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Neurotrophin mRNA expression in the central nervous system of the brain stem-spinal cord regenerating model, *Anguilla anguilla,* the European eel.

Victoria Stephanie Peart

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy to the University of Dublin, Trinity College

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Candidate's Declaration

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Victoria Stephanie Peart

August 2006

Dedicated to my Granny, Rita.

She was a true inspiration to me.

Summary

Neuronal regeneration is extremely limited in the central nervous system (CNS) of mature amniotes. On the other hand, many anamniotes, such as some fish, display rapid morphological and functional recovery after CNS injury, a process that has often been compared to neuronal development. Neurotrophins are a group of molecules that are crucial in development, as well as in the maintenance and plasticity of the adult nervous system. They are also thought to play an important role in neuronal regeneration. Neurotrophin mRNA and protein are upregulated in the cell bodies and target regions of injured neurons that lie in the mammalian peripheral nervous system (PNS), and which do regenerate after axonal injury. Neurotrophins are also present in the anamniote CNS but little is known about their spatial expression patterns or their involvement in regeneration after neuronal insult in this group of animals. Based on the results of mammalian studies, the hypothesis was formulated that neurotrophin expression may be upregulated in the anamniote CNS after injury in order to aid in neuronal regeneration. To test this hypothesis, neurotrophin and neurotrophin receptor mRNA expression was investigated in the eel CNS at various timepoints before and after spinal cord transection, and during regeneration and functional recovery.

The model chosen for the present study was the European eel, *Anguilla anguilla*, a fish that recovers rapidly after a complete spinal cord transection, regaining normal swimming within approximately 35 days. The eel brain stem contains the cell bodies of spinally projecting neurons whose reconnection with their target is essential for the restoration of regular locomotion after axotomy; changes in neurotrophin mRNA expression in the eel brain were, therefore, the main focus of this study. Whilst the procedures were already in place to monitor the morphological and functional facets of regeneration in the eel in our laboratory, methods for monitoring the intrinsic processes such as changes in gene expression had not yet been established for this model. The technique of non-radioactive *in situ* hybridisation (ISH) was optimised for eel brain tissue and employed to examine the mRNA expression pattern of brain derived neurotrophic factor (BDNF). Riboprobes were prepared based on a partial cDNA sequence for *A. anguilla* BDNF (GenBank accession number AY762996), that was amplified using degenerate primers, cloned and sequenced. Cells expressing BDNF mRNA were found to be widespread throughout the eel brain. Its expression pattern in the eel brain was then compared to that reported for the mammalian brain and other vertebrate species. This comparison may provide insights into the differences between regenerating and non-

regenerating species. As in other vertebrates, in the eel, BDNF mRNA expression was seen in the telencephalon, hypothalamus, tectum, many primary and secondary sensory centres, and cranial motor nuclei. However, in contrast to mammals, BDNF mRNA expression was observed in groups of brain stem nuclei such as the reticular formation that are important in the control of movement and that contain the cell bodies of neurons that project down the spinal cord, and whose axons are damaged by spinal cord injury.

Semiquantitative RT-PCR was used to examine the effect of complete spinal cord transection on the mRNA expression of BDNF, a nerve growth factor (NGF)-like gene and the neurotrophin receptor, trk B, in the eel brain and target region in the spinal cord. A range of timepoints pre- and post-injury were chosen for examination to coincide with phases in the spinal cord regeneration process where neurotrophins and their receptors may be important in promoting recovery. The spatial expression pattern of BDNF mRNA in the eel brain was also assessed before and after injury using ISH.

No distinctive changes in BDNF, the NGF-like factor and trk B mRNA levels in the eel CNS were detected at any timepoint after cord transection; however, a slight but significant decrease from BDNF mRNA levels at one day post transection (DPT) was found at 15 DPT in the eel brain. In addition, the spatial expression pattern of BDNF mRNA in the eel brain appeared unchanged at 10 days after injury even though there has been considerable axonal regrowth. It may be the case that due to the continual growth of anamniotes, neurotrophins are present in supraspinal neurons and possibly in the target spinal cord at sufficient levels to promote neuronal regeneration negating the need for an upregulation in their expression after injury.

Acknowledgements

Firstly, my sincerest thanks and gratitude to my supervisors Dr Suzanne Borich and Prof. Barry Roberts for all your invaluable help, advice and input throughout the course of my research. Thank you to Suzanne for your time, effort, advice and encouragement. Thank you to Prof. Roberts for the trips over from the US to meet with me and for the speedy replies with excellent advice to my numerous emails.

A special thanks to Dr Paula Murphy for all the helpful advice throughout the course of my project. I would also like to thank Dr Celia Holland for her encouragement, advice and kind words over the years. Thanks too to Dr Mark Brown for his advice on statistics.

Jackie, thanks a million for all your help and for being a great friend in an otherwise very lonely lab!

Thanks so much to Dr Adrian Dervan for your help and advice over the past few years and especially for introducing me to the delights of Adobe Photoshop.

A big thanks to Peter for all the journeys to get eels and help in the lab. No task was too big or too small! Thanks also to Alison, Martyn and Dick for all your help and advice. Thank you also to Fiona.

Thanks a million to Paddy F (the best office buddy ever), Clare, Laura, Graham, Samina, Áine, Kristen, Victor, Paddy K, Gillian, Val, Karen and all the other postgrads in Zoology for making my time in the department such fun. Thanks also to all the staff in the Zoology department for providing a relaxed, friendly and fun atmosphere in which to work.

Thank you to everyone over in Kevin Mitchell's lab for letting me use the Vibratome. You were always so obliging and accommodating. I really appreciate it.

I also wish to thank Russell Poole and Damien Murray for their help in sourcing eels.

I gratefully acknowledge the receipt of financial support from Enterprise Ireland.

To Elizabeth, Noelle, Lisa, Ruth, Katie, Elaine, Sarah, Kamelia, Paul, Nikki, Ian, John (Fishy), McGarry, Paul G, Mario, Rob, and to all my closest friends, thanks so much for all the fun over the years and for keeping me sane. I'm going to miss you all so much. The 2nd Sept. is going to be a day and a night to remember.

Thanks to Joan, John, Karen, Emma and all the Daltons for all their love, support, advice and all the hot meals! Thanks to Phyllis for keeping me in your prayers.

Thank you to Geraldine for all the lovely lunches and for all your love and support during my time in Trinity.

Thanks to my Godmother, Mary, who has been a great help to me in many ways and who provided me with many needed and greatly appreciated getaways during the course of my study.

To my family, words can't express how thankful I am to you. To my parents, you really are the best parents anyone could ever ask for. I could not have done this without you. Mum, thanks for all your love, support, patience and encouragement over the years. Thanks for putting up with me when I was in a bad mood and for keeping me going. Dad, thanks for believing in me, for all your support and concern. Thanks to Tasha for being my oldest friend, for always making me laugh and for providing somewhere for me to write up! Rossy, thanks for all the walks and trips into college on the DART. It was so nice to have you in Trinity at the same time as me. You're going to be a great OT! Felicity (Jamie), thanks a million for all your help and love, for cheering me up when I was down and for all the fashion advice!

Finally, thank you to my husband, Jonathan. Again, I will never be able to find the words to express how thankful and grateful I am to you. You are my best friend and my rock. I really could not have done this without you. Thanks for all your love, help, support, patience, encouragement and most of all for believing in me. I can't wait to start our new life together downunder. All my love, Tori.

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Abbreviations

ARP	acidic ribosomal phosphoprotein
BCIP	5-bromo-4-chloro-3-indolyl-phophate
BDNF	brain derived neurotrophic factor
CNS	central nervous system
CV	coefficient of variation
DIG	digoxigenin
DPT	days post spinal cord transection
DRG	dorsal root ganglion
ELISA	enzyme-linked immunosorbent assays
TBE	tris borate EDTA
GAP 43	growth associated protein 43
GAPDH	glyceraldehyde-3-phophate dehydrogenase
ISH	*in situ* hybridisation
LTP	long term potentiation
MS 222	tricaine methane sulfonate
NBT	4-nitro blue tetrazolium chloride
NCBI	National Centre for Biotechnology Information
NGF	nerve growth factor
NMDA	N-methyl-D-asparate
nt	nucleotide
NT-3	neurotrophin 3
NT-4/5	neurotrophin 4/5
NT-6/7	neurotrophin 6/7
PBS	phosphate buffered saline
PFA	paraformaldehyde
PNS	peripheral nervous system
rRNA	ribosomal RNA
TH	tyrosine hydroxylase
Trk/trk	tyrosine kinase receptor (Trk refers to the receptor protein and trk, to the receptor mRNA)
WISH	wholemount *in situ* hybridisation

A list of abbreviations specific to chapter 3 are provided in Figure 3.2, pp. 67- 82.

Chapter 1

General Introduction

1. Introduction

Regeneration of the central nervous system (CNS) in amniotes such as mammals is extremely limited following embryological development (Nicholls and Saunders, 1996; Horner and Gage, 2000; Jacobs and Fehlings, 2003). In contrast, however, regeneration of neurons in the CNS occurs readily throughout the life span of many anamniotes e.g. teleost fish and amphibians (Bernhardt, 1999; Ferretti *et al.*, 2003). A greater understanding of the extrinsic factors (e.g. identity of neuronal guidance cues) and intrinsic elements (e.g. expression of genes coding for growth promoting molecules) involved in axonal regeneration in fish and amphibians may lead to a breakthrough in treatment of spinal cord injuries and neurodegenerative diseases in humans (Caroni, 1997; Conner and Dragunow, 1998; Murer *et al.*, 2001; Ferretti *et al.*, 2003; Gillespie, 2003; Selzer, 2003; Wintzer *et al.*, 2004; Roest Crollius and Weissenbach, 2005).

1.1 Extrinsic factors affecting regeneration in the mammalian CNS

Following injury to the amniote CNS, a number of factors contribute to its lack of regeneration (Fig. 1.1). Immediately after injury, neurons are susceptible to death due to the effects of secondary injury processes that follow the initial mechanical injury. These processes include the influx of cations and inflammatory mediators, free radical-mediated cell injury and glutamatergic excitotoxicity, and ultimately lead to necrosis and apoptosis (Crowe *et al.*, 1997; Lu *et al.*, 2000; Sekhon and Fehlings, 2001; Kerschensteiner *et al.*, 2005). Neurons that do survive are provided with a hostile environment for regeneration. Oligodendrocytes release at least three neurite growth inhibitors, which include Nogo-A, myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein (Schwab, 1990, Fawcett and Geller, 1998; Fawcett, 2002; Bandtlow, 2003). These molecules interact with the Nogo receptor leading to growth cone collapse (Watkins and Barres, 2002; Ferraro *et al.*, 2004; Schwab, 2004). Another challenge faced by injured CNS neurons is the glial scar, which is formed at the site of lesion. Not only does the scar form a physical barrier to regenerating axons, but also its cellular components release axon-neuronal repulsive molecules which include members of the chondroitin sulfate proteoglycan and semaphorin family (Kruger *et al.*, 1986; Rudge and Silver, 1990; Jakeman and Reier, 1991; McKeon *et al.*, 1991; Song and Poo, 1999; Fawcett, 2002; Jones *et al.*, 2003; Küry *et al.*, 2004; Klapka *et al.*, 2005).

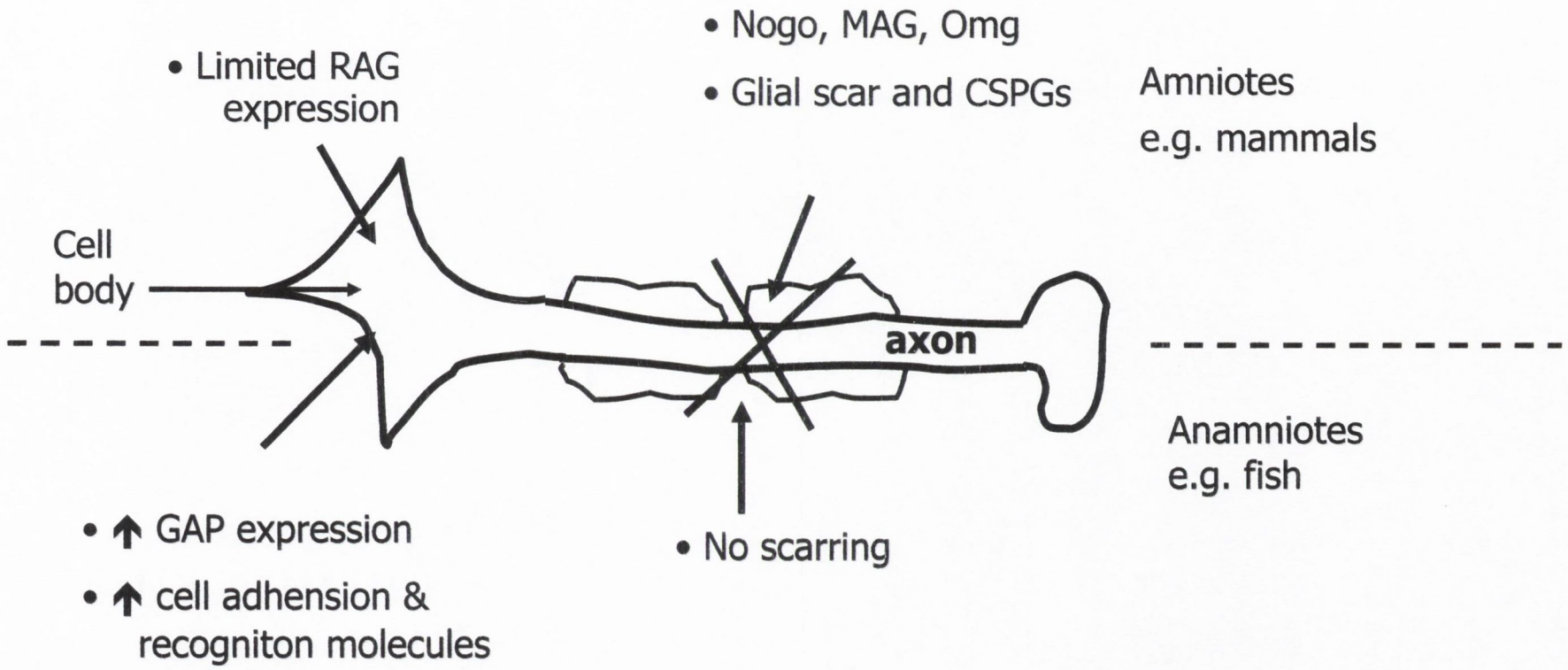


Figure 1.1. Comparison of CNS responses to injury in amniotes (above broken line) and anamniotes (below broken line). The X marks the site of axonal injury. After injury in amniotes limited regeneration associated gene expression (RAG) is seen and axonal regrowth is prevented by the presence of Nogo, myelin associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (Omg) and chondroitin sulfate proteoglycan (CSPG) in the surrounding environment. On the other hand, growth associated protein (GAP) expression is increased in anamniotes after neuronal insult as are cell adhesion and recognition molecules. Little or no glial scarring is seen.

Lack of directional information for injured neurons also appears to be a factor involved in the limited regeneration seen in the amniote CNS after injury (Kerschensteiner *et al.,* 2005). In the lamprey, which successfully regenerates its spinal cord after injury, regrowing axons display directional specificity and form synapses with the neuronal types with which they were originally connected suggesting the presence of guidance cues (Yin *et al.,* 1984; Mackler *et al.,* 1986; Mackler and Selzer, 1987). The non-random pathway choices and ability of regenerating retinal ganglion cell axons in the goldfish optic pathway to reach appropriate targets in the tectum also suggests the presence of guidance cues during CNS regeneration in this fish (Bernhart, 1989). On the other hand, an *in vivo* imaging study examining axonal regrowth after injury in the mouse spinal cord showed that neurons that did mount a regenerative response displayed an erratic growth pattern that did not advance axons closer to their targets (Kerschensteiner *et al.,* 2005). The theory that neuronal regeneration is hindered in the mammalian CNS due to a lack of directional information is further supported by the fact that application of neurotrophins, which promote axonal pathfinding, at the site of injury greatly improves neuronal survival and regrowth (Schnell *et al.*, 1994, Bregman *et al.*, 1997, Song and Poo, 1999; Novikova *et al.,* 2002; Gillespie, 2003).

1.2 Intrinsic factors affecting regeneration in the mammalian CNS

In addition to the extrinsic factors listed above, intrinsic factors can influence the ability of a neuron to regenerate after injury (Fawcett, 1992; Chen *et al.,* 1995; Caroni, 1997; Barron, 2004; Cafferty *et al.,* 2004; Chuckowree *et al.,* 2004; Neumann *et al.,* 2005; Blackmore and Letourneau, 2006). For example, some neurons when provided with a favourable environment in which to regenerate, such as a peripheral nerve graft, can regrow their axons at least within the graft (Richardson *et al.,* 1980; David and Aguayo, 1981; Bregman *et al.*, 1997; Ye and Houle, 1997; Levi *et al.,* 2002). However, other neurons including certain groups of retinal and thalamic neurons, still fail to regrow in this situation which suggests that the failure of CNS neurons to regenerate can in some cases be due to intrinsic factors within the neurons (Benfey *et al.,* 1985; Chen *et al.,* 1995). Regeneration occurs readily in the peripheral nervous system (PNS) and an intrinsic regenerative programme is set in motion after injury (Raivich and Kreutzberg, 1993; Bregman, 1998; Snider *et al.,* 2002). This programme typically includes an upregulation in the expression of regeneration associated genes such as α- and β-tubulin, growth associated protein 43 (GAP 43), actin (Skene, 1989; Caroni, 1997; Bregman, 1998) and neurotrophins such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Raivich and Kreutzberg, 1993; Tonra *et al.,* 1998; Michael *et al.,* 1999; Hammarberg *et al.,* 2000;

Obata *et al.*, 2006). Dorsal root ganglion (DRG) neurons provide a model system in which comparison between intrinsic responses governing PNS and CNS regeneration can be made because from each cell body one process extends into the PNS and another into the CNS. The centrally projecting branch, fails to regenerate after injury, but does so if the peripheral branch, which does regenerate, has been previously lesioned (Neumann and Woolf, 1999). The peripheral "conditioning" lesion appears to have initiated the intrinsic regenerative growth programme normally associated with the PNS and provided the CNS neurons with the capability to regenerate (Neumann and Woolf, 1999; Neumann *et al.*, 2005).

The neuronal cell type and ability to sustain expression of certain regeneration associated genes after injury may be important factors governing the result of the intrinsic response to injury (Tetzlaff *et al.*, 1991, McKerracher *et al.*, 1993). Tetzlaff *et al.* (1991) conducted a study examining mRNA expression of regeneration associated genes, such as GAP 43 and various cytoskeletal proteins, in the cell bodies of facial and rubrospinal neurons. The cell bodies of facial neurons lie within the CNS but project to the PNS and their axons successfully regrow when lesioned. On the other hand, the axons of rubrospinal neurons, which project solely within the CNS, do not. Tetzlaff *et al.* (1991) found that both types of neuron mounted a similar response to injury by upregulating GAP 43 mRNA and mRNAs for the cytoskeletal proteins tubulin and actin. By the second week after axotomy however, while GAP 43 expression remained elevated in both cell types, mRNA expression for the cytoskeletal proteins in rubrospinal neurons was reduced to subnormal levels and could be correlated with neuronal atrophy.

The site of lesion may also be important in a neuron's intrinsic response to injury. Corticospinal neurons lesioned close to the cell body upregulate mRNA expression for a range of regeneration associated genes such as GAP 43 and SCG10 whereas distal axotomy of corticospinal neurons within the spinal cord leads to no change in mRNA expression for regeneration associated genes (Mason *et al.*, 2003).

In addition, neuronal age can affect a neuron's intrinsic ability to regenerate (Fawcett, 1992; Chen *et al.*, 1995; Blackmore and Letourneau, 2006). For example, using explant cocultures of retina and tectum from the hamster, Chen *et al.* (1995), demonstrated that the ability of retinal axons to regrow is lost when the retina is removed from animals on or after postnatal day two, even when the tectum serving as the target is embryonic. On the other hand, embryonic neurons can regenerate into a tectum of any age (Chen *et al.*, 1995). Similarly in the chick, the regeneration potential of spinally projecting brain stem neurons decreases with age (Blackmore and Letourneau, 2006).

In summary, CNS neurons in some cases do appear to be able to mount an intrinsic response to injury (Tetzlaff *et al.*, 1991; Mason *et al.*, 2003). Factors such as the type of neuron i.e. whether it projects to the PNS or CNS (Tetzlaff *et al.*, 1991, Neumann and Woolf, 1999), the ability of a neuron to sustain the expression of certain regeneration associated genes (Tetzlaff *et al.*, 1991), the lesion distance from the cell body (Mason *et al.*, 2003) and neuronal age (Fawcett, 1992; Chen *et al.*, 1995; Blackmore and Letourneau, 2006) can play a role in determining the resulting regenerative response.

1.3 Neurotrophins and neuronal regeneration

The challenge to regenerating neurons is similar to that faced by developing neurons extending axons to their targets and so regeneration in the CNS has been described as a recapitulation of development (Fawcett, 1992; Aubert *et al.*, 1995). A regenerating neuron must first survive the initial injury, then it must regrow and find its way successfully to its correct target and when there, must form synaptic connections which lead to a regain in function (Cohen *et al.*, 1988). During development, a group of molecules called the neurotrophins are important in regulating neuronal survival, promoting axonal pathfinding and elongation towards targets and support of synaptic plasticity (Castrén *et al.*, 1992; Cohen-Cory and Fraser, 1995; Henderson, 1996; Katz and Shatz, 1996; McAllister, 1999; Song and Poo, 1999; Fariñas *et al.*, 2002; Huang and Reichardt, 2001; Blesch *et al.*, 2002; Lom *et al.*, 2002; Gillespie, 2003; Cohen-Cory and Lom, 2004). Neurotrophins are also found in the adult nervous system where they appear to be involved in neuronal plasticity and survival (Altar *et al.*, 1997; Bhattacharyya *et al.*, 1997; Conner *et al.*, 1997; Conner and Dragunow, 1998; McAllister *et al.*, 1999; Mufson *et al.*, 1999; Kelly *et al.*, 2000; Schinder and Poo, 2000; Murer *et al.*, 2001; Gooney *et al.*, 2002; Blum and Konnerth, 2005; Tapia-Arancibia *et al.*, 2004) and where they are also thought to play a significant role in neuronal regeneration (Raivich and Kreutzberg, 1993; Tonra *et al.*, 1998; Zhang *et al.*, 2000; Boyd and Gordon, 2001; Lee *et al.*, 2001a; Gillespie, 2003). They are ideal candidates to support neuronal regeneration since their activity promotes all the tasks described above that a regenerating neuron must complete in order to regenerate successfully (Cohen *et al.*, 1988; Davies and Wright, 1995; Henderson, 1996; Bregman, 1998; McAllister *et al.*, 1999; Song and Poo, 1999; Huang and Reichardt, 2001; Markus *et al.*, 2002; Gillespie, 2003; Cohen-Cory and Lom, 2004).

Neurotrophins constitute a family of structurally and functionally related molecules whose members include NGF, BDNF, neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5), and also neurotrophin 6 (NT-6) and neurotrophin 7 (NT-7) found only in fish (Lewin and Barde, 1996; Hallböök, 1999; Heinrich and Lum, 2001; Dethleffsen *et al.*, 2003). They

can be synthesised by and act on both neuronal and non-neuronal cells (Wetmore and Olson, 1995; Tonra *et al.*, 1998; Lee *et al.*, 2001a). They exert their effects by binding two classes of neurotrophin receptors- the low affinity p75 receptor and the high affinity tyrosine kinase receptors (Trk; Lewin and Barde, 1996; Heinrich and Lum, 2000). All neurotrophins bind to the p75 receptor with equal affinity whereas NGF preferentially binds Trk A, BDNF and NT-4/5 activate Trk B, and NT-3 binds to Trk C (Lewin and Barde, 1996). The actions of neurotrophins on these two different types of receptors were until recently thought to be opposing, i.e. activation of Trk receptors was thought to promote cell survival and neuronal plasticity whilst activation of the p75 receptor was thought to bring about cell death by apoptosis (Yoon *et al.*, 1998; Majdan and Miller, 1999; McAllister *et al.*, 1999; Boyd and Gordon, 2002; Kalb, 2005; Nykjaer *et al.*, 2005). In some cases, however, p75 activation can promote cell survival (Irie *et al.*, 1999; Roux *et al.*, 2001; Kalb, 2005; Nykjaer *et al.*, 2005). Trk activation on the other hand has been shown to promote cell death in cultured medulloblastoma and cortical cells (Muragaki *et al.*, 1997; Kim *et al.*, 1999; Kim *et al.*, 2003). It has also emerged that p75 receptors can be activated by neurotrophins in their precursor form as pro-neurotrophins, leading to cell survival or death depending on the downstream signalling cascade initiated (Lee *et al.*, 2001b; Kalb, 2005; Lu *et al.*, 2005).

Neurotrophin application after injury to neurons in the mammalian CNS has been shown to prevent atrophy of axons, promote axonal regeneration and increase expression of growth associated molecules (Schnell *et al.*, 1994; Xu *et al.*, 1995; Sawai *et al.*, 1996; Bregman *et al.*, 1997; Kobayashi *et al.*, 1997; Ye and Houle, 1997; Menei *et al.*, 1998; Liu *et al.*, 1999; Jin *et al.*, 2002; Kwon *et al.*, 2002; Novikova *et al.*, 2002; Sayer *et al.*, 2002; Zhou and Shine, 2003). Ye and Houle (1997), for example, demonstrated that regeneration of supraspinal neurons into a peripheral nerve graft after a spinal cord hemisection in the rat was significantly increased following BDNF and NT-3 treatment. Synthesis of neurotrophins and their receptors are increased during neuronal regrowth in the mammalian PNS capable of regeneration after injury (Meyer *et al.*, 1992; Funakoshi *et al.*, 1993; Raivich and Kreutzberg, 1993; Sebert and Shooter, 1993; Tonra *et al.*, 1998; Michael *et al.*, 1999; Shen *et al.*, 1999; Lee *et al.*, 2001a; Obata *et al.*, 2006). Furthermore, PNS axonal regrowth and functional recovery are severely comprised in rats treated with neutralising antibodies to BDNF (Zhang *et al.*, 2000) and in heterozygous null mice for the BDNF/NT-4/5 receptor, trk B (Boyd and Gordon, 2001; Irintchev *et al.*, 2005).

During regeneration, neurotrophins have the potential to function as extrinsic, target derived, axonal pathfinding, growth and synaptogenesis promoting factors (Levi-

Montalcini, 1987; McAllister *et al.*, 1999; Song and Poo, 1999; Miller and Kaplan, 2001; Ginty and Segal, 2002; Markus *et al.*, 2002; Gillespie, 2003; Zweifel *et al.*, 2005). They can also be considered as intrinsically produced, survival, regeneration and plasticity promoting molecules since they can be synthesised and released by injured neurons themselves and have the capability to act in an autocrine (Kokaia *et al.*, 1993; Ghosh *et al.*, 1994; Acheson *et al.*, 1995; Davies and Wright, 1995; Sebert and Shooter, 1993, Kobayashi *et al.*, 1996) or paracrine manner (Altar *et al.*, 1997; Heymach and Barres, 1997; Conner *et al.*, 1997; Tonra *et al.*, 1998; McAllister *et al.*, 1999; Caleo *et al.*, 2000; Spalding *et al.*, 2002; Menna *et al.*, 2003). Neurotrophins also have to potential to regulate appropriate target reconnection, which is critical for a regain of functional ability after injury (Cohen *et al.*, 1988), since their actions can also bring about cell death (Kalb, 2005; Lu *et al.*, 2005; Nykjaer *et al.*, 2005).

