor supplementation. TrkB mRNA concentration is expressed as a percentage of the β-actin mRNA concentration. Refer to Figure 5.3

| E8 Treated Embryos | | | E10 Treated Embryos | | | E15 Treated Embryos | | |
|---|---|---|---|---|---|---|---|---|
| Vector Control | BDNF | NT-3 | Vector Control | BDNF | NT-3 | Vector Control | BDNF | NT-3 |
| 21.187 | 22.212 | 2.624 | 54.151 | 27.435 | 4.289 | 17.963 | 8.291 | 4.490 |
| 24.764 | 27.752 | 5.436 | 63.851 | 33.778 | 14.937 | 18.886 | 9.205 | 6.143 |
| 43.581 | 51.879 | 19.175 | 92.642 | 45.992 | 23.379 | 33.337 | 26.897 | 18.744 |
| 29.844 | 33.947 | 9.078 | 70.214 | 35.735 | 14.201 | 23.395 | 14.797 | 9.792 |
| ±3.472 | ±4.553 | ±2.556 | ±5.778 | ±2.722 | ±2.761 | ±2.488 | ±3.027 | ±2.250 |