One of the factors affecting the outcome of neurotrophin activity may be the location of the activated receptor (Miller and Kaplan, 2001; Heersen and Segal, 2002; Lom *et al.*, 2002). Trk receptors, for example, can be located at neuronal terminals, along the axon and at the cell body (Kokaia *et al.*, 1993; Acheson *et al.*, 1995; Davies and Wright, 1995; Miller and Kaplan, 2001). In their role as target derived molecules, neurotrophins are thought to mediate their Trk-related effects by activation of receptors expressed at the terminal. Locally, at the terminal, the activated Trks in turn switch on signalling pathways, which promote axonal growth. Neurotrophin-bound activated Trks are also retrogradely transported to the cell body where they too activate signalling cascades (Miller and Kaplan, 2001, Ginty and Segal, 2002, Heersen and Segal, 2002; Markus *et al.*, 2002; Howe and Mobley, 2005; Zweifel *et al.*, 2005). In their role as autocrine molecules, neurotrophins activate receptors expressed on the cell body (Kokaia *et al.*, 1993; Ghosh *et al.*, 1994; Acheson *et al.*, 1995; Davies and Wright, 1995). It is not clear whether binding of neurotrophins at the cell body leads to the activation of signalling cascades similar to those activated when neurotrophins are target derived and retrogradely transported to the cell body (Heersen and Segal, 2002; Miller and Kaplan, 2001; Zweifel *et al.*, 2005). Application of neurotrophins to the cell body (Sawai *et al.*, 1996; Kobayashi *et al.*, 1997; Cheng *et al.*, 2002; Mo *et al.*, 2002; Salie and Steeves, 2005) and axonal terminals (Schnell *et al.*, 1994; Bregman *et al.*, 1997; Ye and Houle, 1997) does lead to cell survival of injured neurons in both cases. On the other hand, the results of a study by Lom *et al.* (2002) showed that BDNF application at the axonal terminals of retinal ganglion cells (RGCs) increases dendritic arborisation whereas application at the cell body decreases dendritic arborisation. The signal transduction pathways which are activated downstream

of neurotrophin receptor activation are only emerging and include mitogen-activated protein kinase and Akt/phosphatidylinositol 3-kinase pathways for Trk receptors and Jun amino-terminal kinase, p53 and NF-κB pathways for the p75 receptor (Kaplan and Miller, 2000; Heersen and Segal, 2002; Markus *et al.*, 2002; Segal, 2003).

1.4 Fish model of CNS regeneration

In contrast to amniotes, anamniotes following CNS injury display little or no astrocytic scarring, less prominent expression of myelin-associated inhibitory molecules e.g. Nogo A, and limited axotomy-induced cell death (Bastmeyer *et al.*, 1991; Becker *et al.*, 1997, Bernhardt, 1999, Schwab, 2004, Diekmann *et al.*, 2005). There is also a rise in the production of growth associated proteins e.g. GAP 43, as well as cell adhesion and cell recognition molecules, e.g. NCAM, L1.1 and L1.2, which can be correlated with axonal regrowth in the adult anamniote (Giordano *et al.*, 1997; Jacobs *et al.*, 1997; Becker *et al.*, 1998, Bormann *et al.*, 1998, Bernhardt, 1999, Murphy *et al.*, 2000, Becker *et al.*, 2004; Haenisch *et al.*, 2005).

Spinal cord regeneration accompanied by functional recovery has been reported in fish such as the lamprey (Cohen *et al.*, 1988), goldfish (Coggeshall *et al.*, 1982; Sharma *et al.*, 1993) and zebrafish (Becker *et al.*, 1997; Van Raamsdonk *et al.*, 1998). The chosen model for this study is the European eel, *Anguilla anguilla*, which also displays rapid morphological and functional regeneration after a complete spinal cord transection with a regain in swimming ability seen approximately 35 days after injury (Doyle *et al.*, 2001). The advantages of using *A. anguilla* as an experimental model for studies of CNS regeneration in fish are its relatively large body size (approximately 300 – 600 mm in length) which facilitates experimental manipulation and the detailed background knowledge of anguilliform swimming (Doyle *et al.*, 2001; Doyle and Roberts, 2004a). In addition, the timing of the processes involved in regeneration has been thoroughly characterised in the eel model (Doyle *et al.*, 2001; Dervan and Roberts 2003a, b).

Retrograde tracing studies have revealed that functional recovery in the zebrafish (Becker *et al.*, 1997), goldfish (Sharma *et al.*, 1993) and eel (Bosch, Maslam and Roberts, unpublished observations) is correlated with the reinnervation of the spinal cord lying caudal to the transection, by spinally projecting brain stem nuclei such as the nucleus of the medial longitudinal fasciculus, reticular formation and magnocellular octavolateral nucleus. Re-axotomy of regenerated fibres in the lamprey spinal cord results in the loss of regained swimming ability (Cohen *et al.*, 1988). Furthermore, during fictive locomotion in the lamprey elicited in the isolated, regenerated spinal cord, discharges in ventral root fibres on opposing sides of the healed transection are coordinated (Cohen *et al.*, 1988). In

the eel, inhibition of reinnervation of the caudal stump of the spinal cord by the placing of a rubber block at the site of transection prevents functional recovery (Doyle *et al.*, 2001). Axonal regrowth of brain stem neurons across the lesion site in the eel has also been confirmed by electrophysiological responses recorded caudal to the injury elicited by brain stem stimulation (Doyle, 2002). Taken together, these experiments illustrate that brain stem neurons reconnect with their spinal targets after injury and that this event must take place in order for functional recovery to occur. It appears that in the eel, regenerating descending projections need only re-establish contact with a certain minimum number of cells caudal to the transection site in order for the animal to regain swimming ability (Doyle *et al.*, 2001). This is in agreement with studies of spinal cord regeneration in the lamprey and zebrafish (Wood and Cohen, 1979, 1981; Mackler and Selzer, 1985, 1987, Becker *et al.*, 1997).

The basic swimming pattern is produced in the eel spinal cord by neuronal networks called central pattern generators (CPGs) (Grillner *et al.*, 1981; Dale and Roberts; 1985; Grillner and Wallén, 1985; Grillner *et al.*, 1995; Reith and Sillar, 1998; Delvolvé *et al.*, 1999; Doyle *et al.*, 2001; Yamaguchi, 2004). In order for a fish to change the speed, direction or force of swimming and indeed to initiate it, sensorimotor input from the brain is required (Roberts and Mos, 1992; Grillner *et al.*, 1995; Guertin and Dubuc, 1997; Deliagina *et al.*, 2000; Doyle *et al.*, 2001; Deliagina *et al.*, 2002, Matsuyama *et al.*, 2004). The interface between higher brain processing centres and neurons in the spinal cord is provided by the brain stem (Whelan, 1996; Deliagina *et al.*, 2000; Bosch and Roberts, 2001; Deliągina *et al.*, 2002; Drew *et al.*, 2004). The neurons of the brain stem extend throughout the medulla oblongata and into the tegmentum (Prasada Rao *et al.*, 1987; Lee *et al.*, 1993; Prasada Rao *et al.*, 1993; Bosch and Roberts, 1994; Wullimann *et al.*, 1996; Becker *et al.*, 1997; Bosch and Roberts, 2001; Mukuda and Ando, 2003). In the lamprey, the main descending system transmitting commands to the spinal cord is the reticulospinal system. Study of this simple organisation has led to insights into the control of movement by the brain stem (Grillner *et al.*, 1995; Guertin and Dubuc *et al.*, 1997; Deliagina *et al.*, 2002). For example, Deliagina *et al.* (2000) examined the activation of brain stem neurons after sensory stimuli such as illumination of the eyes, which evokes swimming. Initiation of locomotion is always preceded by a bilateral activation of reticulospinal neurons. The reticulospinal neurons are glutamatergic and activate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and N-methyl-D-aspartate (NMDA) receptors on the CPGs leading to a wave of activity down the spinal cord (Grillner *et al.*, 1995).

1.5 Neurotrophins and fish

Despite the fact that neurotrophin nucleotide and amino acid sequences are highly conserved between vertebrate species, limited information is available on their mRNA and protein distribution patterns, and the functions of their biological activity in teleosts are yet to be fully revealed (Martin *et al.*, 1995; Hallböök, 1999; Heinrich and Lum, 2000, Dethleffsen *et al.*, 2003). A few studies have briefly described neurotrophin and neurotrophin receptor expression in the fish CNS (Benowitz and Shashoua, 1979, Götz *et al.*, 1992, Martin *et al.*, 1995, Hashimoto and Heinrich, 1997, Caminos *et al.*, 1999, Hannestad *et al.*, 2000, Lum *et al.*, 2001). BDNF expression has been confirmed in the brain of *Xiphophorus* (platyfish) by northern blot hybridisation (Götz *et al.*, 1992), in the developing zebrafish brain and retina by *in situ* hybridisation (ISH) (Hashimoto and Heinrich, 1997; Lum *et al.*, 2001), and in the tench retina by ISH and immunohistochemistry (Caminos *et al.*, 1999). Five neurotrophin receptors have been described in zebrafish (Martin *et al.*, 1995). Two of these receptors have been described as isoforms of the Trk B receptor and their mRNA is widely distributed throughout the brain and spinal cord of larval zebrafish six days post fertilisation (Martin *et al.*, 1995). Hannestad *et al.* (2000) reported immunoreactivity for Trk B in the adenohypophysis, striatum and retina of the developing teleost *Dicentrarchus labrax* (European seabass). The protein expression pattern for NGF was described in the goldfish brain by Benowitz and Shashoua (1979). They saw immunoreactivity to anti-NGF sera in the optic tectum and in the ependyma and fibre layers of the vagal lobe.

As in mammals, in fishes NGF application to the CNS promotes neuronal outgrowth *in vitro* (Turner *et al.*, 1982; Levi-Montalcini, 1987) and *in vivo* (Yip and Grafstein, 1982; Fernandez *et al.*, 1993). NGF purified from the submandibular gland in mice, injected into developing zebrafish increases neuronal number in dorsal root ganglia. On the other hand, treatment with NGF antiserum decreases DRG neuronal number suggesting an involvement for NGF in neuronal development in zebrafish (Weis, 1968), as is the case in mammals (Smeyne *et al.*, 1994). Recombinant fish NGF and BDNF proteins have also been shown to promote the survival of embryonic chick sensory neurons (Götz *et al.*, 1992).

In summary, nucleotide and amino acid sequences for neurotrophins are highly conserved between vertebrate species (Heinrich and Lum, 2000; Dethleffsen *et al.*, 2003); their activity in fish does appear to be important during neuronal development (Weis, 1968; Martin *et al.*, 1995; Hashimoto and Heinrich, 1997; Hannestad *et al.*, 2000; Lum *et al.*, 2001), and they are also expressed in the adult fish CNS (Götz *et al.*, 1992; Caminos *et*

al., 1999). Application of NGF derived from the mouse can induce neuronal outgrowth in the fish CNS (Turner *et al.,* 1982; Yip and Grafstein, 1982) and, conversely, recombinant fish neurotrophins promote the survival of chick neurons (Götz *et al.,* 1992). Taken together these results suggest that the function of neurotrophin activity in the fish nervous system may be similar to that in other vertebrates (Klein *et al.,* 1993; Jones *et al.,* 1994; Smeyne *et al.,* 1994; Henderson, 1996; Conner *et al.,* 1997; Kobayashi *et al.*, 1997; Ye and Houle, 1997; Heinrich and Lum, 2000; Huang and Reichardt, 2001; Murer *et al.,* 2001).

As highlighted in section 1.3, neurotrophins are probably important in the process of nervous system regeneration although little is known about their role in this process in fish (Turner *et al.,* 1982; Yip and Grafstein, 1982; Caminos *et al.,* 1999). Some indirect evidence suggesting a role for neurotrophins in spinal cord regeneration in the eel comes from studies on the effect of activity on regeneration (Doyle and Roberts, 2004 a,b; Doyle and Roberts, 2006). Supraspinal neurons have been shown to grow faster in eels after overall activity in spinally transected animals is increased by L-DOPA injections (Doyle and Roberts, 2004a) or through an exercise regime (Doyle and Roberts, 2006). Elsewhere, neurotrophin and in some cases neurotrophin receptor expression has been shown to be regulated by neuroelectric activity generated by for example exercise (Neeper *et al.,* 1996; Widenfalk *et al.,* 1999; Gomez-Pinilla *et al.,* 2002; Skup *et al.,* 2002; Griesbach *et al.,* 2004; Huang *et al.,* 2005; Radak *et al.,* 2006), sensory stimulation (Castrén *et al.,* 1992; Herzog *et al.,* 1994; Bozzi *et al.,* 1995; Rocamora *et al.,* 1996; Rossi *et al.,* 1999; Ichisaka *et al.,* 2003) and electrical stimulation (Al-Majed *et al.,* 2000; Hartmann *et al.,* 2001). Molteni *et al.,* (2004) carried out a study linking the improved axonal regeneration after injury in exercised animals with an upregulation in BDNF mRNA expression. They reported that DRG neurons showed improved neuronal outgrowth in culture when taken from exercised rats as opposed to sedentary animals, an effect that could be blocked when animals were treated with a Trk inhibitor during exercise. Higher BDNF mRNA levels were seen in sensory ganglia removed from exercised animals than those from controls. It was also noted that exercised conditioned animals showed improved regeneration *in vivo* (Molteni *et al.,* 2004). It is possible therefore that an increase in overall physical activity following spinal cord transection in the eel may lead to a rise in neurotrophin synthesis and possibly Trk receptor expression, which in turn aids regeneration and results in a shorter recovery time after injury (Doyle and Roberts, 2006).

1.6 Aims of current research

Evidence from the regenerating mammalian PNS (Sebert and Shooter, 1993; Tonra *et al.*, 1998; Michael *et al.*, 1999; Shen *et al.*, 1999; Lee *et al.*, 2001a; Irintchev *et al.*,

2005) and the non-regenerating CNS (Bregman *et al.*, 1997; Kobayashi *et al.*, 1997; Ye and Houle, 1997; Menei *et al.*, 1998; Liu *et al.*, 1999; Jin *et al.*, 2002), strongly suggests that changes in the expression of neurotrophins and their receptors are important in neuronal regeneration after injury. Studies suggest that neurotrophins may also play a role in CNS regeneration in fish (Turner *et al.*, 1982; Yip and Grafstein, 1982; Götz *et al.*, 1992; Caminos *et al.*, 1999; Doyle and Roberts, 2004 a, b; Doyle and Roberts, 2006).

The eel brain stem houses the cell bodies of the neurons that project down the spinal cord to control locomotor activity, and whose reconnection with their spinal targets is essential for functional recovery after injury (Bosch and Roberts, 1994; Bosch and Roberts, 2001; Doyle *et al.*, 2001). Neurotrophins synthesised in the cell bodies of these neurons after injury may promote their own regeneration by acting in an autocrine (Kokaia *et al.*, 1993; Ghosh *et al.*, 1994; Acheson *et al.*, 1995; Davies and Wright, 1995; Kobayashi *et al.*, 1996) or paracrine manner (Altar *et al.*, 1997; Heymach and Barres, 1997; Conner *et al.*, 1997; Tonra *et al.*, 1998; Caleo *et al.*, 2000; Spalding *et al.*, 2002; Menna *et al.*, 2003). It is possible therefore that injury may lead to changes in the neurotrophin mRNA expression pattern in the eel brain, for example, mRNA may be expressed in new or different neuronal populations after cord transection or expressed at higher levels (Kobayashi *et al.*, 1996; Tonra *et al.*, 1998; Michael *et al.*, 1999; Obata *et al.*, 2006). Kobayashi *et al.* (1996), for example, demonstrated differences in the mRNA levels and expression pattern for BDNF in the rat facial nucleus before and after axotomy. As well as the injured neuronal cell body, neuronal and non-neuronal cells in the target region caudal to the site of transection may also act as an important source of retrogradely derived neurotrophins after injury (Lee *et al.*, 2001a; Markus *et al.*, 2002). In addition, neurotrophin activity after injury may also be enhanced by the upregulation in neurotrophin receptor expression (Turner *et al.*, 1982; Frisén *et al.*, 1992, Raivich and Kreutzberg, 1993, Caminos *et al.*, 1999; Lee *et al.*, 2001a).

Based on the evidence described above, the hypothesis was formulated that neurotrophins are upregulated in the fish CNS after spinal cord transection and are involved in the regeneration process. To test this hypothesis, neurotrophin and neurotrophin receptor mRNA expression was examined in the eel CNS at different timepoints before and after cord transection, and during regeneration and functional recovery. In order to achieve this goal, it was first necessary to develop and establish the molecular tools and processes with which to do this.

The neurotrophin, BDNF, was chosen as the main focus for this study. BDNF is the most widely distributed and highly expressed neurotrophin in the mammalian CNS

(Ceccatelli *et al.*, 1991; Conner *et al.*, 1997; Yan *et al.*, 1997b; Friedman *et al.*, 1998; Murer *et al.*, 2001; Nakamura and Bregman, 2001). Its activity is important for neuronal development (Conover *et al.*, 1995; Liu *et al.*, 1995; Henderson, 1996; Huang and Reichardt, 2001) and the survival and functioning of the CNS of adult animals (Connor and Dragunow, 1998; Murer *et al.*, 2001). In mammals, BDNF application in the CNS after injury promotes neuronal regeneration (Kobayashi *et al.*, 1997; Liu *et al.*, 1999; Jin *et al.*, 2002) and, its expression is upregulated in neuronal cell bodies (Kobayashi *et al.*, 1996; Hirsch *et al.*, 2000; Chidlow *et al.*, 2005; Suneja *et al.*, 2005) and target regions following axotomy (Ikeda *et al.*, 2001; Uchida *et al.*, 2003). As in mammals, BDNF activity appears to be important in the developing zebrafish CNS (Martin *et al.*, 1995; Hashimoto and Heinrich, 1997; Huang and Reichardt, 2001; Lum *et al.*, 2001). Neuronal regeneration is improved after spinal cord transection, when overall physical activity is increased above normal levels (Doyle and Roberts, 2004a; Doyle and Roberts, 2006). Since neurotrophins, particularly BDNF, are upregulated in an activity dependent manner (Neeper *et al.*, 1996; Oliff *et al.*, 1998; Al-Majed *et al.*, 2000; Skup *et al.*, 2002; Ichisaka *et al.*, 2003), it is possible that increasing physical activity in the eel may lead to a rise in BDNF synthesis and possibly Trk receptor expression, which in turn could aid regeneration (Doyle and Roberts, 2006). On the other hand, neuronal regeneration is slowed by up to 60 days when activity in the target region of the spinal cord is disrupted by the application of the NMDA receptor blocker, AP5, in this area (Doyle and Roberts, 2004b). BDNF has been shown to be downregulated after a lack of activity (Widenfalk *et al.*, 1999) and this may be a factor in the slowing of regeneration seen in the eel after AP5 treatment (Doyle, 2002; Doyle and Roberts, 2004, b).

A secondary reason for focussing on BDNF was that at the time this study was started (2001), more sequence data was available for fish BDNF than for any other fish neurotrophin. The full length BDNF cDNA sequences were available for zebrafish, carp, and platyfish NCBI GenBank accession numbers NM_131595, L27171 and X59942 respectively and degenerate primers with which zebrafish BDNF cDNA fragments had been cloned, were also published (Hashimoto and Heinrich, 1997). Only one full length sequence and some partial sequences were available for fish NGF and only partial sequences were available for fish NT-3 (Heinrich and Lum, 2000). NT-4/5 has only recently been described in fish (Dethleffsen *et al.*, 2003). Little is known about the function of NT-6 and NT-7 in fish, and orthologues have not been found in mammalian species (Heinrich and Lum, 2000).

Whilst the methods and procedures to monitor the morphological and functional aspects of spinal cord regeneration in the eel had been well established in our laboratory (Doyle *et al.,* 2001; Doyle, 2002; Dervan and Roberts 2003a,b), techniques such as ISH and semiquantitative RT-PCR, with which to examine the intrinsic process of mRNA expression, had not yet been put into place. The study of mRNA expression changes in a tissue is extremely valuable since, in many cases, they reflect downstream processes such as protein expression (Kobayashi *et al.,* 1996; Giordano *et al.,* 1997; Kind, 2000; Bareyre and Schwab, 2003; Adlard *et al.,* 2004; Wintzer *et al.,* 2004; Huang *et al.,* 2005). As outlined in chapter 2 of this thesis, the first aim of this study, therefore, was to establish a protocol for ISH by which the spatial mRNA expression pattern for molecules of interest could be examined in the eel CNS before and after injury. In doing this, an *A. anguilla* BDNF cDNA fragment was cloned in order to generate a genetic tool for further research and also to provide neurotrophin sequence information for a primitive teleost which may be useful in future functional genomic studies and molecular phylogenetic analyses of evolutionary relationships between species (Kullander *et al.,* 1997; Dethleffsen *et al.,* 2003; Roest Crollius and Weissenbach, 2005).

Once the tools were developed, the first approach used was to visualise directly the mRNA expression pattern for BDNF since determining where mRNA for a particular gene is expressed may give an indication of its function or alternatively the function of the region in which it is expressed (Wilkinson, 1998; Wintzer *et al.,* 2004). Chapter 3 describes the expression pattern of BDNF mRNA in the normal eel brain and draws comparisons with other vertebrate species. BDNF appears to be involved in many processes in the normal mammalian brain such as neuronal survival and maintenance (Conner and Dragunow, 1998; Mufson *et al.,* 1999; Murer *et al.,* 2001), synaptic plasticity (McAllister *et al.,* 1999; Schinder and Poo, 2000; Blum and Konnerth, 2005; Bramham and Messaoudi, 2005) and may be involved in the release of neuropeptides and neuromodulators in the hypothalamus (Tapia-Arancibia *et al.,* 2004). The functions of neurotrophins in the fish CNS however have not been defined and a detailed study has not been carried out of the mRNA expression pattern of any of the neurotrophins in the fish brain to date. By comparison with homologous regions in the brains of amniotes that display limited CNS regeneration, elucidation of the BDNF mRNA expression pattern in the eel brain may point towards the possible functions of neurotrophins in the fish CNS and to further our understanding of the ability of fish to regenerate their spinal cord after injury.

In chapter 4, the spatial expression pattern of BDNF mRNA in the normal eel brain was compared with that in the eel brain 10 days after a complete spinal cord transection using ISH. A second technique, semiquantitative RT-PCR, was also set-up and used to compare mRNA levels for BDNF, an NGF-like gene and trk B before and after spinal cord transection, in the eel brain and at the target region in the cord. Timepoints of one, 10, 15 and 20 days post spinal cord transection were chosen for analysis in order to correlate with stages in spinal cord regeneration in the eel where neurotrophins and their receptors may have important roles in promoting recovery.

In summary the main aims of this thesis are:

- To clone an *A. anguilla* BDNF cDNA fragment in order to provide a genetic tool for further research and to make BDNF cDNA sequence information available for a primitive teleost.
- To establish a protocol for ISH by which the spatial mRNA expression pattern for molecules of interest could be examined in the eel CNS before and after injury.
- To describe the expression pattern of BDNF mRNA in the normal eel brain drawing comparisons with other vertebrate species.
- To compare the spatial expression pattern of BDNF mRNA in the normal eel brain with that in the eel brain after the spinal cord has been transected.
- To compare mRNA levels of BDNF, an NGF-like gene and trk B in the eel CNS at different timepoints before and after cord transection, and during regeneration and functional recovery.

Chapter 2

Cloning of a 467 bp cDNA fragment for *Anguilla anguilla* brain derived neurotrophic factor and establishment of *in situ* hybridisation protocols for use in the eel CNS

2.1 Introduction

As mentioned in section 1.6, whilst the procedures had been developed in our laboratory to monitor the morphological and functional recovery of the eel after spinal cord transection, the tools with which to examine gene expression after injury at the molecular level had not yet been established. In order to develop these protocols, not only were the cDNA sequence data required but also it was necessary to optimise standard molecular protocols appropriately for the gene in question and the study organism.

At the time this study was being developed (2001), no *A. anguilla* neurotrophin nucleotide or amino acid sequence data were available. The first step in studying the expression of a gene at the mRNA level is to determine its sequence. In order to study BDNF mRNA expression in the eel, therefore, it was necessary to clone a cDNA fragment specific for *A. anguilla* BDNF.

2.1.1 Organisation and structure of the BDNF gene

The genomic organisation of all the neurotrophins is similar (reviewed by Heinrich and Lum, 2000). All neurotrophin genes possess a major coding exon of approximately 1 kb in length. This exon encodes the signal sequence, a pro-peptide and the mature neurotrophin. Two highly conserved regions are present in the prepropeptide division of the major BDNF exon in many species. The first region consists of 20 amino acids which represent the signal sequence and are identical in all species except for a conservative leucine to valine substitution, implying that BDNF secretion is under stringent and highly conserved control (Hashimoto and Heinrich, 1997; Lessmann *et al.*, 2003). The second highly conserved region is made up of a 35 amino acid segment and is situated upstream of but in close proximity to the cleavage site that generates the mature BDNF peptide (Hashimoto and Heinrich, 1997). It has been suggested that this region may play a role in the biological functioning of BDNF as a pro-neurotrophin (see section 1.3; Chao and Bothwell, 2002; Lee *et al.*, 2001b; Nykjaer *et al.*, 2005).

One or more smaller, untranslated exons are located upstream of the major neurotrophin coding exon. In the case of BDNF, the coding exon in rats and humans is preceded by at least four additional, untranslated exons (1a, b, c and d), each with a separate promoter (Fig. 2.1; Malkovska *et al.*, 2006). Mature BDNF mRNA transcripts, therefore, are made up of two exons, the first being one of the 5' untranslated exons and the second being the major BDNF-coding exon. In rats, alternative splicing and the use of two alternative polyadenylation sites results in at least eight BDNF transcripts (Ohara *et al.*, 1992). A fifth 5' untranslated exon has recently been discovered in the mouse (NCBI

GenBank accession number AY057907; Liu *et al.*, 2006a). Due to the existence of these non-coding exons, it is important to target primers to the major BDNF-coding exon if cloning BDNF by degenerate primers. Figure 2.1 is a diagrammatic representation showing the exon/intron organisation of the BDNF gene.

2.1.2 In situ hybridisation

In situ hybridisation (ISH) is a technique that allows the direct visualisation of the spatial expression pattern of nucleic acid sequences in tissues (Brown, 1998; Wilkinson, 1998) and can be employed to monitor spatial gene expression changes before and after a biological event such as a CNS lesion (Swain *et al.*, 1994; Jacobs *et al.*, 1996; Jacobs *et al.*, 1997; Wintzer *et al.*, 2004). A probe, complementary to the nucleic acid sequence of interest, is labelled with a reporter molecule and is then allowed to hybridise *in situ* with the target sequence thus elucidating the spatial location of its expression in a tissue sample. ISH can be carried out with isotopically or nonisotopically labelled probes, however ISH protocols involving nonisotopic labelling techniques have a number of advantages over isotopic methods. These include improved histological resolution, improved safety and reduced need for specialised facilities, shorter exposure times, greater probe stability and reusability of probes.

A range of different non-radioactive probe labelling methods are available which can be combined with direct or indirect methods of detection of the reporter molecule (Wilkinson, 1998). The procedures described in this chapter were carried out using an indirect method of detection for digoxigenin (DIG) labelled RNA probes. RNA probe synthesis for ISH is generally carried out by *in vitro* transcription from a DNA template in the presence of RNA nucleotides, one of which is conjugated with the hapten molecule. The DNA template for *in vitro* transcription can be amplified by PCR (Gandrillon *et al.*, 1996; David and Wedlich, 2001). On the other hand, probes can be generated from a cDNA fragment cloned into a vector containing RNA polymerase initiation sites such as T7 and SP6, located upstream and downstream of the poly-cloning site (Fig. 2.2; Wilkinson, 1998). In order to generate an RNA transcript that is complementary to and therefore binds to the target mRNA sequence, (i.e. the antisense probe, the plasmid is linearised with a restriction enzyme possessing a cut site), which is located 5' of the cDNA insert and *in vitro* transcription is carried out by an RNA polymerase with an initiation site located 3' of the insert. Control probes for ISH, i.e. sense probes, are synthesised by *in vitro* transcription using an RNA polymerase with an initiation site 5' of the insert. The resulting RNA transcript will be identical to the target mRNA sequence and therefore should not hybridise to it.

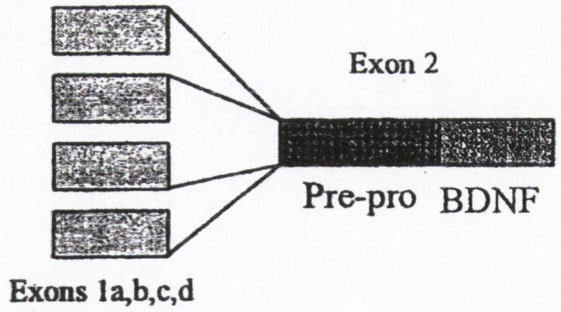


Figure 2.1. The exon/intron organisation of the brain derived neurotrophic factor (BDNF) gene. The boxes represent exons and the lines introns that will be removed by alternative splicing. Mature BDNF transcripts have two exons, the first is one of the four upstream non-coding exons (exons 1a, b, c or d; pale grey boxes) and the second is always the BDNF-encoding major exon (exon 2) which is made up of regions encoding for the pre-pro (black box) and mature (dark grey box) form of the protein. This diagram is adapted from Heinrich and Lum (2000).

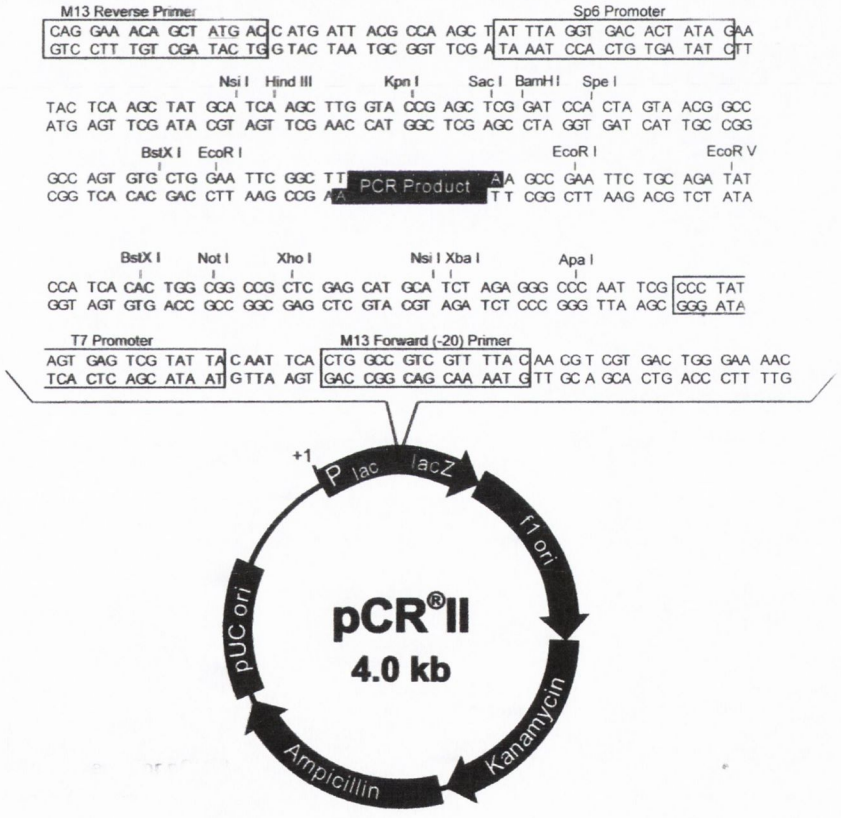


Figure 2.2. Plasmid map of pCR II vector (Invitrogen) used for cloning brain derived neurotrophic factor and tyrosine hydroxylase cDNA fragments. The map shows T7 and SP6 RNA polymerase initiation sites (boxed regions labelled SP6 and T7 promoter) and the poly-cloning site (region marked PCR product). Restriction enzyme (e.g. BamH I, EcoR V) cut sites are also shown.

Bioinformatics analysis of the DNA template sequence for probe generation should be carried out to search for regions of homology with other gene sequences. Some polypeptide families, such as the neurotrophins, possess regions of protein sequence that are highly conserved between members (Heinrich and Lum, 2000). For this reason, ideally ISH should not be performed with a probe for the gene of interest that has a high level of similarity (> approximately 80%) with another gene sequence (Rattray and Michael, 1998). Furthermore, the use of species-specific probes in ISH is preferable. Problems can be encountered when using cross-species probes since base mismatch decreases the stability of probe-target hybrids and may require a decrease in the stringency of the hybridisations conditions. This in turn may lead to non-specific binding of the probe with closely related sequences in other genes (Wilkinson, 1998).

In addition to probe design and detection considerations, owing to inherent tissue differences such as fat content, cell composition and developmental stage, the numerous steps in the ISH protocol may also require optimisation when developing an ISH protocol for a particular model organism (Breitschopf *et al.*, 1992; Strähle *et al.*, 1994; Swain *et al.*, 1994; Kanazir *et al.*, 1997; Braissant and Wahli, 1998; Xu and Wilkinson, 1998; Shifman and Selzer, 2000; Plenz *et al.*, 2002; Vanholme *et al.*, 2002). For example, the optimal fixation time for a particular tissue is important to determine since the greater the cross-linking, the less accessible the target, but insufficient fixation may lead to RNA degradation and/or poor histological quality of tissue (Guiot and Rahier, 1995; Baumgart *et al.*, 1997; Jin and Lloyd, 1997; Brown, 1998; Fernandez-Santos and Martin-Lacave, 2000). In order to aid penetration of the probe into the tissue, proteinase K treatment and optimisation of digestion time are regarded as essential in some ISH protocols (Guiot and Rahier; 1995; Baumgart *et al.*, 1997; Xu and Wilkinson, 1998; Fernandez-Santos and Martin-Lacave, 2000). On the other hand, proteinase K treatment has been deemed unnecessary by others (Swain *et al.*, 1994; Shifman and Selzer, 2000). The necessity for proteinase K treatment of a particular tissue should also be determined therefore and digestion time optimised if it is deemed necessary (Xu and Wilkinson, 1998). Furthermore, for the hybridisation step, it is critical to provide the optimal conditions for annealing of the probe to the target nucleic acid whilst preventing non-specific binding of the probe to other RNA species present in the tissue. Factors that affect the stringency of the hybridisation include the temperature at which the reaction is performed and the composition of the hybridisation solution (e.g. monovalent cation concentration, presence or absence of formamide and type of blocking agents used) in which the probe is applied

(Brown, 1998; Roche Applied Science DIG application manual for nonradioactive *in situ* hybridization, 2002).

2.1.3 In situ hybridisation to wholemounted and sectioned tissue preparations

ISH performed with non-radioactively labelled probes can be carried out on wholemounted tissue preparations. The advantages of wholemount *in situ* hybridisation (WISH) over ISH on sectioned tissue are that WISH can be carried out on many wholemounted samples at once and gives a direct visualisation of the spatial pattern of mRNA expression in three dimensions. As a result, a complete picture of gene expression can be obtained without the need to prepare, hybridise and process a large number of sections. The success of WISH can be affected by the complexity and size of the tissue being examined since penetration of probes and reagents is hindered in larger tissues. This technique is mainly restricted therefore to the study of patterns of gene expression in the developing embryos (e.g. Giordano *et al.*, 1997; Hashimoto and Heinrich, 1997; Murphy and Kolstø, 2000; Lum *et al.*, 2001; Schön *et al.*, 2006). However, a protocol for WISH has been developed for the adult lamprey brain (Swain *et al.*, 1994) and developmental and regeneration-related increases in the mRNA expression pattern of the neurofilament protein NF-180 have been described using this technique (Swain *et al.*, 1994; Jacobs *et al.*, 1996; Jacobs *et al.*, 1997). Owing to the advantages of WISH over ISH on sectioned tissue, and since WISH had been successfully carried out in the brain of another adult fish (Swain *et al.*, 1994), ISH was attempted in the wholemounted eel brain. Furthermore, facilities for carrying out optical projection tomography (OPT) were available in our laboratory. This procedure aids in gene expression analysis since it can be used to create a computer generated 3D reconstruction of a tissue after WISH showing the distribution of the gene expression pattern of interest (Sharpe *et al.*, 2004). The reconstruction can be rotated through 360° and virtual sections may also be created in any plane (Sharpe *et al.*, 2004).

Protocols for ISH to slide-mounted cryostat sections (25-30 μm) and free-floating vibrating microtome sections (100 μm) of eel brain are also described in this chapter. ISH to sections allows the examination of nucleotide expression patterns in large embryos and more complex adult tissues. ISH to cryostat sections allows excellent histological resolution but may yield a weaker signal than wholemounted tissue after ISH since less RNA is available to the probe in thinly sectioned material (Xu and Wilkinson, 1998). Although histological resolution is compromised slightly in thicker sections, ISH to free-floating vibrating microtome sections provides a more sensitive method than ISH to slide-mounted cryostat sections since more RNA is available to the probe and since reagents are free to penetrate the tissue from both sides.

2.1.4 Aims of chapter 2

In this chapter, the cloning and characterisation of a 467 bp cDNA fragment for *A. anguilla* BDNF is described and the BDNF sequence is compared with that of other vertebrate cDNA sequences for BDNF and related neurotrophin family members. The optimisation of ISH protocols suitable for use with eel CNS tissue is also detailed.

2.2 Materials and methods

2.2.1 Animals

This study is based on European eels, *Anguilla anguilla* L. (Total n=24) of unknown sex obtained from Co. Mayo in a non-migratory, immature stage of the life cycle. Animals ranged in length from 300 to 495 mm. They were kept in large aquaria with recirculating water at 25°C.

Experimental eels were anaesthetized in neutralised tricaine methane sulfonate (MS 222; 0.4 g/l, pH 7.4, Sigma, UK). During surgery, their bodies were packed with ice and ice-cold water was pumped over their gills. The spinal cord, at the level of 13 segments caudal to the anus, was carefully exposed by removing a small dorsal part of three vertebrae and then cut with microscissors producing a gap of approximately 1 mm between rostral and caudal stumps. The wound was stitched closed and on recovery from anaesthetic, eels were returned to individual holding tanks for a 10 day recovery period.

For RNA extraction, eels were anaesthetised with MS 222 and alphaxalone (0.4 ml of 9 mg/ml solution, sc; "Saffan", Schering-Plough Animal Health Ireland). Fresh brain tissue was then dissected out, snap frozen in liquid nitrogen and stored at –80 °C.

For *in situ* hybridisation, 10 days after surgery, eels were anaesthetised with MS 222 and alphaxalone, and were injected with heparin (0.2 ml of 5000 units/ml solution, sc, Leo Laboratories, Ireland). Eels were then perfused through the heart with phosphate buffered saline (PBS; 0.1 M, pH 7.4) containing procaine (1g/l, Sigma, UK) followed by a fixative solution of 4% paraformaldehyde (PFA; Sigma, UK) in PBS. The brain was dissected out and postfixed in 4% PFA/PBS for 24 hours. Tissue from uninjured, control animals was prepared in the same fashion. After the brain had been removed postmortem from all cord-transected animals, cord regeneration was confirmed by examining bridge formation in the spinal cord.

For WISH, a total of 10 animals were used. A total 9 animals were used of for ISH to cryostat sections. ISH to vibrating microtome sections was optimised on a total of 3 animals.

2.2.2 Selection of a positive control probe for in situ hybridisation- Cloning a 770 bp tyrosine hydroxylase cDNA fragment

Since the expression pattern of BDNF had not been described in the fish brain in detail, and also due to the fact that WISH had not been attempted in the eel before, a probe for tyrosine hydroxylase (TH) mRNA was chosen for use as a positive control to ensure the process of ISH was operating successfully. Its mRNA expression pattern in sectioned eel brain had already been briefly described in Boularand *et al.* (1998). The protein

expression pattern of dopamine has also been described in detail in the eel brain (Roberts *et al.,* 1989). TH is an enzyme which is involved in dopamine synthesis and its mRNA expression pattern on average corresponds with that of dopamine (Boularand *et al.*, 1998).

A 770 bp cDNA fragment was amplified from total eel brain RNA prepared by Dr S. M. Borich, using the QIAGEN OneStep RT-PCR kit according to the manufacturer's instructions. Primer sequences for *A. anguilla* TH taken from Boularand *et al.* (1998) were as follows: forward: 5'-ATGCCTATTTCCAACTCTTCCG- 3' and reverse: 5'-GGGCTGTATCCACAATGTCTCT- 3' and the primer annealing temperature was 58°C. The 770 bp cDNA fragment was cloned into the pCR®II vector using the TA Cloning Dual Promoter Kit (Invitrogen) according to the manufacturer's instructions. The insert was then sequenced on both DNA strands in order to confirm its identity and orientation in the vector.

2.2.3 Cloning of a 104 bp BDNF fragment, establishment of neurotrophin sequence database and bioinformatics analysis

A 120 bp cDNA fragment containing 104 bp of BDNF sequence and 16 bp of sequence coding for restriction enzyme sites, amplified and cloned by Dr Suzanne Borich from total RNA isolated from the eel, was selected for use as a template for RNA probe synthesis for ISH. A 700 bp RNA probe containing this sequence region had previously been used successfully by Hashimoto and Heinrich (1997) to examine BDNF expression in the developing zebrafish using ISH.

The fragment was amplified and subcloned into the pCR®II vector (Invitrogen) as described below. First, the 120 bp cDNA fragment was amplified from plasmid DNA containing the insert prepared by Dr S. M. Borich, using degenerate primers taken from Hashimoto and Heinrich (1997). Primer sequences were as follows: BDNF-3 (forward): 5'-CT**GGATCC**CA*R*TA*Y*TTYT*WY*GA*R*AC-3' and BDNF-4 (reverse): 5'-CA**GAATTC**GACTGGGTAGTTCGGCA-3', where *R* = G and A, *Y* = C and T, *W* = A and T. Note forward and reverse primer sequences included flanking *Bam*H I and *Eco*R I restriction enzyme sites respectively (in bold) and thus added 8 bp to the 5' and 3' ends of the putative eel BDNF cDNA fragment. The primer annealing temperature was 48°C and other PCR cycling conditions were as described in Hashimoto and Heinrich (1997). The 120 bp PCR product was then cloned using the TA Cloning Dual Promoter Kit (Invitrogen) and the insert sequenced on both DNA strands in order to confirm its identity and orientation in the vector.

Bioinformatics analysis was carried out on the 104 bp eel BDNF sequence to search for homology with other neurotrophins. First, a computer based nucleotide and

protein sequence database was created using MacVector™ software (Accelrys), for the neurotrophins BDNF, NGF, NT-3, NT-4/5, NT-6 and NT-7 from a variety of species of fish, birds, amphibians and mammals. Sequences were retrieved from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nih.gov/) GenBank and GenPept databases using the *Entrez* program by typing the neurotrophin name or simply "neurotrophin" and a species name into the *Entrez* search window. Some sequences were also retrieved by carrying out BLASTN (Altschul *et al.,* 1997) homology searches of the NCBI database with the sequence of a particular neurotrophin, e.g. the cDNA sequence for zebrafish BDNF, GenBank accession number NM_131595. In addition, sequences were obtained from Hallböök *et al.* (1991; salmon BDNF and NT-3) and Dethleffsen *et al.,* (2003; fugu and tetraodon NGF, NT-6/7 and NT-4/5 and fugu NT-3).

Nucleotide ClustalW alignments (Higgins *et al.,* 1994) were then carried out with the proposed eel BDNF probe template sequence and other neurotrophin sequences i.e. NGF, NT-3, NT-4/5, NT-6 and NT-7 from other species. It was noted that BDNF-4 (Hashimoto and Heinrich, 1997), the reverse primer used to amplify the BDNF insert, spanned a BDNF specific region of the neurotrophin alignment. A BLASTN search of the NCBI GenBank database was also carried out with the 104 bp cloned sequence to search for homology with other neurotrophin family members and other non-related sequences (Altschul *et al.,* 1997).

2.2.4 BDNF primer design to amplify a 467 bp BDNF cDNA fragment

Degenerate primers were designed using MacVector™ software (Accelrys), based on conserved coding regions of available BDNF cDNA sequences from four teleost species; carp, platyfish, zebrafish and flounder (GenBank accession numbers L27171, X59942, NM_131595 and AY074888 respectively). Resulting degenerate primer sequences were as follows: forward: 5'-CTC*R*CGGGTGATGATCAGCAACCA-3' and reverse: 5'-T*S*CC*Y*CT*Y*TTAATGGTCAATGTGCATA-3', where *S* = C and G, *R* = G and A, *Y* = C and T. BLASTN searches (Altschul *et al.,* 1997) of the NCBI database with primer sequences were carried out to ensure that they were BDNF specific. The optimal annealing temperature for the primers was determined by carrying out PCR reactions on a gradient block (ThermoHybaid) with temperatures ranging from 48 to 62°C at the annealing step during PCR cycling.

2.2.5 Cloning a 467bp BDNF cDNA fragment

Total RNA was extracted from the brain of one cord-transected animal 10 days after surgery using Tri reagent (MRC) and DNase treated using RQ1 RNase free DNase (Promega) according to the manufacturer's instructions in each case. A two-step RT-PCR

approach was employed to amplify a 467 bp cDNA fragment. In the presence of oligo $(dT)_{12-18}$ primers (Invitrogen, UK), single-stranded complementary DNA (sscDNA) was synthesized from 1 μg of total RNA using PowerScript™ Reverse Transcriptase (BD Biosciences, UK) according to the manufacturer's instructions.

PCR was carried out using 1% of the sscDNA template, 0.6 μM of each BDNF primer, 2 units *Taq* DNA polymerase (New England Biolabs, Inc) and 0.2 mM dNTPs (Invitrogen, UK). Cycling conditions were as follows: 95°C for 1 min followed by 35 cycles of 95°C for 30 s, 62°C for 40s, 72°C for 1 min. PCR products were cloned using the TA Cloning Dual Promoter Kit (Invitrogen, UK) and the insert sequenced on both DNA strands. The corresponding amino acid sequence was predicted using sequence analysis software, MacVector™, (Accelrys). In order to confirm the identity of the cloned putative eel BDNF sequence, BLASTN searches of the NCBI database (Altschul *et al.*, 1997) were carried out. ClustalW alignments (Higgins *et al.*, 1994) with the eel BDNF cDNA sequence, excluding degenerate regions amplified by the primers, were also carried out with BDNF and other neurotrophin family member sequences from the neurotrophin database already established (see section 2.2.3). The corresponding amino acid sequence was predicted using MacVector™ software, (Accelrys). ClustalW alignments (Higgins *et al.*, 1994) with the predicted amino acid sequence for eel BDNF, excluding degenerate regions amplified by the primers, were then performed with BDNF and other neurotrophin family member sequences. The BDNF cDNA sequence was submitted to the NCBI GenBank database.

2.2.6 Antisense and sense RNA probe synthesis

For all RNA work, standard precautions were taken to ensure reagents, consumables and equipment were free from RNases (Sambrook and Russell, 2001).

Plasmid DNA, containing BDNF and TH inserts, was linearised with appropriate restriction enzymes (i.e. for BDNF 104 bp cDNA sequence: *Hind* III and *Xho*I; for BDNF 467 bp sequence and TH: *EcoR* V and *Bam*H I;). Antisense and sense digoxigenin-labelled RNA probes were transcribed, according to insert orientation, from 1 μg of linearised plasmid from SP6 and T7 promoter sites in the pCR®II vector. DNA template was degraded by incubation of probes with RNase free DNase (Roche, Germany). The probes were then purified on G25 columns (Amersham Biosciences) according to the manufacturer's instructions. Probe concentrations were determined by spectrophotometry and probes were then stored at – 20°C.

2.2.7 Wholemount in situ hybridisation (WISH)

After postfixation, brains were either left intact or cut in half down the midline in order to aid probe penetration (Xu and Wilkinson, 1998). The forebrain area rostral to the tegmentum was removed from brains to be probed for BDNF. Tissue samples were dehydrated through a series of methanol/PBT (PBT: 0.1% Triton X-100 in PBS; 1 X 10 min 25, 50, 75%) washes, followed by 2 X 10 min 100% methanol washes. Tissue was then stored in 100% methanol at -20°C until needed.

For WISH, tissue was rehydrated through a series of methanol/PBT (75, 50, 25%; 1 X 5 min) washes at 4°C. The sections were treated with proteinase K (Sigma, UK; 10 μg/ml in PBT) for 30 min at room temperature. Tissue was then washed twice in PBT and fixed for 45 min in 4% PFA/PBS followed by 3 X 5 min PBT washes.

Tissue was equilibrated for 15 min at room temperature in hybridisation solution consisting of 2% blocking reagent (Roche, Germany), 50% deionised formamide, 5 X SSC, 50 μg/ml Heparin, 100 μg/ml yeast tRNA, 0.1% Triton X-100 and 5 mM EDTA (Sigma, UK). Tissue was then prehybridised for 2 hours at 55°C in fresh hybridisation solution. The BDNF probe for WISH was synthesised from plasmid DNA containing the 120 bp insert. Antisense and sense TH and BDNF probes were denatured at 80°C for 3 min and then diluted to a concentration of 1 ng/μl in hybridisation solution. Prehybridisation solution was replaced with hybridisation solution containing diluted probe and sections were hybridised at 55°C overnight. All prehybridisation, hybridisation and posthybridisation washes were carried out in a waterbath.

Posthybridisation washes were then carried out at 55°C in the following order:

- 1 X 10 min in 75% wash 1(50% formamide, 5 X SSC, 0.5% CHAPS)/25% wash 2 (2 X SSC, 0.1% CHAPS)
- 1 X 10 min in 50% wash 1/50% wash 2
- 1 X 10 min in 25% wash 1/75% wash 2
- 2 X 30 min in 100 % wash 2
- 2 X 30 min in 0.2 X SSC, 0.1% CHAPS.

Posthybridisation washes were followed by 2 X 10 min in TNT (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Triton X-100) at room temperature.

Tissue was pre-blocked at 4°C overnight in 2% BSA, 15% goat serum in TNT, with rocking. Brains were then incubated overnight in fresh blocking solution containing a 1:2000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche, Germany), at 4°C with rocking. Postantibody washes were carried out at room

temperature and were as follows 2 X 5 min in 0.1% BSA/TNT, 4 X 1 hour in 0.1% BSA/TNT. Washing continued overnight at 4°C in 0.1% BSA/TNT.

On the day the signal was developed, tissue samples were washed in 3 changes of NTMT (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min each. The chromogenic reaction was carried out in NTMT containing 338 µg/ml 4-nitro blue tetrazolium chloride (NBT; Roche, Germany) and 350 µg/ml 5-bromo-4-chloro-3-indolyl-phophate (BCIP; Roche, Germany). Tissue was developed in the dark at room temperature with rocking for approximately 2 hours. The signal was fixed in 4% PFA/PBS for 2 hours. Tissue samples were cleared in 50% followed by 80% glycerol/PBT solutions until they sank.

2.2.8 Optimisation of protocol for in situ hybridisation to cryostat sections

Three methods for ISH were followed in order to identify a suitable protocol for ISH to cryostat sections in our laboratory. For method I, the protocol for ISH with hapten labeled probes to tissue sections on slides was followed from Xu and Wilkinson (1998) using a modification of the hybridisation solution described for mouse, chick or *Xenopus* embryos. Method II consisted of a protocol described for cryostat sections of the eel brain by Kapsimali *et al.* (2000). For method III, "protocol 4" for WISH from Xu and Wilkinson (1998), was adapted for cryostat sections and is detailed in section 2.2.9. Important differences between methods I-III were hybridisation solution compositions and the variation in blocking procedures prior to probe detection, and are compared in Table 2.1.

Table 2.1. Comparison of hybridisation solution compositions and antibody blocking procedures in methods I, II and III for ISH to cryostat sections.

Hybridisation solutions *		
Method I	**Method II §**	**Method III**
2% blocking reagent (Roche, Germany)	N/P	2% blocking reagent (Roche, Germany)
100 μg/ml Yeast tRNA	1 mg/ml Yeast tRNA	1 μg/ml Yeast tRNA
50 μg/ml Heparin	N/P	500 μg/ml Heparin
N/P	1 X Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 10 mg/ml bovine serum albumin)	N/P
0.05% CHAPS	N/P	0.5% CHAPS
0.1% Triton X 100	N/P	0.1% Tween 20
N/P	10 % dextran sulphate	N/P
Antibody pre-block and incubation conditions		
Method I	**Method II**	**Method III**
Antibody pre-block: 1 X 30 min in 5% serum in PBT	Antibody pre-block: 1 X 60 min in 20% serum/2% blocking reagent/0.1 M maleic acid/0.15 M NaCl/0.1% Tween 20 (Sigma)	Antibody pre-block: 1 X 60 min in 0.1 M maleic acid/0.15 M NaCl (Sigma)/3% blocking reagent (Roche, Germany)
Antibody incubation solution: as above	Antibody incubation solution: 0.1 M maleic acid/0.15 M NaCl/0.1% Tween 20	Antibody incubation solution: as above

N/P = not present

* All hybridisation solutions contained 50% formamide, 5 mM EDTA and 5 X SSC.

§ The hybridisation solution components for method II were diluted in a solution of 0.006 M phosphate buffer, 0.2 M NaCl, 0.01 M Tris (pH 7.5).

Signals for ISH were not attained using methods I or II despite attempts at protocol optimisation by variation of proteinase K treatment (method I only) and tissue fixation times, which are important factors in the success of ISH (Guiot and Rahier, 1995; Baumgart *et al.*, 1997; Xu and Wilkinson, 1998; Fernandez-Santos and Martin-Lacave, 2000). Clear signals were achieved however using method III, the protocol for which is described below in section 2.2.9.

2.2.9 In situ hybridisation- cryostat sections (method III)

Optimising experiments were carried out on cryostat sections with BDNF probes synthesised from plasmid DNA containing the 120 bp insert and the 467 bp insert.

After postfixation, brains were equilibrated in 30% sucrose/PBS overnight at 4°C and frozen in optimal cutting temperature compound (BDH) on Dry Ice the following day. Serial horizontal sections (25-30 μm) were cut on a cryostat (Bright), mounted on Superfrost plus slides (BDH) and stored at -80°C. Whilst cutting in various regions of the

brain, consecutive sections were placed on two alternate slides so that one slide could serve as a sense control.

On the first day of ISH, slides were allowed to come to room temperature. After 2 X 10 min washes in PBS, the sections were fixed for 20 min in 4 % PFA/PBS. After 2 X 5 min washes in PBS, the sections were treated with proteinase K (Sigma, UK; 10 μg/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA) for 5 min at room temperature. After proteinase K treatment, sections were washed twice in PBS and fixed for 20 min in 4% PFA/PBS. Fixation was followed by 2 X 5 min washes in PBS at RT and then sections were dehydrated through a series of ethanol (ETOH)/0.85% NaCl washes as follows: 1 X 5 min 0.85% NaCl, then 1 X 2 min 30% and 50%, 1 X 5 min 70% and 1 X 2 min 90% ETOH/0.85% NaCl, and lastly 2 X 2 min 100% ETOH. Antisense and sense TH and BDNF probes were denatured at 80°C for 3 min before use. Hybridisation was carried out in 100 μl hybridisation solution and containing 100-200 ng of probe in 2% blocking reagent (Roche, Germany), 50% formamide, 5 X SSC, 0.5% CHAPS, 500 μg/ml Heparin, 1 μg/ml Yeast tRNA, 0.1% Tween 20 and 5 mM EDTA (Sigma, UK). Slides then were coverslipped, placed in a humid chamber containing 50% formamide/5 X SSC and incubated overnight in an oven at 55 °C.

After hybridisation, coverslips were removed in 50% formamide/5 X SSC in a waterbath at 65°C. Posthybridisation washes were then carried out in the following order:

- 2 X 30 min in 50% formamide/2 X SSC at 65°C
- 3 X 10 min in 2 X SSC at 65°C
- 3 X 10 min in 0.2 X SSC at room temperature.

Posthybridisation washes were followed by 2 X 10 min in KTBT (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween 20) at room temperature.

Sections were preblocked with blocking buffer (0.1 M maleic acid, 0.15 M NaCl (Sigma), 3% blocking reagent (Roche, Germany)) for 1 hour at room temperature. The sections were then incubated overnight in fresh blocking buffer containing a 1:2000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche, Germany) in a humid chamber containing KTBT at 4°C.

Postantibody washes were as follows 2 X 5 min PBS/0.1% Tween 20 and 3 X 20 min PBS/0.1% Tween 20. Before staining sections were washed NTMT (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 3 X 5 min. The chromogenic reaction was carried out in NTMT containing 270 μg/ml NBT and 125 μg/ml BCIP. Slides were

developed in the dark at room temperature for approximately 6 hours. The signal was fixed in 4% PFA/PBS for 30 min.

2.2.10 Counterstaining cryostat sections

Cryostat sections were counterstained with a 0.1% nuclear fast red (BDH)/ 5% aluminium sulphate solution (Roubert *et al.*, 2001). Before use, the staining solution was boiled for approximately 15 min, cooled and then filtered. Sections were stained for approximately 15 s, washed in deionised water and mounted with Aquapolymount (Polysciences, Inc).

2.2.11 In situ hybridisation- vibrating microtome sections

ISH was carried out with vibrating microtome sections (100 μm) in order to improve the sensitivity of the protocol for the 467 nucleotide (nt) BDNF probe. After postfixation, brains were mounted in 5% agar/PBS (similarly to Strähle *et al.*, 1994) and 100 μm sections cut in the horizontal plane with a vibrating microtome (VT1000S, Leica). Sections were dehydrated through a series of methanol/PBT (25, 50, 75%; 1 X 5 min) washes, followed by 2 X 5 min 100% methanol washes and stored at -20°C in 6-well plates in 100% methanol until needed.

On the first day of ISH, sections were rehydrated through a series of methanol/PBT (75, 50, 25%; 1 X 5 min) washes at 4°C. After 2 X 10 min washes in PBT, the sections were treated with proteinase K as described in section 2.2.9, Method III for cryostat sections. Following proteinase K treatment, sections were washed twice in PBT and fixed for 20 min in 0.2% glutaraldehyde/4% PFA in PBT. Fixation was followed by 3 X 5 min washes in PBT at room temperature. Sections were then transferred to 7 ml, flat-bottomed Bijou tubes and washed for 30 min in PBT at 55°C. Sections were prehybridised at 55°C for 15 min in hybridisation solution (composition identical to that described in section 2.2.9, Method III for cryostat sections). Sections were then left to prehybridise overnight at 55°C in fresh hybridisation solution.

Antisense and sense BDNF and TH probes were denatured at 80°C for 3 min and then diluted to a concentration of 2 ng/μl in hybridisation solution. Prehybridisation solution was replaced with hybridisation solution containing diluted probe and sections were left at 55°C overnight. Posthybridisation washes were carried out at 60°C as follows:

- 2 X 10 min in 2 X SSC
- 3 X 20 min in 2 X SSC/0.1% CHAPS
- 3 X 20 min in 0.2 X SSC/0.1% CHAPS.

All prehybridisation, hybridisation and posthybridisation washes were carried out in a waterbath.

Sections were then washed for 2 X 10 min in KTBT at room temperature and then preblocked in blocking buffer (0.1 M maleic acid, 0.15 M NaCl (Sigma), 3% blocking reagent (Roche, Germany)) for 3 hours at 4°C. Sections were incubated overnight in fresh blocking buffer containing a 1:1000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche, Germany) at 4°C with rocking.

Postantibody washes consisted of 5 X 1 hour washes at room temperature in KTBT. Sections were then left overnight in KTBT at 4°C with rocking. On the day the signal was developed, sections were washed in 3 changes of NTMT for 15 min each. The chromogenic reaction was carried out in NTMT containing 175 μg/ml NBT and 62.5 μg/ml BCIP. Sections were developed in the dark at RT with rocking for 6-8 hours. The signal was fixed in 4% PFA/PBS for 1 hour. Vibrating microtome sections did not require counterstaining since their thickness provided adequate contrast for identification of brain structures and regions. Sections were mounted with Aquapolymount (Polysciences, Inc).

2.3 Results

2.3.1 Cloning 770 bp TH and 104 bp BDNF cDNA fragments

cDNA fragments of 770 bp for TH and 104 bp for BDNF with 8 bp flanking regions for restriction enzyme cut sites added by PCR primers (see Fig. 2.2 for BDNF primer binding sites) were amplified (data not shown) and cloned successfully into the pCR®II vector (Invitrogen). Insert identities and orientations were confirmed by sequencing (data not shown).

2.3.2 Bioinformatics analysis- 104 nt BDNF probe

The percentage identity of the BDNF 104 bp cDNA sequence with nucleotide sequences for other neurotrophins was not greater than 62% therefore the probe was unlikely to cross hybridise with other neurotrophin family members (Rattray and Michael, 1998; Wilkinson, 1998; Hasegawa *et al.,* 2005). Furthermore, all hits returned after a BLASTN search (Altshcul *et al.,* 1997) of the NCBI GenBank database with the 104 bp cDNA template sequence were for BDNF orthologues from other species (data not shown). On the basis of this bioinformatics analysis, the probe was deemed suitable for ISH according to the criteria described by Rattray and Michael (1998) and Wilkinson (1998).

2.3.3 Partial 467 bp cDNA sequence for A. anguilla BDNF, GenBank accession number AY762996

The optimal annealing temperature at which the BDNF-specific degenerate primers described in section 2.2.4. produced a band of the correct size, free from non-specific PCR products, was determined to be 62°C. Using these primers, a partial 467 bp cDNA fragment was successfully amplified, cloned and sequenced (Fig. 2.3). In pairwise ClustalW alignments (Higgins *et al.,* 1994) with cDNA sequences for coding regions of BDNF from other teleost species, the cloned cDNA sequence showed 87% identity with carp and 88% identity with flounder, platyfish and zebrafish, GenBank accession numbers L27171, AY074888, X59942, NM_131595 respectively (alignments not shown). Pairwise alignments (data not shown) were carried out with the predicted amino acid sequence for the cDNA fragment and amino acid sequences coding for BDNF orthologues (see Table 2.2 for percentage identities and similarities with conservative substitutions). A multiple alignment of the predicted amino acid sequence with other BDNF amino acid sequences revealed that the cloned fragment spans part of the propeptide and also the mature region of the BDNF protein (Fig. 2.4). Pairwise alignments (data not shown) were also conducted between the predicted amino acid sequence for the cDNA fragment and other neurotrophins from various species (see Table 2.3). In a multiple alignment between the predicted amino acid sequence and other neurotrophin amino acid sequences, it was

observed that the cloned fragment encompasses the five variable regions that exist between the mature forms of the peptide sequences for the different neurotrophin family members (Fig. 2.5).

Table 2.2. Percentage identities and similarities with conserved substitutions determined from pairwise alignments of the predicted amino acid sequence (NCBI GenPept accession number AAV31512) for the cloned *A. anguilla* BDNF 467 bp cDNA fragment with BDNF amino acid sequences from other species.

Species	**NCBI GenPept accession number/sequence source**	**Identities**	**Similarities §**
Flounder	AAL71888	97%	100%
Platyfish	CAA42567	97%	99%
Salmon	Hallböök *et al.* (1991)	96%	100%
Tetraodon	CAF92094	96%	99%
Zebrafish	NP_571670	95%	99%
Carp	AAA49204	95%	99%
Ray	P25430	79%	98%
Xenopus	P25432	86%	96%
Chicken	AAC42220	88%	97%
Rat	AAH87634	91%	96%
Pig	CAA34685	91%	97%
Human	AAT74399	91%	97%

§ Sequence similarities with conservative substitutions

Table 2.3. Percentage identities and similarities with conserved substitutions determined from pairwise alignments of the predicted amino acid sequence (NCBI GenPept accession number AAV31512) for the cloned *A. anguilla* BDNF 467 bp cDNA fragment with amino acid sequences for NGF, NT-3, NT-6/7 and NT-4/5 from other species.

Species and gene	NCBI GenPept accession number/sequence source	Identities	Similarities §
Eel NGF	AAO72445	49%	67%
Carp NGF	AAB63508	48%	64%
Platyfish NGF	CAA42566	44%	63%
Zebrafish NGF	AAO31814	43%	60%
Salmon NGF	AA072444	50%	61%
Fugu NGF	Dethleffsen *et al.* (2003)	49%	65%
Tetraodon NGF	Dethleffsen *et al.* (2003)	42%	56%
Xenopus NGF	CAA39249	49%	67%
Rat NGF	P25427	46%	66%
Human NGF	AAH32517	48%	64%
Fugu NT-3	Dethleffsen *et al.* (2003)	58%	69%
Zebrafish NT-3	AAH92731	46%	70%
Salmon NT-3	Hallböök *et al.* (1991)	55%	71%
Carp NT-3	AAB63510	55%	69%
Ray NT-3	P25434	49%	79%
Xenopus NT-3	AAB17723	52%	72%
Rat NT-3	NP_112335	53%	75%
Human NT-3	AAA63231	53%	75%
Carp NT-6/7 *	AAC25632	47%	60%
Swordtail NT-6/7	AA61922	47%	63%
Platyfish NT-6/7	AA61923	47%	63%
Zebrafish NT-6/7	AAO31821	46%	65%
Salmon NT-6/7	AAO72443	41%	61%
Fugu NT-6/7	Dethleffsen *et al.* (2003)	40%	51%
Tetraodon NT-6/7	Dethleffsen *et al.* (2003)	40%	50%
Sculpin NT-6/7	AAQ63407	31%	51%
Bullhead NT-6/7	AAQ63408	31%	46%
Tetraodon NT-4/5	Dethleffsen *et al.* (2003)	43%	61%
Fugu NT-4/5	Dethleffsen *et al.* (2003)	42%	57%
Xenopus NT-4/5	CAA82906	55%	75%
Rat NT-4/5	AAA41728	53%	73%
Human NT-4/5	AAV38176	52%	74%

§ Sequence similarities with conservative substitutions

* NT-6 and NT-7 were originally considered to be different genes but have been shown to have evolved from a single ancestral gene. The term NT-6/7 is used in Table 2.3 to refer to NT-6 or NT-7 as suggested by Dethleffsen *et al.* (2003).

The cloned insert was deemed to be a partial cDNA fragment coding for *A. anguilla* BDNF. This conclusion was based on the high level of nucleotide (87-88% with other fish) and protein (≥79% for all species examined, see Table 2.2 above) identity of the cloned sequence with other BDNF sequences from various species and the lower identity (≤58%, see Table 2.3) with other neurotrophins. Furthermore, all results retrieved by a BLASTN search (Altshcul *et al.,* 1997) of the NCBI GenBank database with the 467 bp cDNA sequence were BDNF sequences from other species (data not shown). Out of 531 hits retrieved by a BLASTP search (Altshcul *et al.,* 1997) of the NCBI GenPept database with the predicted amino acid sequence for the cloned fragment, the first 210 amino acid sequences coded for BDNF from other species. On submission to the NCBI GenBank database, the partial eel BDNF cDNA sequence was assigned the accession number AY762996. The predicted amino acid sequence was assigned the GenPept accession number AAV31512.

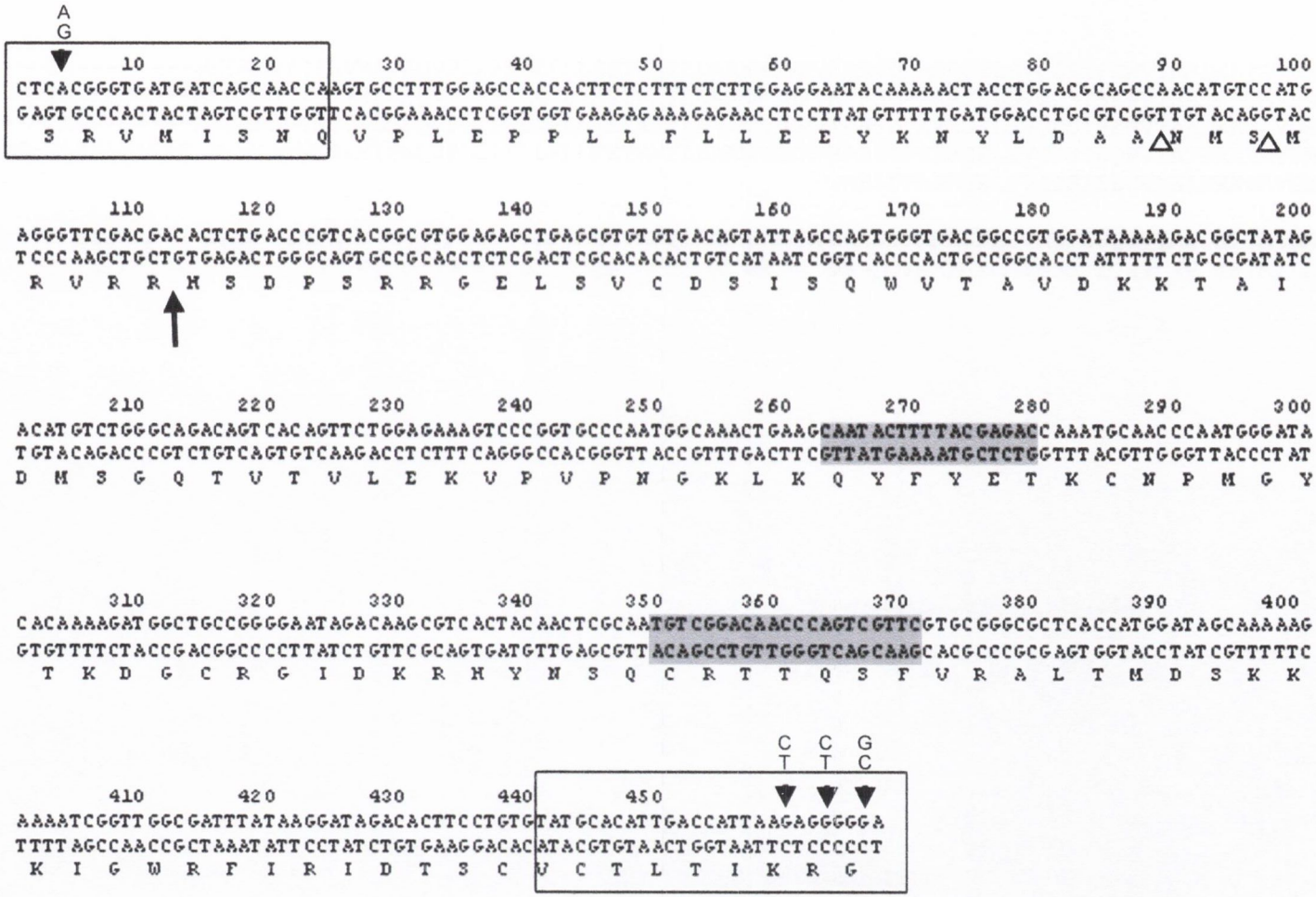


Figure 2.3. Partial 467 bp cDNA sequence for *Anguilla anguilla* BDNF (GenBank accession number AY762996) with predicted amino acid sequence (GenPept accession number AAV31512). The binding positions of the forward and reverse degenerate primers designed to amplify the 467 bp BDNF fragment are indicated by the open boxes. Positons of degenerate bases are marked by the closed arrowheads. Degenerate nucleotide choices are included at the relevant positions. The open arrowheads indicate the position of a consensus sequence for an N-glycosylation site. The arrow marks the site at which the precursor is cleaved to form mature BDNF. The binding sites for BDNF-3 and BDNF-4 (taken from Hashimoto and Heinrich *et al.* (1997)), the forward and reverse primers respectively, used to amplify the 120 bp BDNF sequence are indicated by the shaded boxes.

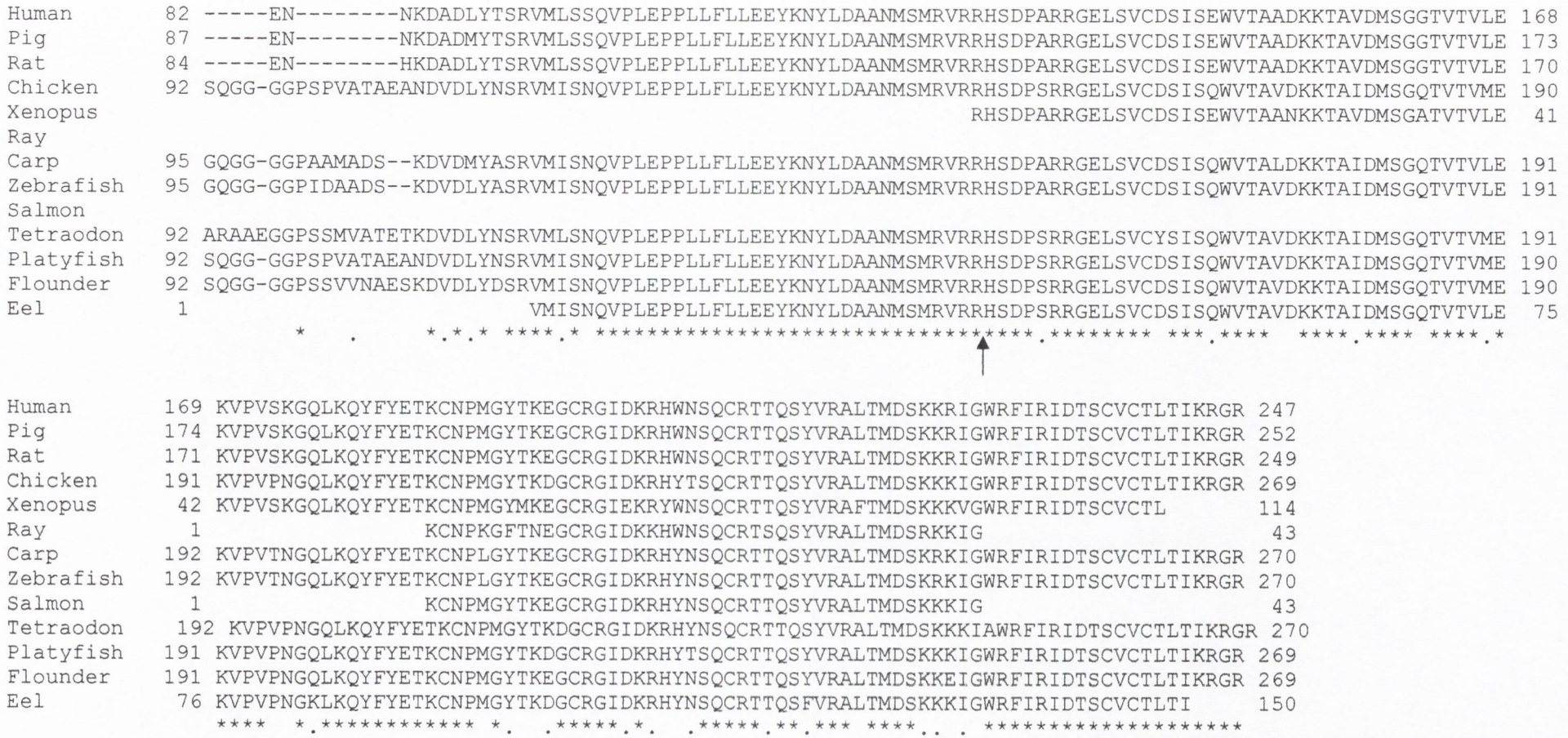


Figure 2.4. Multiple alignment of the predicted BDNF amino acid sequence for the eel (GenPept accession number AAV31512) and BDNF amino acid sequences for human (AAT74399), pig (CAA34685), rat (AAH87634), chicken (AAC42220), xenopus (P25432), ray (P25430), carp (AAA49204), zebrafish (NP_571670), salmon (Hallböök *et al.*, 1991), tetraodon (CAF92094), platyfish (CAA42567) and flounder (AAL71888). The arrow indicates the site where the mature form of BDNF is cleaved from the precursor. * = identical amino acids; . = conservative substitutions.

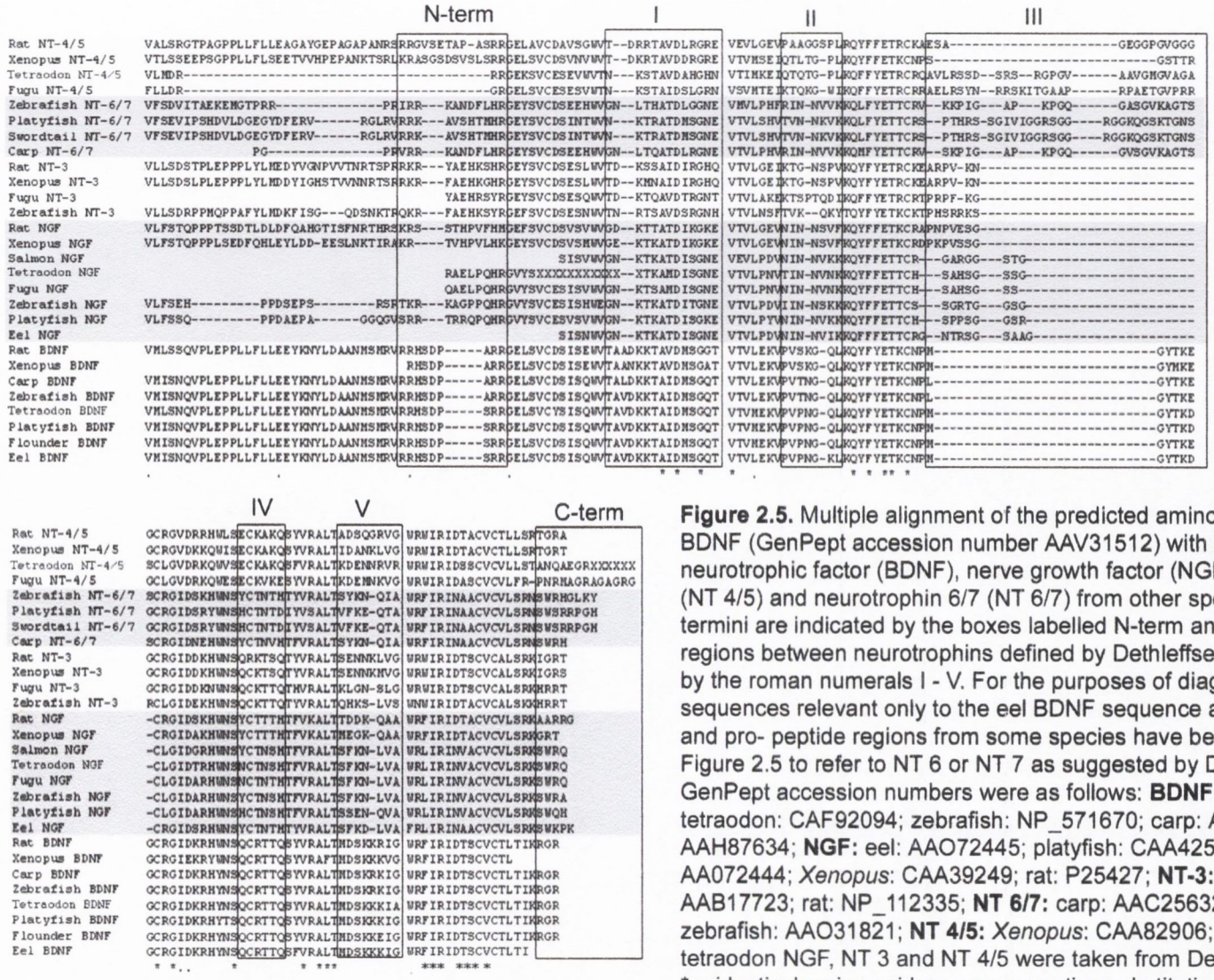


Figure 2.5. Multiple alignment of the predicted amino acid sequence for *Anguilla anguilla* BDNF (GenPept accession number AAV31512) with amino acid sequences for brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT 4/5) and neurotrophin 6/7 (NT 6/7) from other species. The amino and the carboxyl termini are indicated by the boxes labelled N-term and C-term respectively. The variable regions between neurotrophins defined by Dethleffsen *et al.* (2003) are boxed and marked by the roman numerals I - V. For the purposes of diagramatic clarity regions of amino acid sequences relevant only to the eel BDNF sequence are included therefore portions of prepro- and pro- peptide regions from some species have been excluded. The term NT 6/7 is used in Figure 2.5 to refer to NT 6 or NT 7 as suggested by Dethleffsen *et al.* (2003).
GenPept accession numbers were as follows: **BDNF**: flounder: AAL71888; platyfish: CAA42567; tetraodon: CAF92094; zebrafish: NP_571670; carp: AAA49204; *Xenopus*: P25432; rat: AAH87634; **NGF:** eel: AAO72445; platyfish: CAA42566; zebrafish: AAO31814; salmon: AA072444; *Xenopus*: CAA39249; rat: P25427; **NT-3:** zebrafish: AAH92731; *Xenopus:* AAB17723; rat: NP_112335; **NT 6/7:** carp: AAC25632; swordtail: AA61922; platyfish: AA61923; zebrafish: AAO31821; **NT 4/5:** *Xenopus*: CAA82906; rat: AAA41728. Sequences for fugu and tetraodon NGF, NT 3 and NT 4/5 were taken from Dethleffsen *et al.* (2003).
* = identical amino acids; . = conservative substitutions

2.3.4 Wholemount in situ hybridisation (WISH)

After WISH experiments, tissue remained opaque despite clearing, and was distorted and curled due to heating during the hybridisation wash steps. The opaque nature of the tissue rendered signal visualisation unsatisfactory and determination therefore of adequate probe penetration was unfeasible. Staining did seem to be apparent in some regions however.

Staining could not be verified as being specific for TH since a satisfactory comparison of staining patterns in the brains hybridised with the antisense and sense probes was not possible due to the opacity of the tissue. However, the olfactory bulb appeared to be more strongly stained in brains hybridised with the antisense TH probe as opposed to the sense control probe (Fig. 2.6B) and a distinctive rugby ball shaped region of staining was observed in the diencephalon, rostral to the pituitary gland (Fig. 2.6A). These findings are in agreement with those of Roberts *et al.* (1989) and Boularand *et al.* (1998).

Staining, although stronger for the antisense probe (Fig. 2.7A), was seen in the tegmentum after WISH with sense and antisense BDNF probes (Fig, 2.7). Staining was not obvious in other brain regions (e.g. the rhombencephalon) due to the opaque nature of the tissue (Fig. 2.7).

2.3.5 In situ hybridisation to cryostat and vibrating microtome sections - 770 nt TH probe

Methods I and II for ISH to cryostat sections yielded no signal for TH (data not shown). Using method III, counterstaining of cryostat sections with nuclear fast red was necessary to visualise brain areas that were not stained by ISH and to aid identification of brain regions. Distinctive cellular staining for the antisense TH probe was seen after ISH to cryostat (Fig. 2.8A, C and E) and vibrating microtome (Fig. 2.9A, C and D) sections. Areas of staining included the olfactory bulb (Fig. 2.9), telencephalon (Fig. 2.9), diencephalon (Fig. 2.8 and Fig. 2.9) and some cells in the reticular formation (Fig. 2.8). The results were in agreement with those reported by Boularand *et al.* (1998) for TH mRNA expression. No signal was seen on examination of corresponding sense controls (Fig. 2.8B and D and F; Fig. 2.9B).

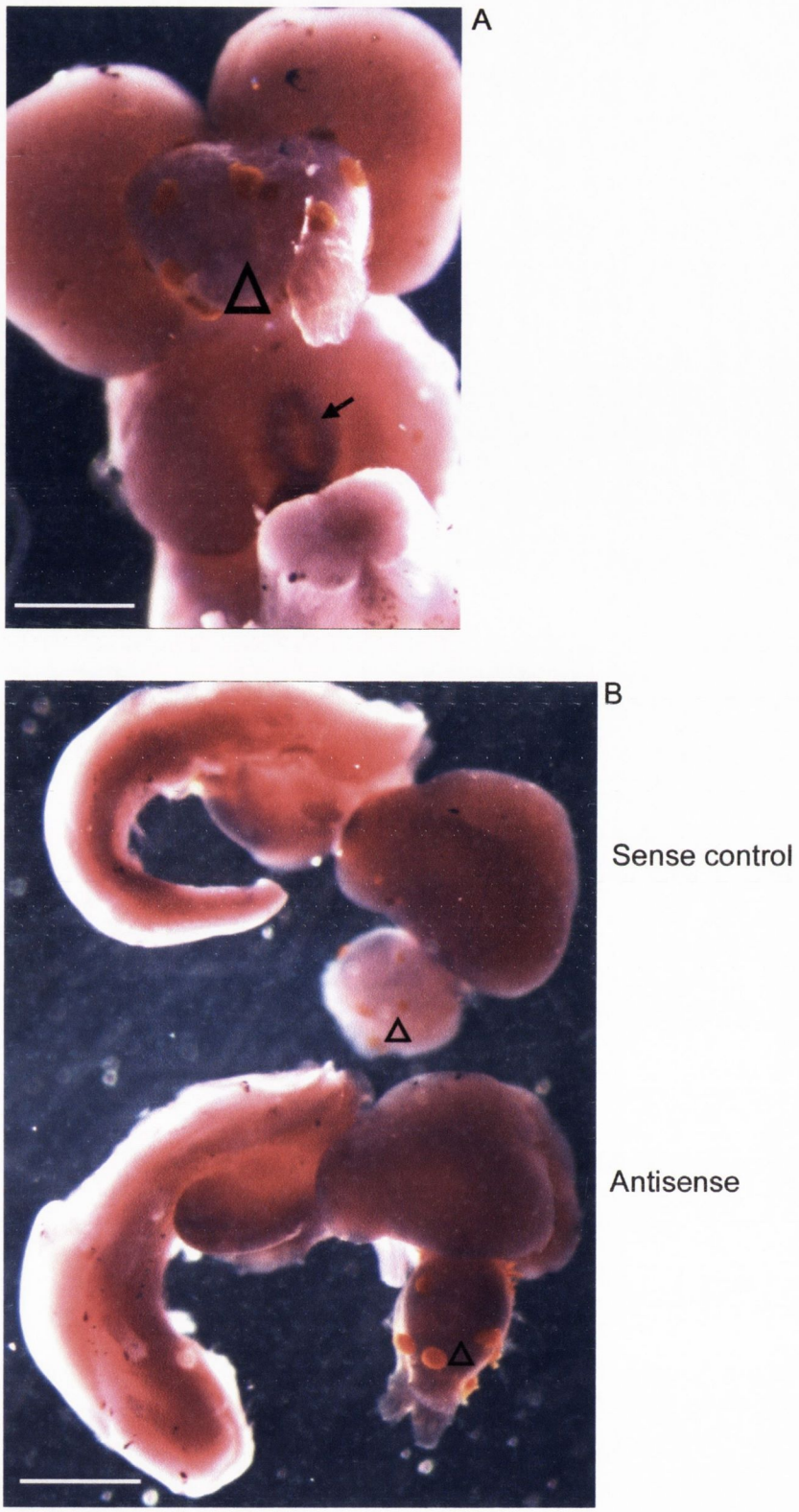


Figure 2.6. Results of wholemount *in situ* hybridisation (WISH) with tyrosine hydroxylase (TH) antisense and sense probes. Photographs are taken under dark field microscopic illumination. A: View of the ventral surface of a whole brain after WISH with TH antisense probe. The regions of the olfactory bulb and diencephalon where staining was obvious, are indicated by the open arrowhead and arrow respectively. B: Lateral view of brains cut down the midline. The olfactory bulb (indicated by the open arrowheads) is more strongly stained in the antisense brain (bottom) than in the sense control (top). Note the curling of the tissue in A and B.
Scale bars = 1.5 mm.

Figure 2.7. Wholemount *in situ* hybridisation (WISH) results for 104 nt BDNF antisense (A) and sense (B) probes. Both brains are shown in lateral view. Prior to WISH, brains were cut down the midline and the forebrain area rostral to tegmentum was removed. Staining was seen in the tegmentum (region indicated by arrows) following WISH with the antisense probe but was also present, although fainter, in the brain probed with the sense probe. Presence or absence of staining in the rhombencephalon (region indicated by arrowheads) could not be determined due to the opaque nature of the tissue. Scale bars = 700 μm.

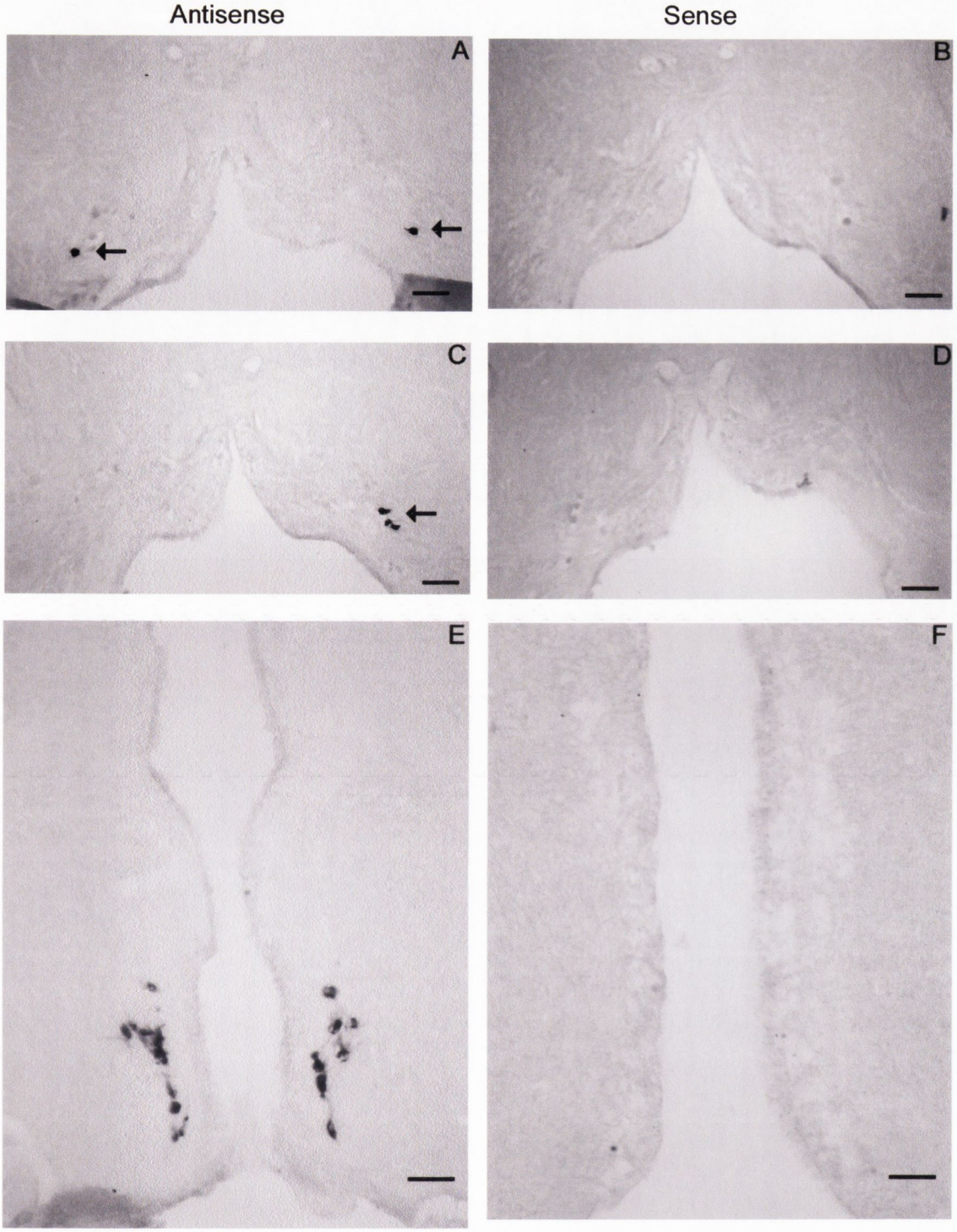


Figure 2.8. In situ hybridisation to cryostat sections with tyrosine hydroxylase antisense (A, C, E) and sense (B, D, F) probes. A-D: Horizontal sections through eel brain stem. Stained cells of the reticular formation in A and C are indicated by black arrows. Note absence of staining in corresponding sense controls, B and D respectively. E, F: Transverse sections through diencephalon. E shows staining in the magnocellular hypothalamic nucleus. Staining is not present in corresponding sense control, F. Scale bars = 200 μm for A, B, C and D and 100 μm for E and F.

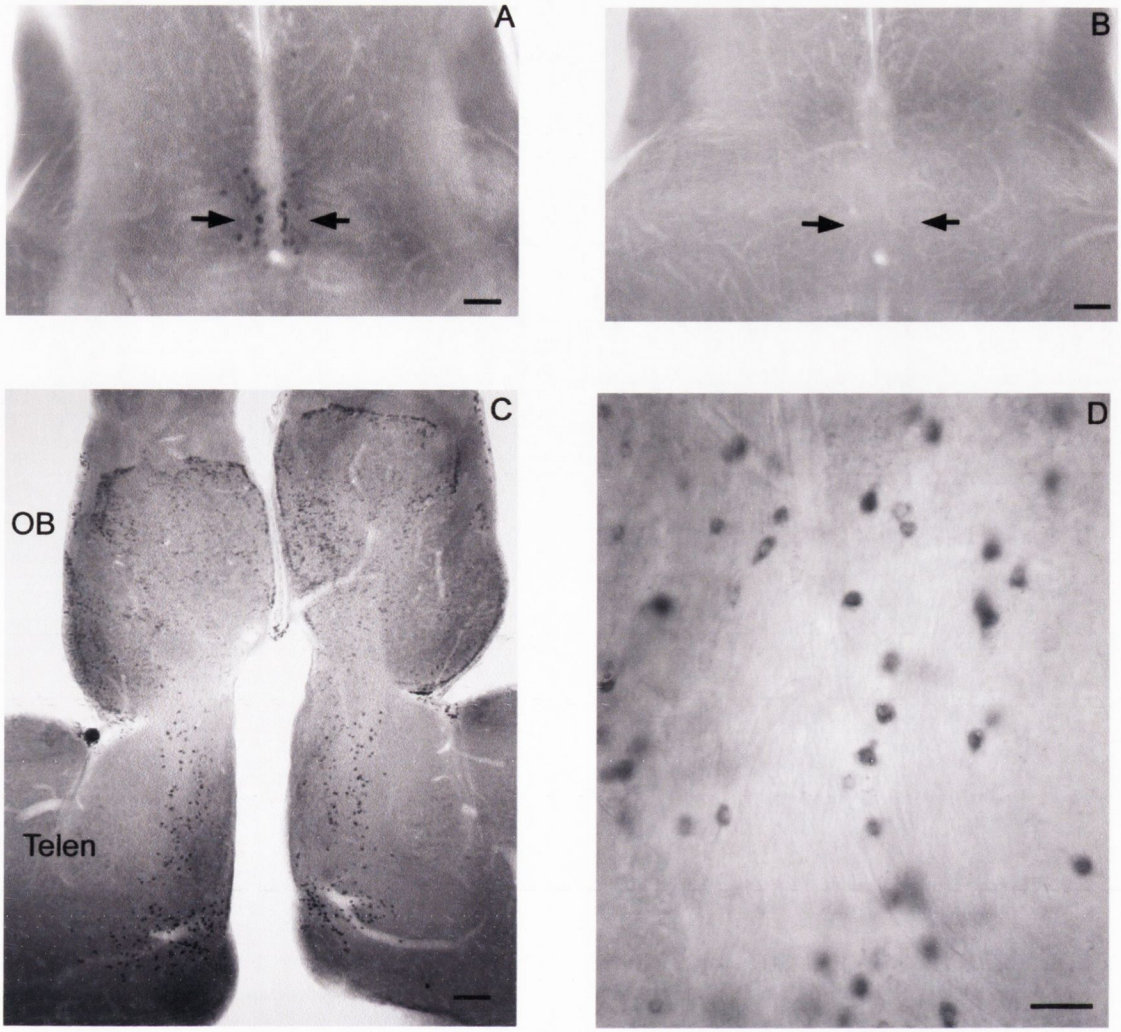


Figure 2.9. Photomicrographs of Vibratome sections following *in situ* hybridisation with tyrosine hydroxylase (TH) antisense (A, C and D) and sense (B) probes. A: TH mRNA expression in the diencephalon marked by arrows. B: Corresponding sense control for A. Note absense of staining in region marked by arrows. C: Low power photomicrograph showing TH mRNA expression in the olfactory bulb (OB) and the telencephalon (Telen). D: High power photomicrograph of staining in C in the telencephalon. Scale bars = 100 μm for A, B and D and 400 μm for D.

2.3.6 In situ hybridisation to cryostat and vibrating microtome sections – 104 nt BDNF probe

As with the TH probe, methods I and II for ISH to cryostat sections yielded no signal for BDNF (data not shown). When Method III was used, staining was seen in the regions of the brain such as the olfactory bulb, telencephalon and brain stem with the 104 nt antisense probe for BDNF and was more obvious in vibrating microtome (Fig. 2.10C and E) than in cryostat sections (Fig. 2.10A). The staining however could not be localised to specific cell bodies. The sense controls sections also appeared to show a fainter but similar pattern of staining when compared to experimental tissue (Fig. 2.10B, D and F). A longer fragment of *A. anguilla* BDNF cDNA was cloned (see section 2.2 and 2.3) in order to improve probe specificity.

2.3.7 In situ hybridisation to cryostat and vibrating microtome sections – 467 nt BDNF probe

Staining could be localised to specific cell bodies when ISH was carried out with the 467 nt antisense probe for BDNF on cryostat sections (25-30 μm). Staining was seen in regions such as the telencephalon (data not shown) and the brain stem (Fig. 2.11A, C and E). Staining was not seen in corresponding sense controls (Fig. 2.11B, D and F). The intensity of staining following ISH to cryostat sections achieved with the 467 nt antisense probe for BDNF was relatively faint however when compared with that seen with the TH probe (see Fig. 2.8).

Distinctive staining patterns could be cellularly localised when ISH was carried out on the thicker (100 μm) vibrating microtome sections (Fig. 2.12A, C, E and G). These patterns were not seen in corresponding sense controls (Fig. 2.12B, D, F and H). BDNF mRNA was seen in the brainstem nuclei such as the reticular formation, in hypothalamic nuclei and in the telencephalon. The overall expression pattern for BDNF mRNA in the eel brain is described in detail in chapter 3.

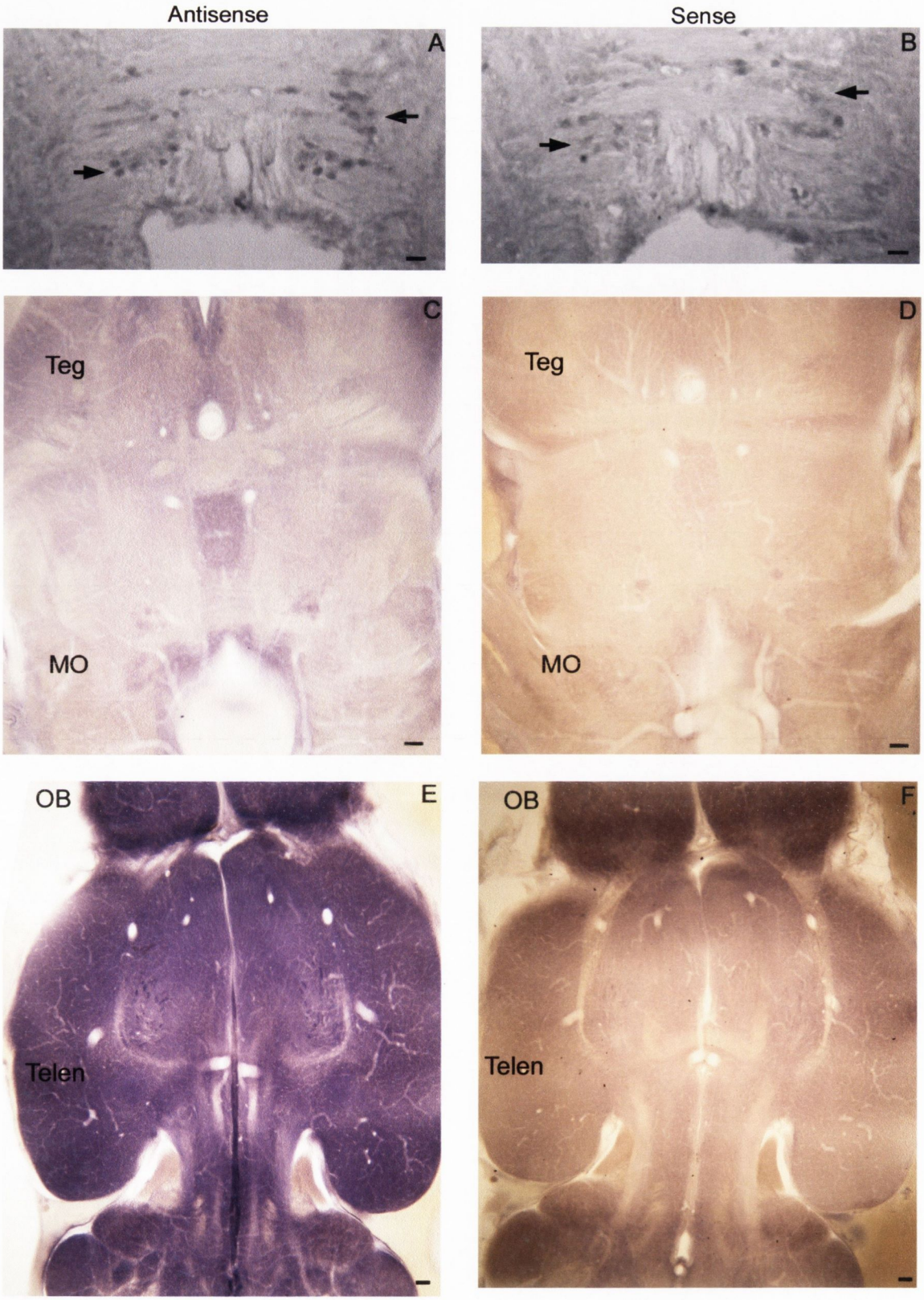


Figure 2.10. Results of *in situ* hybridisation to cryostat (A,B) and Vibratome (C-F) sections with 104 bp BDNF probe. A: Staining of cells indicated by arrows in the reticular formation. B: Corresponding sense control for A. Staining is visible the same cells indicated in A (marked by arrows). C: Staining in the tegmentum (Teg) and medulla oblongata (MO). D: Corresponding sense control for C. Note overall staining pattern is fainter but similar to that in C. E: Staining in olfactory bulb (OB) and telencephalon (Telen). F: Sense control for E. Staining pattern in F is similar to that in E. Scale bars = 100μm.

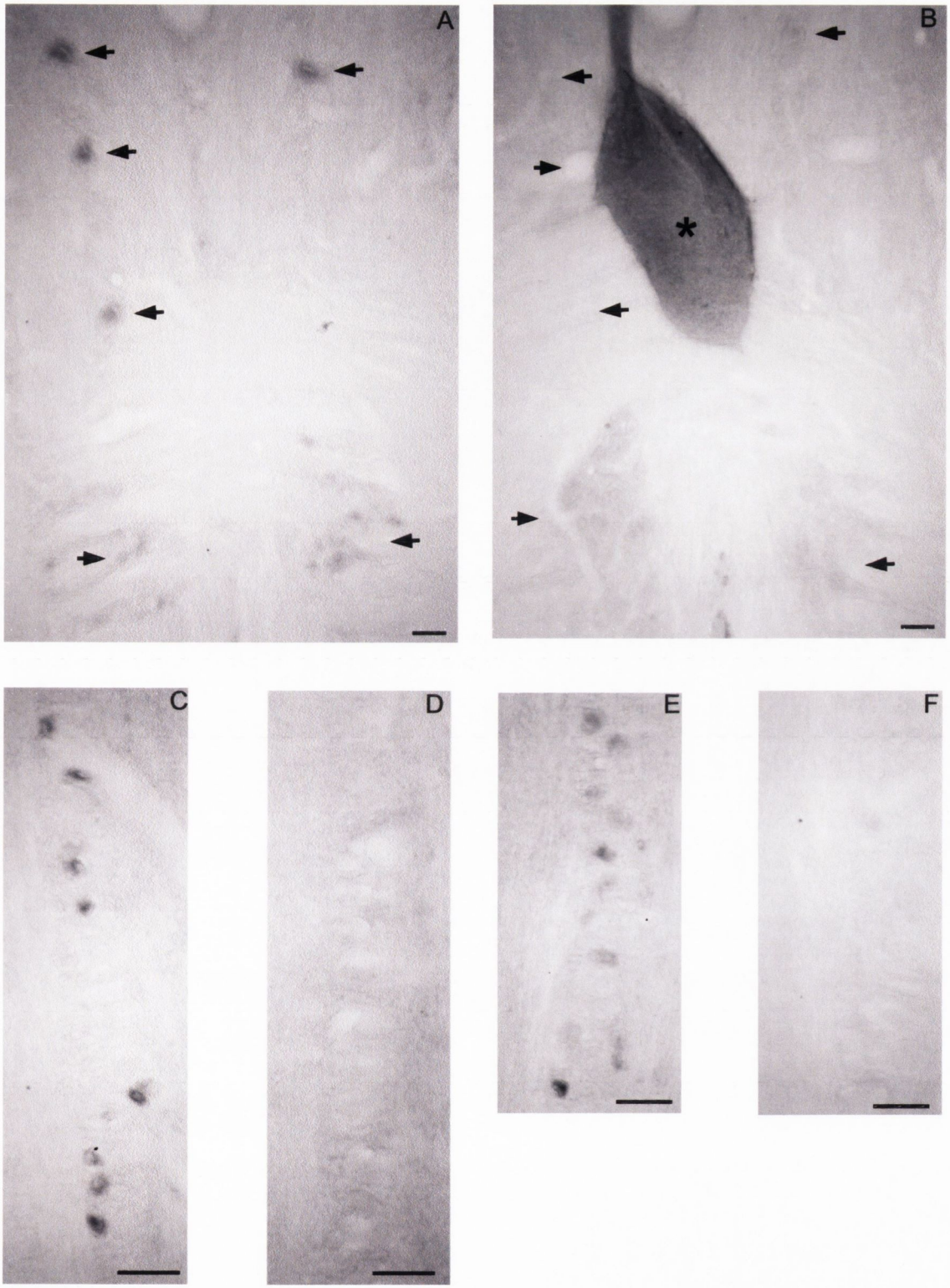


Figure 2.11. *In situ* hybridisation to cryostat sections with the 467 nt BDNF probe. A: Staining of cells in the reticular formation indicated by arrows. B: Corresponding sense control for A. Note lack of staining in regions (indicated by arrows) that are stained in A. A fold in the tissue is marked by *. C, E: BDNF mRNA expression in the caudal medulla oblongata. D and F: Corresponding sense controls for C and E, respectively. Scale bars = 100 μm.

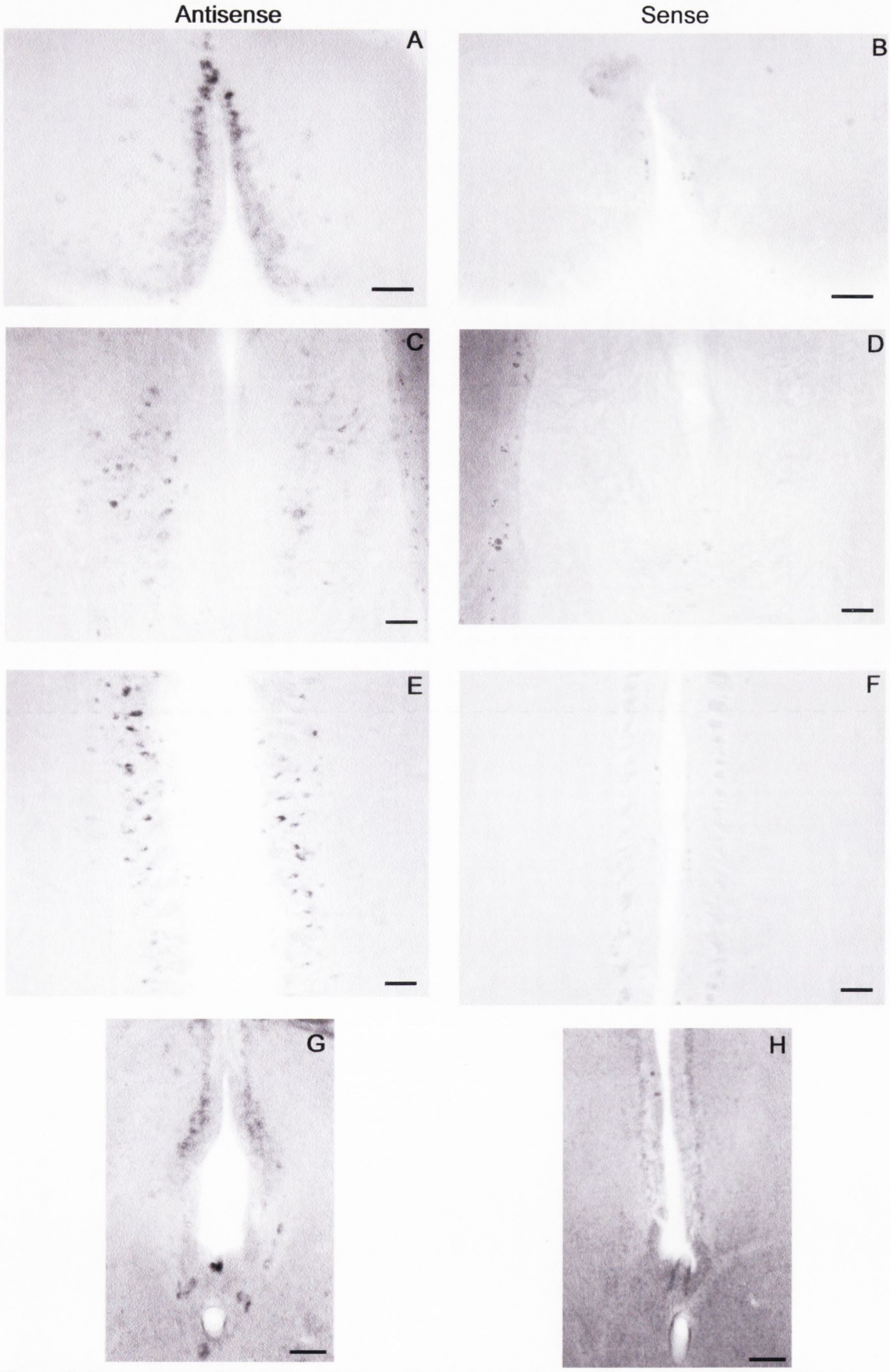


Figure 2.12. ***In situ*** **hybridisation to vibrating microtome sections (100 μm thick) with the 467 nt BDNF probe. BDNF mRNA expression in the periventricular hypothalamus (A and G), reticular formation (C), caudal medulla oblongata (E) is shown. B, D, F and H are the corresponding sense controls for A, C, E and G respectively. Scale bars = 100 μm**

2.4 Discussion

This chapter describes the successful cloning and characterisation of a 467 bp cDNA fragment for *A. anguilla* BDNF. It also covers the trial of WISH with eel brain tissue. In addition, protocols for ISH to cryostat and vibrating microtome sections of the eel brain set up and optimised for use in our laboratory are described in detail.

2.4.1 Cloning and characterisation of a 467 bp cDNA fragment for A. anguilla BDNF

The 467 bp cDNA fragment coding for *A. anguilla* BDNF cloned in this study possessed a high degree of identity with BDNF sequences from various species at the nucleotide (87-88% with other fish) and amino acid (≥79% for all species examined, see Table 2.2; Fig. 2.4) level. The fragment spans the majority of the coding and some of the propeptide region of the major BDNF exon (see Fig. 2.1 and 2.3) including the five variable regions, which exist between all neurotrophins (Fig. 2.5). Five BDNF specific regions will be present therefore in antisense probes transcribed from the 467 bp insert and will help to promote specificity of probe binding to target BDNF mRNA only during ISH.

The cloning of BDNF is the first step towards enabling the expression of BDNF to be studied at the molecular level. Not only will the cloning of this fragment be important for future experiments investigating CNS regeneration in *A. anguilla* but also this sequence information may be important in functional genomic studies in this and other species. For example, sequence comparisons between distantly related organisms such as fish and humans may aid in recognising functionally important regions in a gene sequence (Roest Crollius and Weissenbach, 2005). Furthermore, since neurotrophin sequences are highly conserved between species, they may also be useful in evolutionary and phylogenetic studies (Kullander *et al.*, 1997; Heinrich and Lum, 2000). Kullander *et al.* (1997) for instance, used sequence data for NGF, BDNF and NT-3 to carry out a phylogenetic analysis of Australian marsupials. The results of their study confirmed the previous classification of marsupial orders, which was based on morphological characteristics (Kullander *et al.*, 1997). Some debate exists over the phylogeny of freshwater eels and the dispersal routes of the genus based on studies involving analysis of mitochondrial genes (Bastrop *et al.*, 2000; Aoyama *et al.*, 2001; Lin *et al.*, 2001; Wirth and Bernatchez, 2001; Mank and Avise, 2003; Minegishi *et al.*, 2005). A different approach comparing neurotrophin sequences in different eel species similarly to Kullander *et al.* (1997) and for which degenerate primers that have been shown to amplify BDNF from *A. anguilla* RNA samples are now available may help to further clarify and/or to confirm present findings related to the phylogeny of freshwater eels.

2.4.2 Wholemount in situ hybridisation

For the purposes of this study, it was decided that WISH was not suitable for examination of mRNA expression patterns the eel brain. The opaque nature of the tissue rendered signal visualisation unsatisfactory for further analysis and determination of adequate probe penetration was unfeasible (Fig. 2.6 and Fig. 2.7).

As mentioned in section 2.1, the process of WISH is usually restricted to studies of gene expression patterns during embryogenesis and is not normally feasible in relatively more dense and complex adult tissues (Xu and Wilkinson, 1998). However, WISH has been carried out effectively in the adult lamprey brain (Swain *et al.*, 1994; Jacobs *et al.*, 1996, 1997). Its success in the lamprey may be due to the absence of myelin in the CNS of these animals (Bullock *et al.*, 1984). Myelin has a high lipid content (Lees and Brostoff, 1984) and ISH has been shown to be hindered in fatty tissues (Braissant and Wahli, 1998). The lack of myelin in the lamprey brain therefore probably allows for easier penetration of probes and reagents into neurons than in the eel brain. In addition, the absence of myelin in the lamprey renders the entire CNS translucent which greatly aids in signal visualisation (Bullock *et al.*, 1984; Swain *et al.*, 1994; Shifman and Selzer, 2000).

In order to determine definitively if WISH is feasible in the eel brain, a future experiment could be carried out whereby a tissue sample sectioned after WISH was compared with sections from the same area after ISH. The only advantage of carrying out WISH in the eel brain would be the greater volume of samples that could processed in one experiment since due to the tissue opacity, brains would have to be sectioned for further examination of results. However, tissue distortion and curling after heat treatment may hinder the cutting of sections and this problem would need to be overcome before such an approach would be beneficial.

2.4.3 Probe specificity

Short nucleic acid probes (25-200 bp) have been shown to produce ISH signals (Cox *et al.*, 1984; Wilkinson, 1995; Braissant and Wahli, 1998; Rattray and Michael, 1998; Miura *et al.*, 2002) and may be particularly useful when examining expression of splice variants and closely related members of a particular protein family (Rattray and Michael, 1998; Wilkinson, 1995, 1998). However, both sense and antisense 104 nt BDNF probes bound non-specifically to the eel brain tissue resulting in an all over signal that could not be localised to specific cell bodies (Fig. 2.10). The non-specific signal was deemed likely to be probe related and not protocol or tissue related (Wilkinson, 1998) since the TH probe used in parallel with the BDNF probe produced a specific signal (see Fig. 2.8 and Fig. 2.9). The cause of the non-specific binding of the probe was unlikely to

be due to the cross hybridisation of the probe with other neurotrophin family members such as NGF since a BLASTN search of the NCBI database (Altschul *et al.*, 1997) and ClustalW alignments (Higgins *et al.*, 1994) with the BDNF sequence that served as the probe template revealed low similarity with other neurotrophin family members (see Table 2.3; Rattray and Michael, 1998; Wilkinson, 1998; Hasegawa *et al.*, 2005).

The cause of the non-specific probe binding therefore may have been the presence of the flanking sequence added to the 104 nt BDNF probe by primers during PCR amplification (see section 2.2.3) and the *in vitro* transcription process (Blödorn *et al.*, 1998; Braissant and Wahli, 1998, Xu and Wilkinson, 1998). In the present study, probes were generated from a cDNA template cloned into the pCR®II vector (Invitrogen). T7 and SP6 are the RNA polymerase initiation sites in the pCR®II vector (Fig. 2.2). During *in vitro* transcription from this plasmid, approximately 90 nt of the pCR®II vector sequence corresponding to the region of the poly-cloning site between the chosen RNA polymerase initiation site and the cDNA insert will always be unavoidably included in the probe. In the case of the 104 nt probe used in this study therefore, in addition to the specific BDNF related sequence, the sequence of approximately 90 nt transcribed from the pCR®II vector and 16 nt transcribed from restriction sites added by primers would have been included in the final probe. Flanking regions transcribed from plasmids are not normally a problem when using longer probes (>500 nt) since longer probes will preferentially bind to the target sequence (Braissant and Wahli, 1998). However, the inclusion of a non-specific flanking region in a probe sequence that is approximately the same length as the specific sequence directed towards the target DNA, may have led to the generation of the non-specific signal seen in this study (Blödorn *et al.*, 1998; Braissant and Wahli, 1998; Xu and Wilkinson, 1998).

Non-specific binding of short probes due to the inclusion of flanking plasmid sequences in the RNA probe may be overcome by amplifying the probe template by PCR using gene-specific primers into which an RNA polymerase initiation site has been incorporated at the 5' end. *In vitro* transcription can then be carried out directly from the purified PCR products thus avoiding the inclusion of plasmid sequence (Gandrillon *et al.*, 1996; David and Wedlich, 2001). On the other hand, the use of longer RNA probes (500-1000 bp) for ISH is preferable when possible since they provide greater specificity and a stronger signal since more hapten labeled nucleotides can be incorporated into them during *in vitro* transcription (Wilkinson, 1998). In the current study therefore, it was deemed more appropriate to overcome the problem of non-specific binding of the BDNF probe by

repeating the degenerate RT-PCR using a different primer pair in order to clone a longer cDNA fragment.

2.4.4 Optimisation of ISH protocols for cryostat sections

Three methods for ISH to cryostat sections were attempted. For the eel brain tissue used in this study, despite attempts at protocol optimisation for methods I and II, only method III produced a positive ISH signal for TH and BDNF. An experiment carried out in parallel using method I with cryostat sections of embryonic mouse tissue did result in a positive signal (Dr P. Murphy, personal communication, data not shown). It is difficult, therefore, to pinpoint the specific reason for the success of method III over I and II. However, it is often necessary to develop specific protocols to suit a particular tissue (Breitschopf *et al.*, 1992; Strähle *et al.*, 1994; Swain *et al.*, 1994; Kanazir *et al.*, 1997; Braissant and Wahli, 1998; Xu and Wilkinson, 1998; Shifman and Selzer, 2000; Plenz *et al.*, 2002; Vanholme *et al.*, 2002). A comparison of the three methods used in the present study does reveal some important differences, which may give some insight into the success of method III over I and II.

Although method II was applied by Kapsimali *et al.* (2000) to the eel brain, the animals were at a later stage in their life cycle and had undergone metamophosis from "yellow", fresh water-dwelling to "silver", salt water-dwelling animals and so the tissue composition may have been different at this life stage (Rossi and Palombi, 1976; Yamoto and Hirano, 1978; Ellerby *et al.*, 2001; Aroua *et al.*, 2005). As mentioned in section 2.1, intrinsic differences in tissue composition such as fat content, cell composition and developmental stage may affect the success of an ISH protocol therefore method II may be more suitable for brain tissue from silver eels than yellow eels. In addition, a proteinase K digestion step was not present in the Kapsimali *et al.* (2000) protocol. This treatment permeabilises the tissue and aids probe and reagent penetration. The absence of this step in method II may have led to difficulty in probe and reagent access to the eel brain tissue used in the current study thus reducing the ISH signal.

The hybridisation conditions for the probe e.g. hybridisation temperature and hybridisation solution composition, can affect the success of ISH (Brown, 1998). All hybridisations for methods I-III were carried out 55 °C; the compositions of hybridisation solutions for each method, however, were different, particularly with regards to the concentrations of blocking agents such as CHAPS, heparin and tRNA for methods I and III (see Table 2.1) and the presence of Denhardt's solution and absence blocking reagent (Roche) in the hybridisation solution for method II (see Table 2.1). Another difference

between all three methods was the variation in pre-blocking and blocking procedures prior to and during the antibody incubation step (see Table 2.1).

In conclusion, the presence of a proteinase K treatment step in method III may have been a contributing factor to its success over method II. It may also be the case that the blocking procedures to prevent non-specific binding of the probe during hybridisation and of the antibody prior to signal development described for method III are particularly effective in the eel brain tissue at reducing background, which is known to limit the detection of weak signals (Xu and Wilkinson, 1998). Other factors however, such as developmental stage and subtle differences in pre- or posthybridisation wash procedures may also have contributed to the success of method III and failure of methods I and II in producing a signal for TH and BDNF.

2.4.5 Comparison of ISH procedures to slide-mounted cryostat and free-floating vibrating microtome sections

The protocol for ISH to thin (25 – 30 μm), slide-mounted cryostat sections is slightly less time-consuming and labour-intensive than that for ISH to thick (100 μm), free-floating vibrating microtome sections. It is not as cost effective however since it requires the preparation and utilisation of large volumes (e.g. 200 ml per slide dish versus 7 ml per wash tube for free floating sections) of washing solutions and expensive reagents such as formamide for posthybridisation washes. Although histological resolution is slightly compromised in the thicker vibrating microtome sections, this method is more sensitive than ISH to cryostat sections since more target mRNA in the sectioned material is available to the probe, and in addition, reagent and probe penetration can take place from both sides of the section. In this study, signals obtained after ISH to cryostat sections using method III with the BDNF probe (Fig. 2.11) were not as strong as those obtained using the TH probe (Fig. 2.8). This suggests that BDNF is not as highly expressed in the eel brain as TH. For future studies investigating gene expression patterns in the eel brain, it may be advantageous to first carry out an ISH experiment with thick tissue sections cut on a vibrating microtome. If the gene is highly expressed and greater histological resolution is required for examination of results, it may then be beneficial to carry out an ISH to thinner cryostat sections with the same probe.

2.4.6 Concluding remarks

A 467 bp cDNA fragment for *A. anguilla* BDNF has been cloned, sequenced and is publicly accessible to the scientific community on the NCBI GenBank database (accession number AY762996). A 770 bp fragment for TH has also been cloned and hence, a positive control probe is now available for future ISH experiments in our laboratory to ensure the

process is operating successfully. A protocol for ISH to thin, slide-mounted cryostat sections has been optimised for use with brain tissue from *A. anguilla*. A more sensitive procedure for ISH to thicker, free-floating vibrating microtome sections, particularly useful in the investigation of mRNA transcripts that are not highly expressed in eel CNS tissue has also been established. This protocol was used for studies described in chapter 3 to examine of BDNF mRNA expression in the normal eel brain and in chapter 4 to investigate changes in BDNF expression in the eel brain after axotomy of spinally projecting brain stem neurons.

Chapter 3.

The mRNA expression pattern of brain derived neurotrophic factor (BDNF) in the eel brain.

3.1 Introduction

BDNF mRNA is widely distributed throughout the adult mammalian brain (Wetmore *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997). Its overall expression pattern has been described in detail from the results of ISH experiments with isotopically labelled probes in the rat (Wetmore *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997) and pig (Wetmore *et al.*, 1990) brain. Immunocytochemical and immunohistochemical studies have also revealed the overall expression pattern for BDNF protein in the rat (Kawamoto *et al.*, 1996; Conner *et al.*, 1997; Yan *et al.*, 1997a; Friedman *et al.*, 1998; Furukawa *et al.*, 1998) and human brain (Murer *et al.*, 1999). Studies indicate that BDNF mRNA (Wetmore *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997) and protein (Conner *et al.*, 1997; Yan *et al.*, 1997a; Furukawa *et al.*, 1998) appear to be expressed mainly in cells possessing a neuronal morphology in the adult mammalian brain although Murer *et al.* (1999) noted some BDNF protein expression in glial cell bodies and processes in the human brain.

3.1.1 Overall expression patterns of BDNF mRNA and protein in the adult mammalian brain

In general, expression levels of BDNF mRNA are not uniform throughout the mammalian brain and the strength of ISH labelling is typically described by authors as being light/weak, moderate or heavy/strong (Wetmore *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997). In addition, the cellular pattern of labelling varies from region to region and labelled cells in a nucleus or brain area are usually described as being occasional in number, scattered, moderate in number or densely packed (Wetmore *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997). For example, densely packed pyramidal cells in the hippocampus are described as being moderately to strongly labelled (Wetmore *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997). In contrast, light labelling of occasional or scattered cells has been found in the nucleus brachium and external cortex of the inferior colliculus (Conner *et al.*, 1997). The main regions in which BDNF mRNA is expressed in the adult mammalian brain are mentioned below, progressing through the brain in a rostral to caudal direction where possible.

Moderate to heavy BDNF mRNA expression has been found in higher processing centres of the brain such as various divisions of the cortex including the visual (Castrén *et al.*, 1992; Conner *et al.*, 1997), frontal, cingulate and entorhinal cortices (Castrén *et al.*, 1995; Conner *et al.*, 1997). In the hippocampus, moderate to strong BDNF mRNA expression was described in the stratum pyramidale of the CA1, 2 and 3 regions and the hilus and granule cell layer of the dentate gyrus (Wetmore *et al.*, 1990; Kokaia *et al.*, 1993;

Conner *et al.*, 1997). Light, moderate and strong BDNF mRNA expression was found in some basal forebrain regions such as the septum, some preoptic nuclei and the septofimbrial nucleus (Castrén *et al.*, 1995; Conner *et al.*, 1997). Labelling of BDNF mRNA in the amygdala ranged from light to heavy (Castrén *et al.*, 1995; Conner *et al.*, 1997).

Light to moderate levels of BDNF mRNA expression have been reported in some thalamic nuclei such as the central medial, intermediodorsal, anterodorsal thalamic nuclei and the medial geniculate nucleus (Castrén *et al.*, 1995; Conner *et al.*, 1997). BDNF mRNA expression ranged from light to heavy in the hypothalamic nuclei. For example, a moderate number of cells were moderately labelled in the paraventricular nucleus, lightly to moderately labelled in the dorsomedial nucleus and heavily labelled in the ventromedial hypothalamic nucleus (Castrén *et al.*, 1995; Conner *et al.*, 1997).

In addition, BDNF mRNA expression tended to be light to moderate in primary sensory regions of the brain such as the internal granule and glomerular layers of the olfactory bulb (Conner *et al.*, 1997), the optic nerve layer of the superior colliculus (Wetmore *et al.*, 1990; Conner *et al.*, 1997) and the inferior colliculus in the brain stem which is associated with audition (Castrén *et al.*, 1995; Conner *et al.*, 1997).

Light to heavy labelling of BDNF mRNA has been reported in the nuclei located in the brain stem that are associated with the control of breathing such as the cuneate nucleus, inferior olive, pontine nuclei and spinal trigeminal nucleus (Castrén *et al.*, 1995; Conner *et al.*, 1997). In brain stem nuclei that are involved in somatic motor control, light to moderate BDNF mRNA expression was seen only in the locus coeruleus and the dorsal paragigantocellular nucleus, which forms part of the reticular formation (Castrén *et al.*, 1995; Conner *et al.*, 1997).

BDNF protein expression, for the most part, matches the pattern of BDNF mRNA in the adult mammalian brain. For example, BDNF mRNA and protein have been found at similar expression levels and in similar cellular patterns in most regions of the cortex, the claustrum, regions of the hippocampus such as the granule layer of the dentate gyrus and most thalamic nuclei (Conner *et al.*, 1997; Yan *et al.*, 1997a). Nevertheless, there are some regions, such as the striatum, the magnocellular preoptic nucleus, the intermediate and deep grey layer of the superior colliculus, the spinal vestibular nucleus and the central nucleus and dorsal cortex of the inferior colliculus that contain BDNF protein but show no corresponding mRNA expression (Altar *et al.*, 1997; Conner *et al.*, 1997; Yan *et al.*, 1997a). It is probable that BDNF is anterogradely or retrogradely transported to these areas from other brain regions where BDNF synthesis takes place (Altar *et al.*, 1997; Conner *et*

al., 1997; Mufson *et al.*, 1999). In the case of the striatum, it is likely that BDNF is supplied to this region from anterograde sources. For example, Altar *et al.* (1997) reported that BDNF protein and mRNA are present in striatal afferents but not striatal targets and that lesioning of corticostriatal and nigrostriatal neurons resulted in the depletion of striatal BDNF. Furthermore, Kokaia *et al.* (1998) demonstrated that injury of corticostriatal neurons by focal cerebral ischemia resulted in a decrease in striatal levels of BDNF protein. Anterograde transport of BDNF has also been demonstrated in cortical neurons (Adachi *et al.*, 2005), by neurons of the lateral parabrachial nucleus that project to the amygdala and by septal nuclei projecting to the medial habenular nucleus (Conner *et al.*, 1997). On the other hand, exogenous BDNF has been shown to be retrogradely transported to distinct cell groups in regions such the frontal cortex, hippocampus, entorhinal cortex and the amygdala, from a number of injection sites in the mammalian brain (Mufson *et al.*, 1999). Target-derived retrogradely transported BDNF may account therefore for a BDNF mRNA/protein mismatch in some cases.

3.1.2 Expression patterns of BDNF mRNA and protein in the non-mammalian brain

The overall expression patterns for BDNF mRNA and protein have not been described in the adult brain of any bird or amphibian to date. However, the presence of BDNF mRNA in specific avian and amphibian brain regions has been noted. For example, BDNF mRNA is expressed in the optic tectum, which is the non-mammalian equivalent of the superior colliculus (Torborg and Feller, 2005), in the frog (Duprey-Díaz *et al.*, 2002) and chick (Herzog and von Bartheld, 1998). In addition, BDNF protein expression has been reported in the pigeon (Theiss and Güntürkün, 2001) and frog (Duprey-Díaz *et al.*, 2002) tectum and in the hypothalamo-hypophyseal system of *Xenopus* (Wang *et al.*, 2005). With regard to fish, although BDNF mRNA expression has been confirmed in the platyfish brain by northern blot hybridisation (Götz *et al.*, 1992) and in the brain of the developing zebrafish by ISH (Hashimoto and Heinrich, 1997; Lum *et al.*, 2001), no information is available on the expression pattern of BDNF mRNA or protein in any region of the adult fish brain.

3.1.3 Functions of BDNF in the adult brain

BDNF has the potential to be involved in a number of processes in the adult brain and associated nervous system. The Trk B receptor protein is expressed in virtually all regions of the adult rat brain including areas that lack BDNF mRNA expression (Yan *et al.*, 1997b). As mentioned above BDNF can be retrogradely and anterogradely transported along neurons (Altar *et al.*, 1997; Mufson *et al.*, 1999) and can also act in an autocrine manner (Davies and Wright, 1995).

BDNF may function as an autocrine, anterogradely-transported or target-derived, survival promoting factor for neurons within the brain itself, e.g. pyramidal neurons, and for various neuronal populations within the rest of the CNS such as primary sensory afferents (Kokaia *et al.,* 1993; Davies and Wright, 1995; Altar *et al.,* 1997; Connor and Dragunow, 1998; Mufson *et al.,* 1999; Xu *et al.,* 2000b; Murer *et al.,* 2001). A study by Xu *et al.* (2000b) supports this idea. They created a conditional trk B mutant mouse in which trk B can be deleted in the pyramidal neurons only. They reported severe neuronal loss in the neocortex of mature mutant animals, which suggests that pyramidal neurons are dependent on the trophic effects of Trk B receptor activation for their survival (Xu *et al.,* 2000b).

In addition, BDNF appears to have a role in modulating synaptic transmission, synaptic morphology and connectivity, and may be involved in synaptic plasticity in various regions of the adult brain such as the hippocampus and the amygdala (McAllister *et al.,* 1999; Sherwood and Lo, 1999; Kelly *et al.,* 2000; Schinder and Poo, 2000; Gooney *et al.,* 2002; Vicario-Abejón *et al.,* 2002; Blum and Konnerth, 2005; Rattiner *et al.,* 2004; Hu *et al.,* 2005). For instance, long term potentiation (LTP), a well studied model of synaptic plasticity, is impaired at the hippocampal Schaffer collateral-CA1 synapse in mice heterozygous and homozygous for BDNF and can be rescued by BDNF application to the hippocampal slices (Korte *et al.,* 1995; Patterson *et al.,* 1996). LTP is also impaired in hippocampal slices from animals treated with antibodies against BDNF (Chen *et al.,* 1999) or when BDNF was sequestered with trkB-IgG fusion protein (Figurov *et al.,* 1996; Kang *et al.,* 1997).

BDNF may also modulate the synthesis and release of neuropeptides such as somatostatin in the hypothalamo-hypophysial system (Tapia- Arancibia *et al.,* 2004; Givalois *et al.,* 2006). BDNF has been implicated in the synthesis and release of somatostatin *in vitro* (Rage *et al.,* 1999; Marmigère *et al.,* 2001) and *in vivo* (Givalois *et al.,* 2006), and Trk B receptors have been located on somatostatin producing neurons in hippocampal cultures (Rage *et al.,* 1999). In rats, a continuous administration of BDNF in the lateral ventricle increased mRNA and protein levels of corticotropin releasing hormone and vasopressin in various hypothalamic nuclei (Naert *et al.,* 2006).

3.1.4 Aims of chapter 3

As mentioned above, the overall expression patterns for BDNF mRNA and protein have been described in the brain of mammals such as the rat and pig, and in particular brain regions in birds and amphibians. However, a detailed study of BDNF mRNA or protein expression in the fish brain has not been carried out to date. Since BDNF and its

receptor are highly conserved between species, it is likely that they perform similar functions in all vertebrates including fish (Heinrich and Lum, 2000). A comparison of BDNF expression in homologous regions of the eel brain with other species is a first step in testing this idea. The aim of this study therefore is to provide a detailed description of BDNF mRNA expression in the eel brain and to compare its expression with that in other vertebrates such as mammals. Comparing BDNF mRNA expression in brains of amniotes that display limited CNS regeneration, with expression in the brain of an anamniote such as the eel, may give some insight into the ability of fish to regenerate the injured axons of brain stem neurons projecting to the spinal cord. Elucidating the mRNA expression pattern for BDNF in the eel brain will also help to further our knowledge and understanding of functional anatomy of the teleost brain, where some gaps still exist particularly with regard to the functional organisation of the telencephalon (Northcutt and Braford, 1980; Nieuwenhuys and Meek, 1990).

3.1.5 Overview of the morphology and functional divisions in the eel brain

As in other teleost fish, the eel brain is divided into a number of different regions (Nieuwenhuys and Pouwels, 1983; Mukuda and Ando, 2003; Fig.3.1). The most rostral of these is the telencephalon, which is made up of the olfactory bulbs and the telencephalic hemispheres. The midbrain or mesencephalon then follows and encompasses the tectum and tegmentum. The diencephalic nuclei are located in the ventral region of the telencephalic hemispheres and extend below the caudal portion of the tegmentum. The rhombencephalon is found caudal to the mesencephalon and consists of the medulla oblongata and the cerebellum (Mukuda and Ando, 2003). Ten cranial nerves, an anterior and posterior lateral line nerve and some spinooccipital nerves supply the eel brain and protrude from the basal regions of the brain (Meredith and Roberts, 1987; Mukuda and Ando, 2003).

The higher processing centres of the fish brain include the area dorsalis and area ventralis of the telencephalic hemispheres, the tectum and the cerebellum (Wullimann *et al.*, 1996; Wullimann and Rink, 2002). These centres receive sensory information, in most cases via intermediate nuclei, from the terminal fields of primary sensory afferents and send commands to primary motor nuclei. They process sensory information and ultimately control functions such as movement and feeding (Nieuwenhuys and Meek, 1983; Nieuwenhuys and Meek, 1990; Molist *et al.*, 1993; Wullimann *et al.*, 1996; Herrero *et al.*, 1998; Prechtl *et al.*, 1998; Wullimann and Rink, 2002; Pérez-Pérez *et al.*, 2003; Angeles Luque *et al.*, 2005). Primary sensory and motor centres in the teleost brain are located mainly in the olfactory bulb, the tectum, some diencephalic regions and the mesencephalic

tegmentum and medulla oblongata of the rhombencephalon (Wullimann *et al.,* 1996). The diencephalon, mesencephalic tegmentum and medulla oblongata of the rhombencephalon together make up a functional region brain known as the brain stem (Nieuwenhuys and Pouwels, 1983). The brain stem provides an information relay station between higher brain processing centres and the neurons that project to and from many structures in the periphery (Whelan, 1996; Deliagina *et al.,* 2000; Bosch and Roberts, 2001; Deliagina *et al.,* 2002).

3.2 Materials and Methods

3.2.1 Animals

This study includes uninjured European eels, *Anguilla anguilla* L., (total n = 4) obtained from the wild in Co. Mayo and Co. Westmeath, of unknown sex in a non-migratory, immature stage of the life cycle. Animals ranged in length from 350 to 500 mm. The eels were anaesthetized and the tissue was fixed for ISH as described in section 2.2.1. Serial sections of 100 μm in thickness were cut in the horizontal (n = 2) or transverse plane (n = 1) with a vibrating microtome (VT1000S, Leica).

3.2.2 Histology

The perfused brain of another eel was equilibrated in 30% sucrose/PBS solution overnight at 4 °C and frozen in optimal cutting temperature compound (BDH) embedding medium on Dry Ice the following day. Serial sections (30 μm) were cut horizontally on a cryostat (Bright) and stained with cresyl violet (0.5 %) for the study of cytoarchitecture. A transverse series from the midbrain region (diencephalon and mesencephalon) of another fish, stained with cresyl violet and prepared previously, was also consulted during the identification of the cell masses.

3.2.3 In situ hybridisation

Sense and antisense probes were prepared as described in section 2.2 from plasmid DNA containing a 467 bp BDNF cDNA insert. Probes were also prepared for TH as described in section 2.2. ISH was carried out as described in section 2.2.11. Negative (sense) controls were included in all experiments. Some sections probed for TH served as positive controls for ISH experiments.

3.2.4 ISH analysis - Visual comparisons, reconstructions

Light microscopic images were collected using a microscope (Olympus BX41) and a digital camera and processed using Adobe Photoshop 6.0. The locations of all labeled cell profiles were charted at 40 X magnification from serial transverse sections using a drawing tube attached to a microscope (a camera lucida). Cells were included when cellular staining was clearly visible above background and not present in sense controls. Reconstructions of the overall staining patterns in the telencephalon, mesencephalon, diencephalon and rhombencephalon were compiled from the camera lucida drawings of transverse sections.

In order to designate the cells labeled after ISH, correlations were made between the cresyl violet-stained and ISH sections. Standard nomenclature was used to facilitate comparison with previous studies on teleost fish (Butler and Hodos, 1996), nevertheless, there are some inconsistencies between authors in the nomenclature of various nuclei in the

teleost brain (Nieuwenhuys and Pouwels, 1983; Schellart, 1990; Mukuda and Ando, 2003). Difficulties were encountered therefore in the nomenclature of specific nuclei since a comprehensive brain atlas for *A. anguilla* is not yet available. A brain atlas is available for *A. japonica* (Mukada and Ando, 2003), a species closely related to *A. anguilla* (Minegishi *et al.,* 2005). However, the diagrammatic quality is poor and the authors frequently used different nomenclature from other brain studies on fish (Braford and Northcutt, 1983; Nieuwenhuys and Pouwels, 1983; Meredith *et al.* 1987; Roberts *et al.,* 1989; Molist *et al.* 1993; Wullimann *et al.*, 1996; Bosch and Roberts, 2001). In order to overcome these difficulties, nomenclature was chosen for cellular groups in the eel brain based on studies concerning *A. anguilla* (Meredith *et al.*, 1987; Meredith and Roberts, 1987; Roberts *et al.,* 1989; Molist *et al.*, 1993; Kapsimali *et al.,* 2000; Bosch and Roberts, 2001) and where this was not possible, the brain atlas for *A. japonica* (Mukada and Ando, 2003) was followed.

Tables were compiled in line with Conner *et al.* (1997) so as to summarise the results of microscopic observation of histochemical sections from the three animals used in this study. In all tables, the reader is referred to Figure 3.2 in order to aid in the location of cellular groups mentioned. Staining of brain nuclei was rated on the basis of intensity where "absent" (denoted by -) indicates that there was no staining, "light" (denoted by +), faint staining visible above background, "moderate" (denoted by ++), where staining was clearly visible, and "strong" (denoted by +++), where cells were stained dark purple. The cellular pattern expression pattern was also described on the basis of a rating system where 1 = staining of occasional cells (approximately one or two cells within the nucleus in each section), 2 = staining of some scattered cells (approximately five to six cells within the nucleus in each section), 3 = staining of the majority of cells in the nucleus, 4 = staining of many small densely packed cells in a nucleus.

3.3 Results

Staining for BDNF mRNA with antisense probes was restricted to cells possessing a neuronal morphology. Corresponding staining was not observed in sections hybridised to the BDNF sense control probe (Figs 3.6 b, d and f; Fig. 3.12 b and d; Fig. 3.14 b and d; Fig. 3.16 b and d; Fig. 3.17 b, d and f). Clear signals were observed in sections that served as positive controls, hybridised with the antisense TH probe (data not shown). No signal was seen in sections hybridised to TH sense control probes (data not shown).

Drawings mapping the overall expression pattern of BDNF mRNA in the eel brain were constructed from transverse sections with a camera lucida (Fig. 3.2). Figure 3.1 shows the eel brain in sagittal view with vertical lines indicating the level of the transverse sections (A-X) used in Figure 3.2. As the drawings were compiled from two to five sections, they do not exactly match any one photomicrograph.

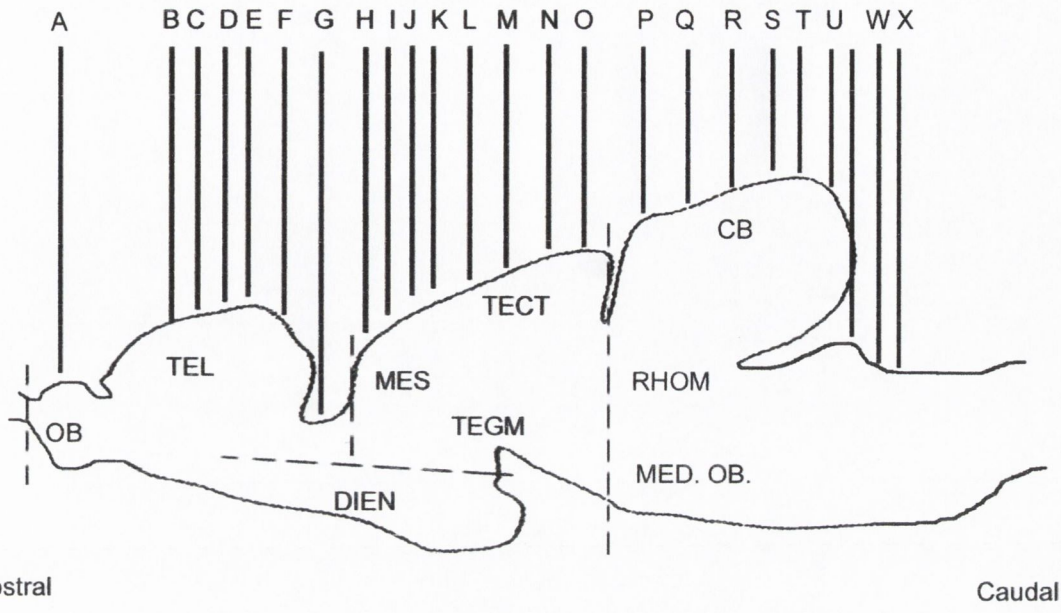
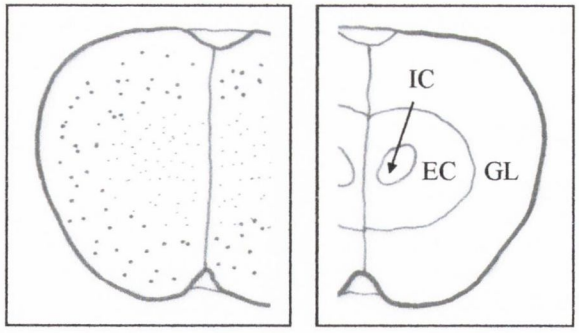


Figure 3.1. A schematic diagram of the eel brain in the sagittal plane. The telencephalon is the rostralmost division of the brain and is made up of the olfactory bulb (OB) and the telencephalic hemispheres (TEL). The mesencephalon (MES) is located caudal and dorsal to the telencephalon. It comprises the tectum (TECT) and the tegmentum (TEGM). The diencephalon (DIEN) is located in the ventral region of the telencephalic hemispheres and extends ventrally to the caudal portion of the tegmentum. The rhombencephalon (RHOM) consists of the cerebellum (CB) and the medulla oblongata (MED. OB.). The anatomical divisions of the telencephalon, mesencephalon, diencephalon and rhombencephalon are roughly demarcated by the broken lines. The brain stem, a functional division of the brain, is made up of the diencephalon, the tegmentum and the medulla oblongata. The vertical lines indicate the level of the transverse sections (A - X) used in Fig. 3.2. The letter V has not been included above its corresponding vertical line for the purposes of diagrammatic clarity.

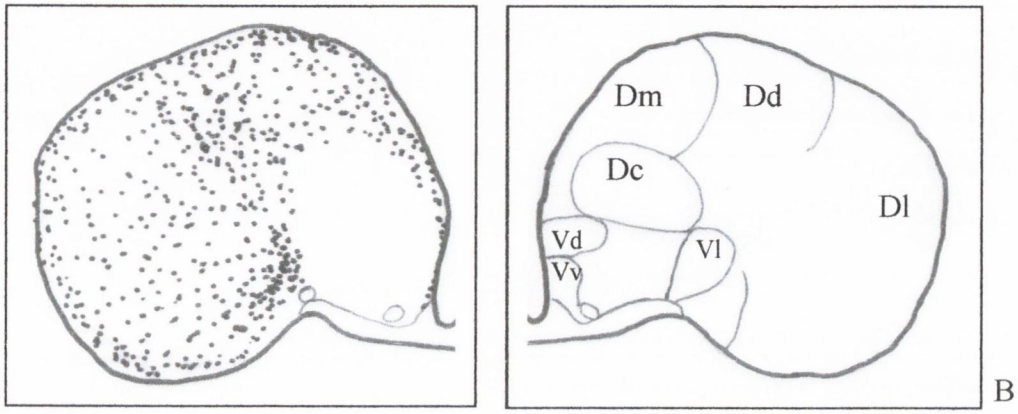
Figure 3.2. Camera lucida drawings showing the overall BDNF mRNA expression pattern in transverse sections of the eel brain. The locations of all labeled cells were charted at 40 X magnification. This figure spans pages 67 - 82 and abbreviations relevant to each drawing page are shown on the opposing page.

Abbreviations for Fig. 3.2A - D

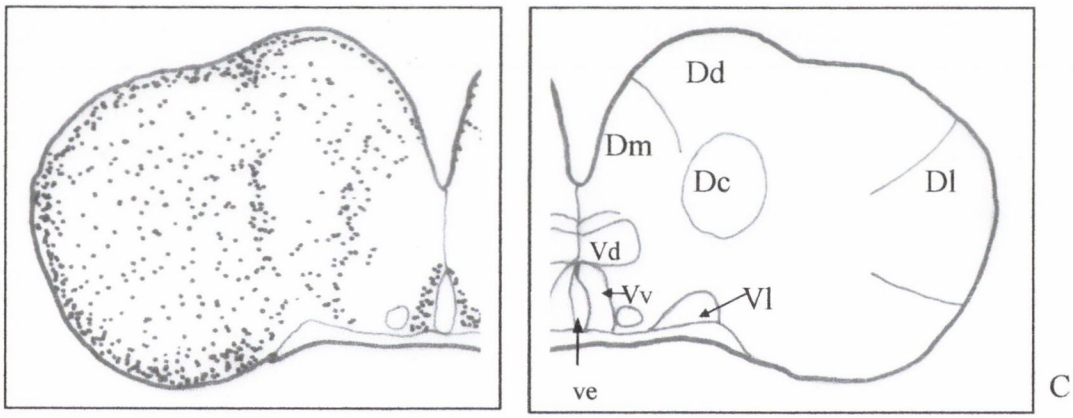
ac	anterior commissure
Dc	central zone of the area dorsalis
Dd	dorsal zone of the area dorsalis
Dl	lateral zone of the area dorsalis
Dld	dorsal portion of lateral zone of the area dorsalis
Dlv	ventral portion of lateral zone of the area dorsalis
Dm	medial zone of the area dorsalis
E	entopeduncular nucleus
EC	external cell layer
GL	glomerulus (olfactory bulb)
LC	locus coeruleus
nT	nucleus taenia
PPa	nucleus preopticus parvocellularis anterior
Vc	commissural nucleus of the area ventralis
Vd	dorsal nucleus of the area ventralis
VL	ventrolateral nucleus of the thalamus
Vs	supracommissural nucleus of the area ventralis
Vv	ventral nucleus of the area ventralis


IC
EC
GL

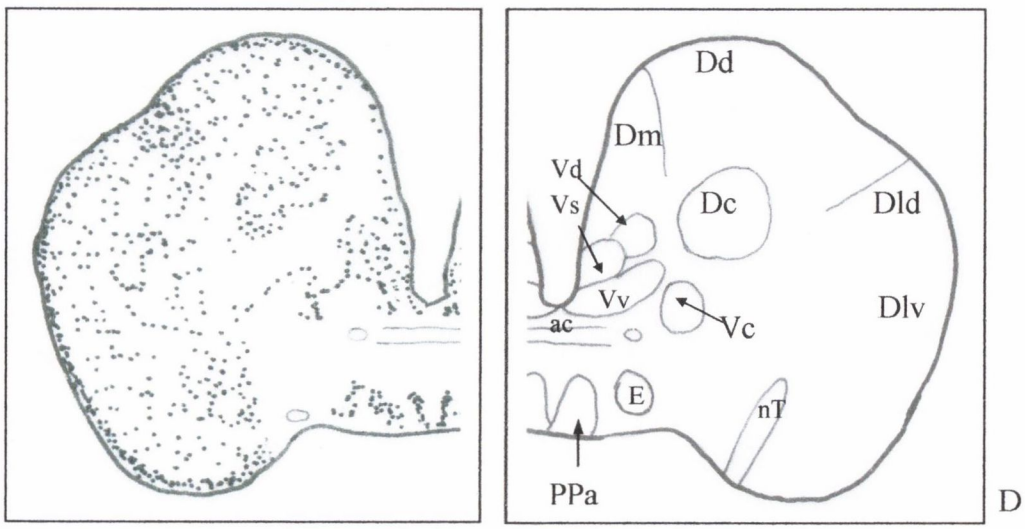
A


Dm
Dd
Dc
Dl
Vd
Vv
Vl

B


Dd
Dm
Dc
Dl
Vd
Vv
Vl
ve

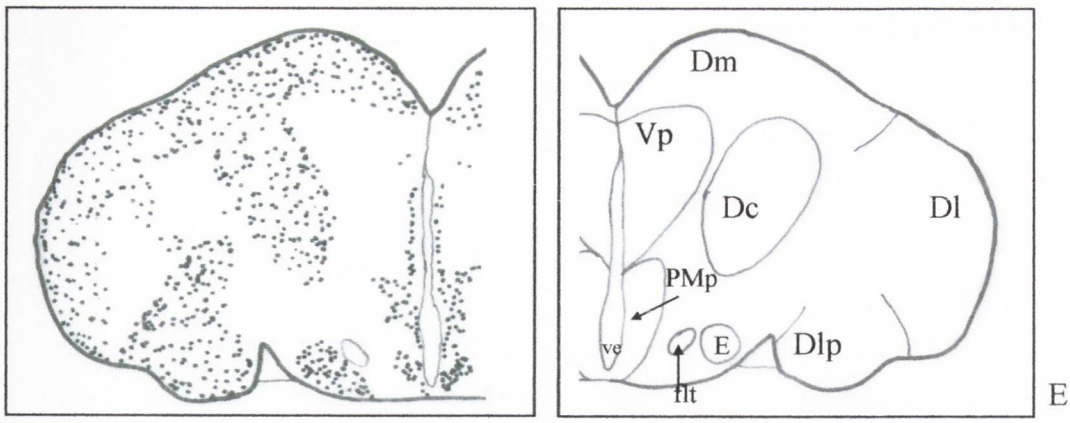
C


Dd
Dm
Vd
Vs
Dc
Dld
Vv
Dlv
ac
Vc
E
nT
PPa

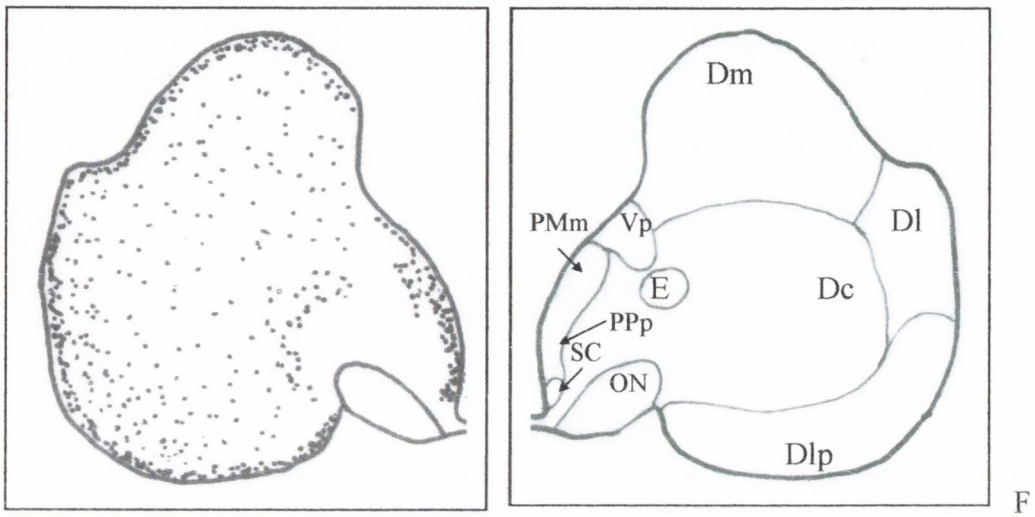
D

Abbreviations for Fig. 3.2E-G

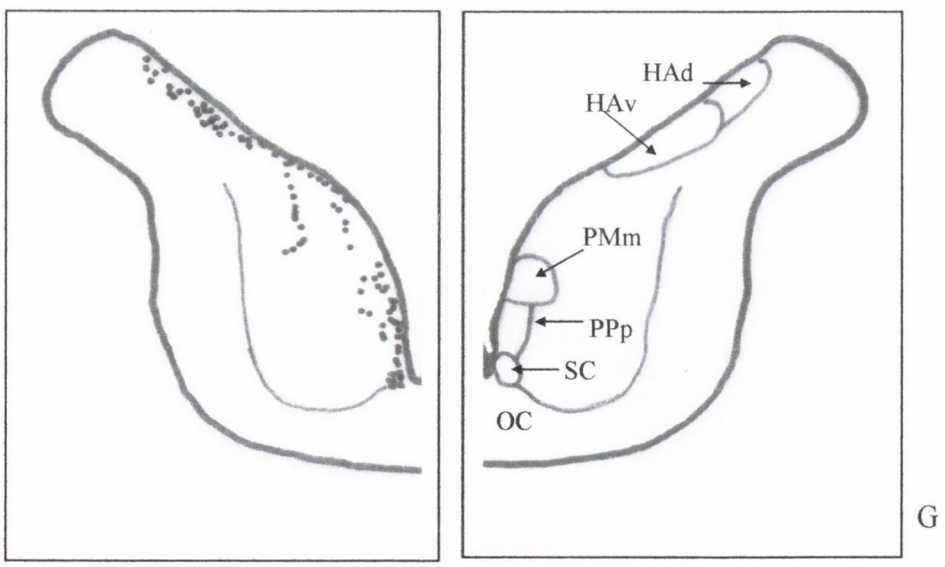
Dc	central zone of the area dorsalis
Dl	lateral zone of the area dorsalis
Dlp	posterior portion of lateral zone of the area dorsalis
Dm	medial zone of the area dorsalis
E	entopeduncular nucleus
flt	telencephalic lateral longitudinal fasciculus
HAd	dorsal habenular nucleus
HAv	ventral habenular nucleus
OC	optic chiasma
ON	optic nerve
PMm	nucleus preopticus magnocellularis, pars magnocellularis
PMp	nucleus preopticus magnocellularis, pars parvocellularis
PPp	nucleus preopticus parvocellularis posterior
SC	suprachiasmatic nucleus
ve	ventricle
Vp	posterior nucleus of the area ventralis


Dm
Vp
Dc
Dl
PMp
ve
E
Dlp
tlt

E


Dm
PMm
Vp
Dl
E
Dc
PPp
SC
ON
Dlp

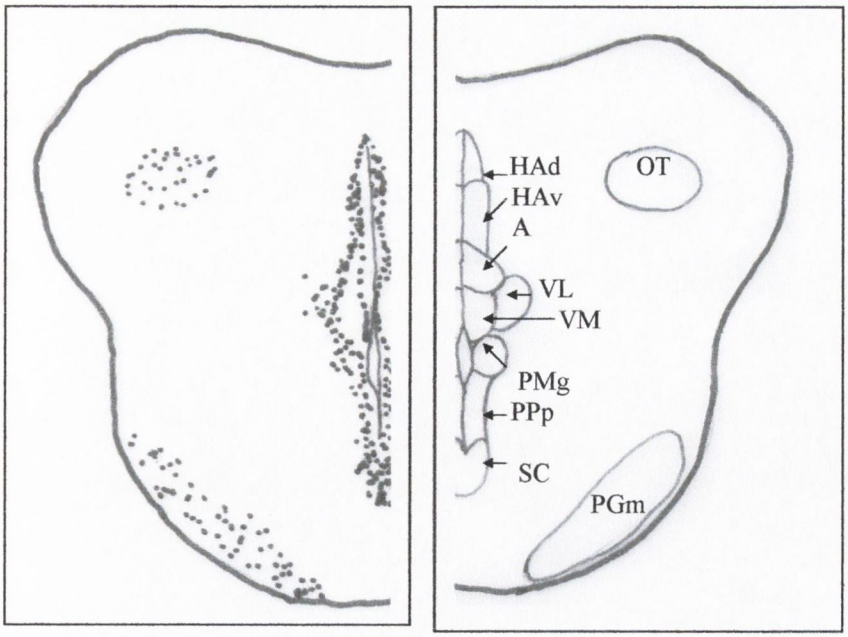
F


HAd
HAv
PMm
PPp
SC
OC

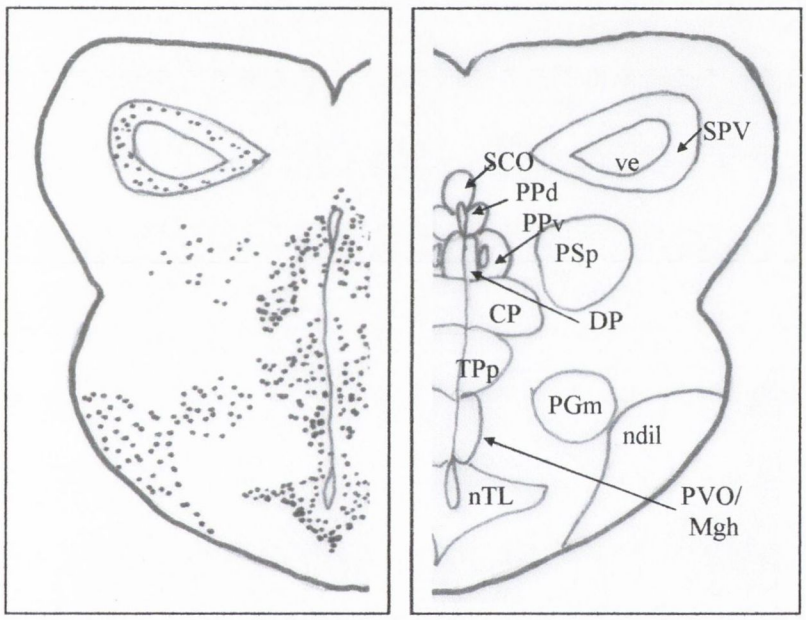
G

Abbreviations for Fig 3.2H-J

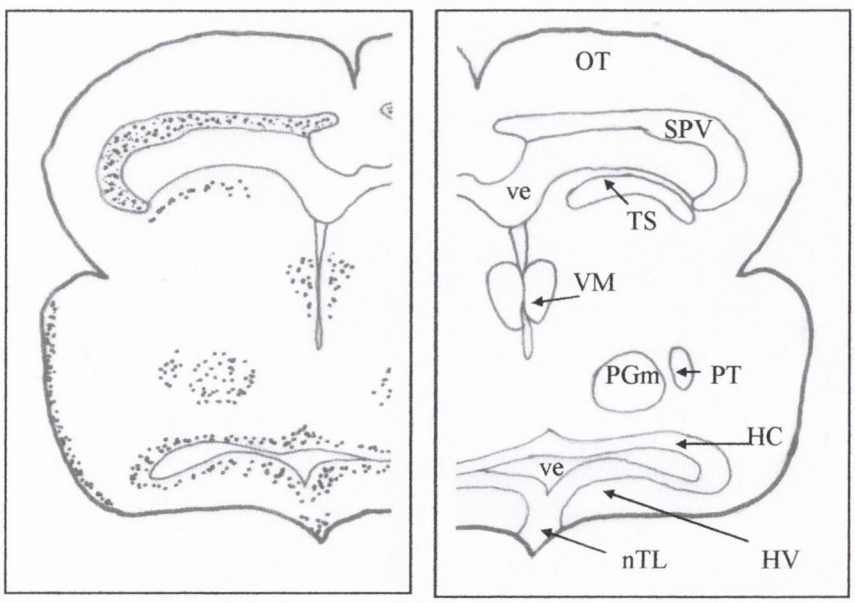
A	anterior thalamic nucleus
CP	central posterior thalamic nucleus
DP	dorsal posterior thalamic nucleus
HAd	dorsal habenular nucleus
HAv	ventral habenular nucleus
HC	caudal zone of periventricular hypothalamus
HV	ventral zone of periventricular nucleus hypothalamus
ndil	diffuse nucleus of inferior lobe
nTL	nucleus lateralis tuberis
OT	optic tectum
PPd	nucleus pretectalis periventricularis, pars dorsalis
PGm	preglomerular nuclear complex
PMg	nucleus preopticus magnocellularis, pars gigantocellularis
PPp	nucleus preopticus parvocellularis posterior
PPv	nucleus pretectalis periventricularis, pars ventralis
PSp	nucleus pretectalis superficialis, pars parvocellularis
PT	posterior thalamic nucleus
PVO	paraventricular organ
SC	suprachiasmatic nucleus
SCO	subcommissural organ
SPV	stratum periventricular
TPp	periventricular nucleus of posterior tuberculum
TS	torus semicircularis
ve	ventricle
VL	ventrolateral nucleus of the thalamus
VM	ventromedial nucleus of the thalamus


HAd
HAv
A
OT
VL
VM
PMg
PPp
SC
PGm

H


SPV
SCO
ve
PPd
PPv
PSp
CP
DP
TPp
PGm
ndil
nTL
PVO/
Mgh

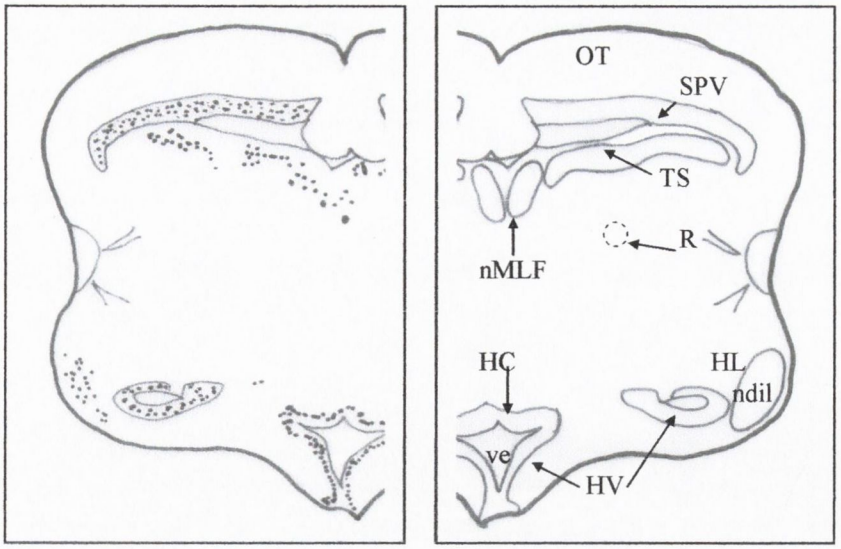
I


OT
SPV
ve
TS
VM
PGm
PT
HC
ve
nTL
HV

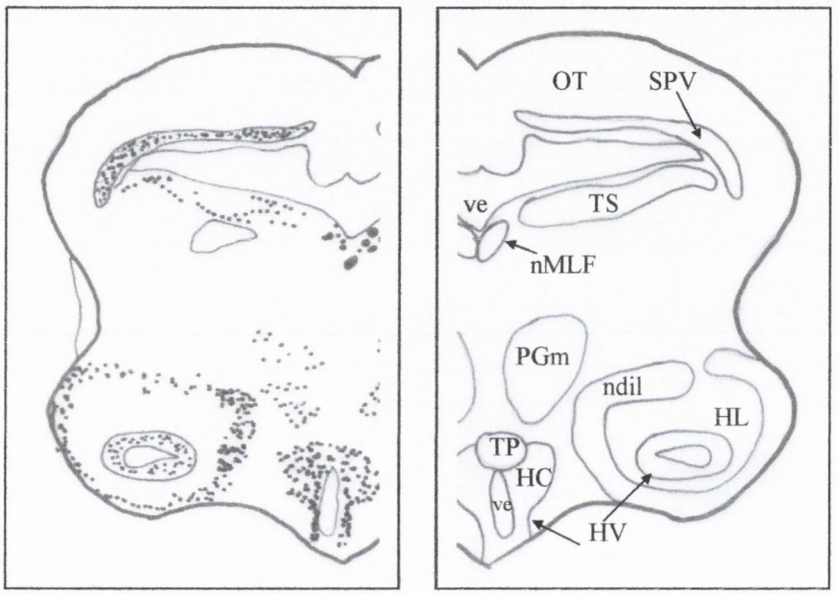
J

Abbreviations for Fig. 3.2K-M

HC	caudal zone of periventricular hypothalamus
HL	inferior lobe of hypothalamus
HV	ventral zone of periventricular nucleus hypothalamus
MLF	medial longitudinal fasciculus
ndil	diffuse nucleus of inferior lobe
nIII	nucleus of oculomotor nerve
nMLF	nucleus of medial longitudinal fasciculus
OT	optic tectum
PGm	preglomerular nuclear complex
R	red nucleus
SPV	stratum periventricular
sv	saccus vasculosus
TP	nucleus posterior tuberis
TS	torus semicircularis
ve	ventricle


OT
SPV
TS
R
nMLF
HC
HL
ndil
ve
HV

K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TS
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PGm
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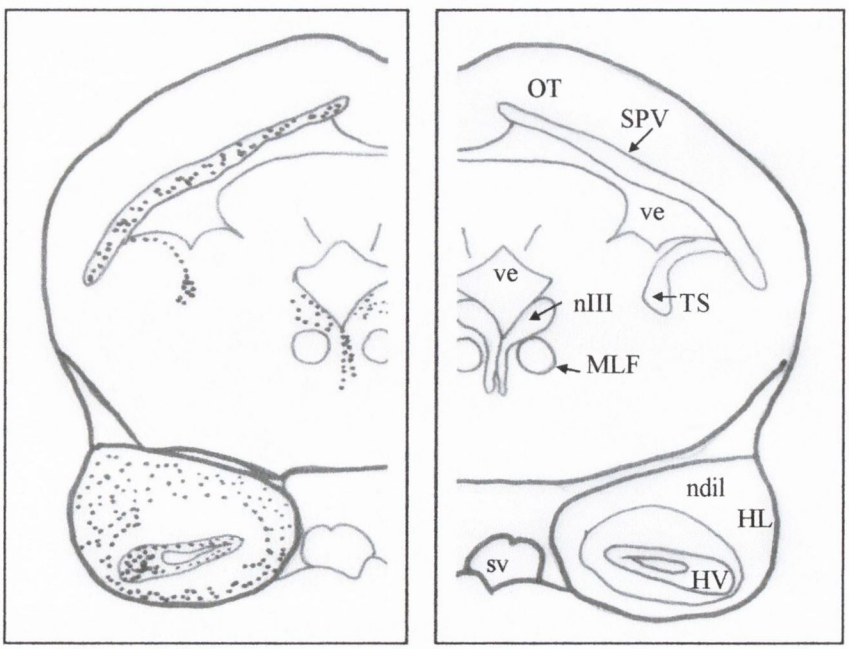
HL
TP
HC
ve
HV

L


OT
SPV
ve
ve
nIII
TS
MLF
ndil
HL
sv
HV

M

Abbreviations for Fig. 3.2N-P

CbSg granular layer of cerebellum

CbSm molecular layer of cerebellum

GS secondary gustatory nucleus

HL inferior lobe of hypothalamus

HV ventral zone of periventricular nucleus hypothalamus

LC locus coeruleus

LL lateral line nerve

LV nucleus lateralis valvulae

MLF medial longitudinal fasciculus

ndil diffuse nucleus of inferior lobe

nI nucleus isthmi

nIV nucleus of trochlear nerve

nMed nucleus medialis

nVm trigeminal motor nucleus

nVs trigeminal sensory nucleus

OT optic tectum

RS superior reticular nucleus

SO superior olive

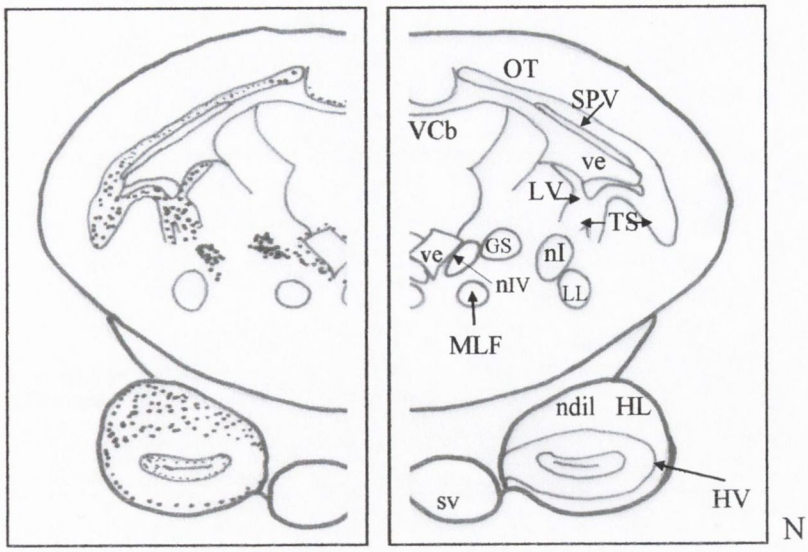
SPV stratum periventricular

SRF superior reticular formation

TS torus semicircularis

Vc commissural nucleus of the area ventralis

VCb valvula cerebelli


OT
SPV
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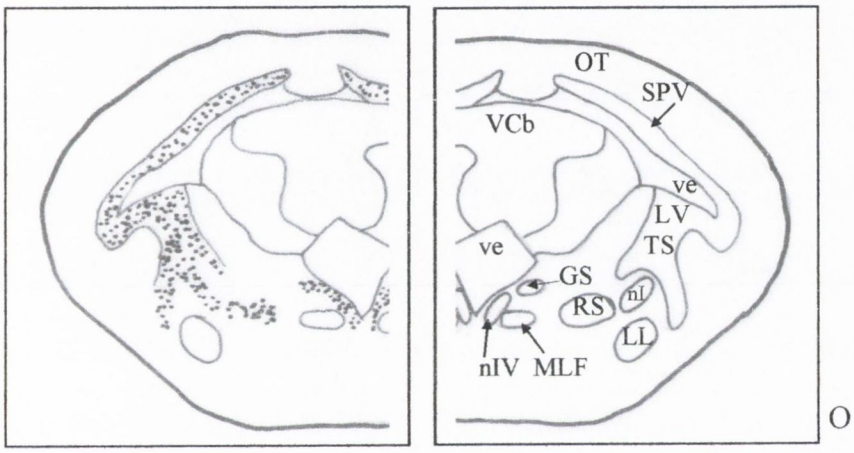
ve
LV
TS
ve
GS
nI
nIV
LL
MLF
ndil
HL
sv
HV

N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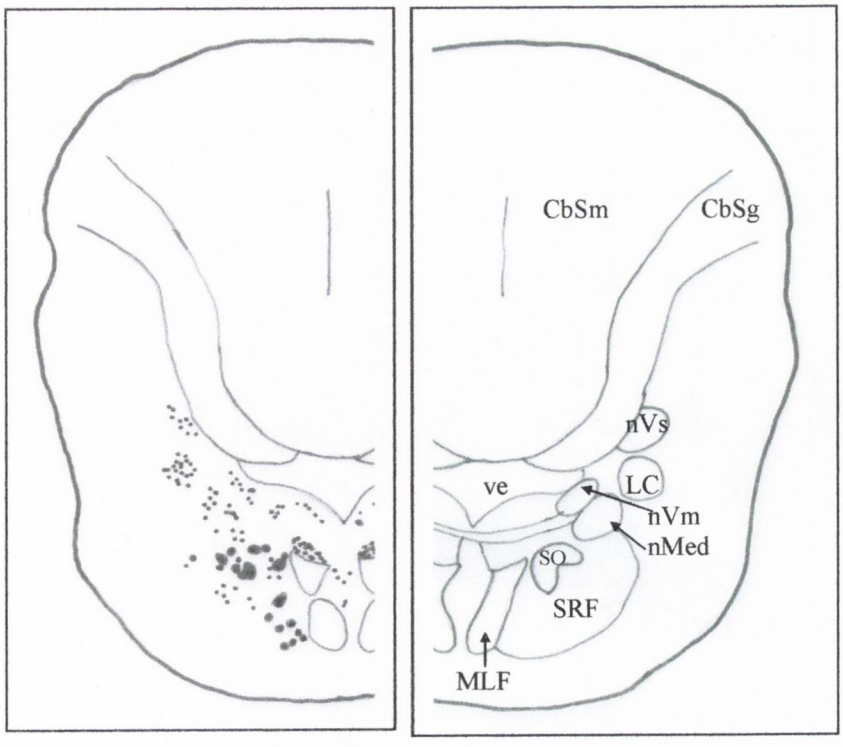
VCb
ve
LV
TS
ve
GS
RS
nI
LL
nIV
MLF

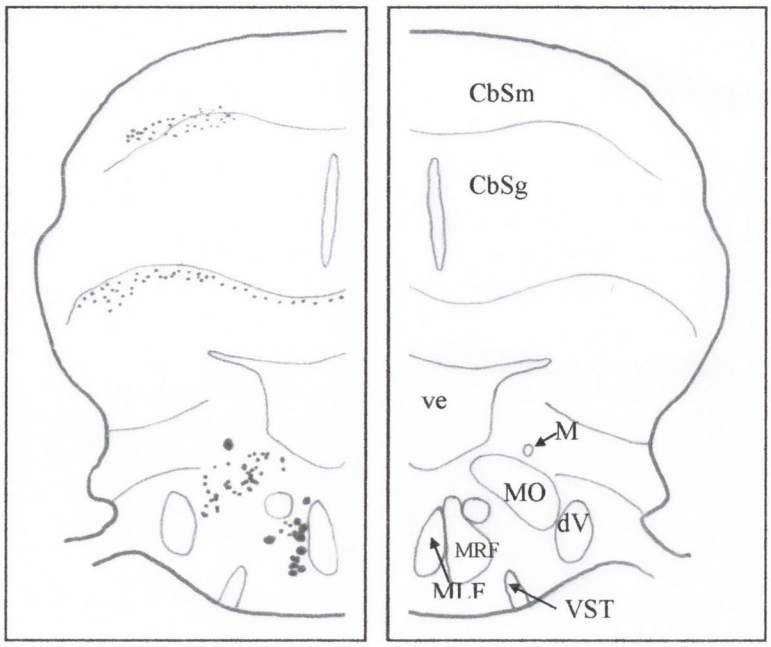
O


CbSm
CbSg
nVs
ve
LC
nVm
nMed
SO
SRF
MLF

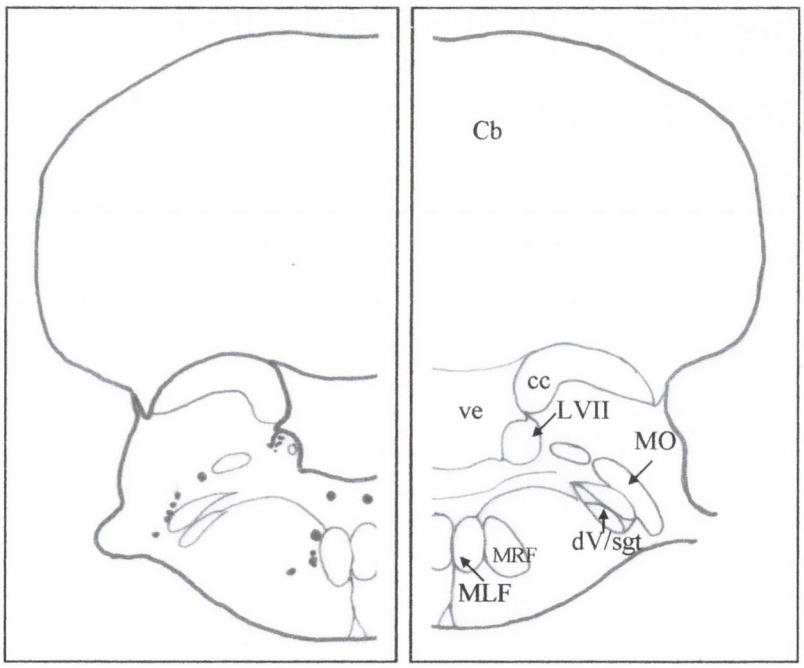
P

Abbreviations for Fig. 3.2Q and R

Cb	cerebellum
CbSg	granular layer of cerebellum
CbSm	molecular layer of cerebellum
cc	cerebellar crest
dV	descending tract of trigeminal nerve
LVII	facial lobe
M	Mauthner cell
MLF	medial longitudinal fasciculus
MO	magnocellular octavolateral nucleus
MRF	medial reticular formation
sgt	secondary gustatory tract
ve	ventricle
VST	vestibulospinal tract



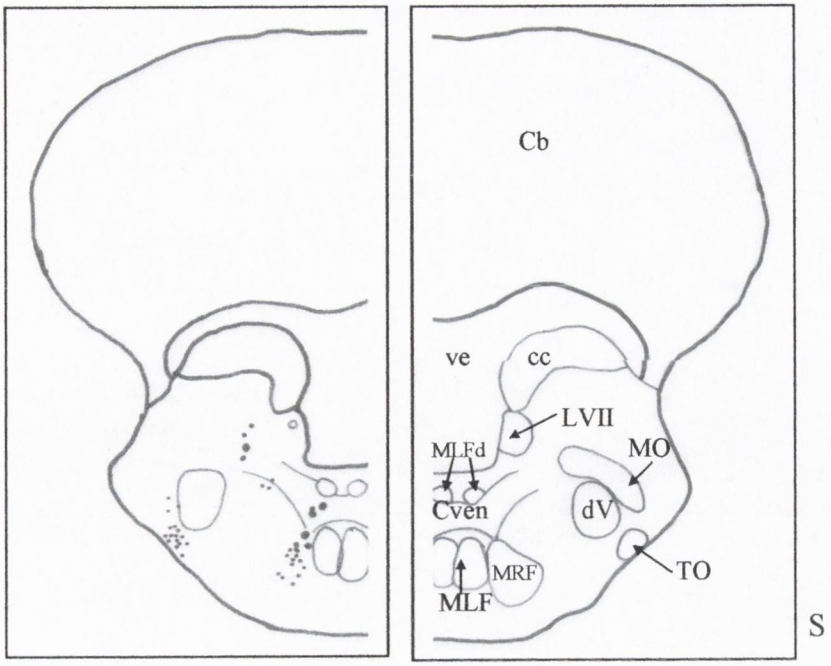
Q



R

Abbreviations for Fig. 3.2S-U

Cb	cerebellum
cc	cerebellar crest
Cven	ventral commissure of rhombencephalon
dV	descending tract of trigeminal nerve
IRF	inferior reticular formation
LLp	posterior lateral line nerve
LVII	facial lobe
MLF	medial longitudinal fasciculus
MLFd	dorsal subdivision of medial longitudinal fasciculus
MO	magnocellular octavolateral nucleus
MRF	medial reticular formation
nVIIm	facial motor nucleus
OEN	octavolateral efferent nucleus
Rinf	inferior raphe nucleus
sgt	secondary gustatory tract
TO	tangential octavolateral nucleus
ve	ventricle
VST	vestibulospinal tract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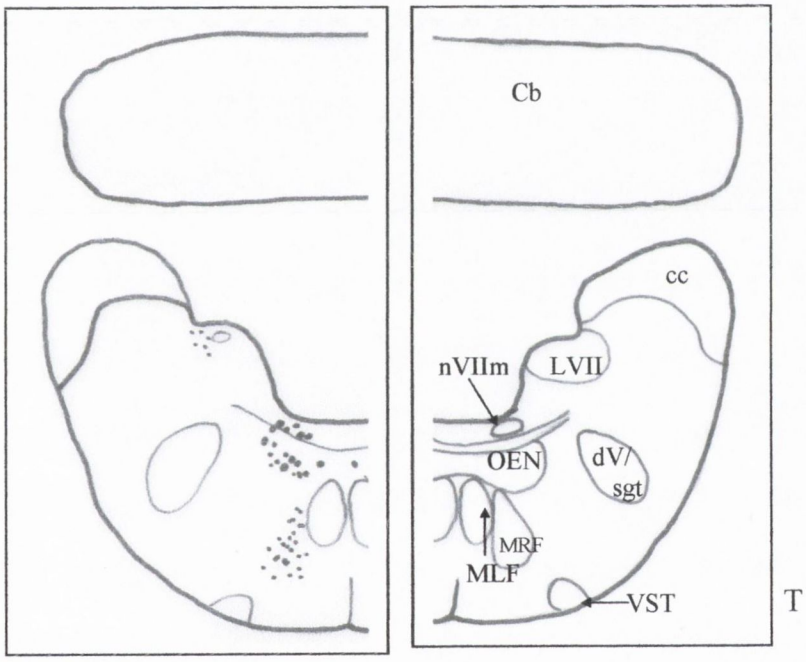
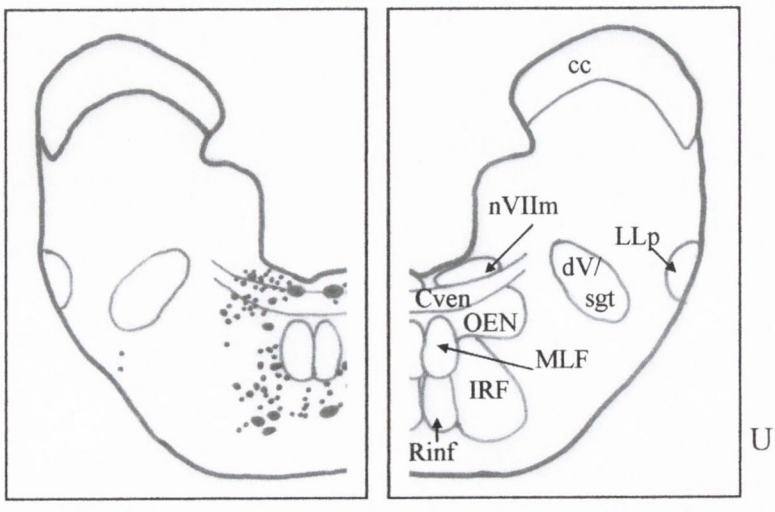
cc
LVII
MLFd
MO
Cven
dV
MRF
TO
MLF

S


Cb
cc
nVIIm
LVII
OEN
dV/
sgt
MRF
MLF
VST

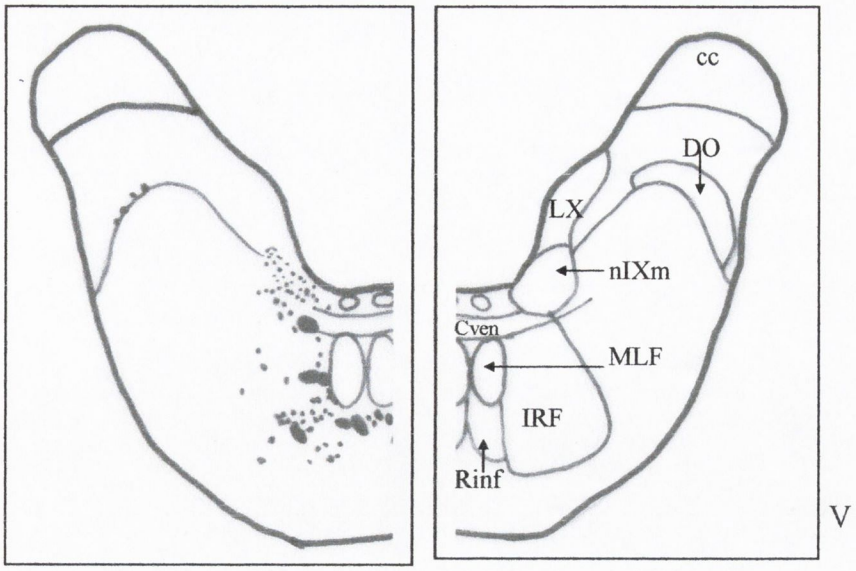
T


cc
nVIIm
LLp
dV/
sgt
Cven
OEN
MLF
IRF
Rinf

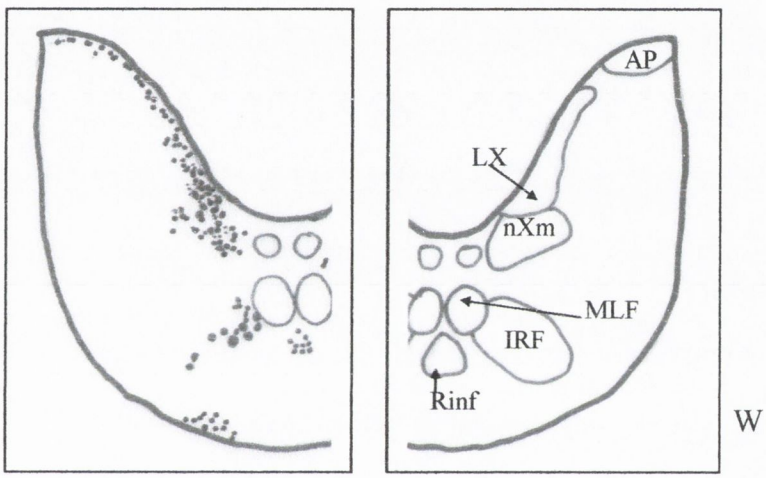
U

Abbreviations for Fig. 3.2V-X

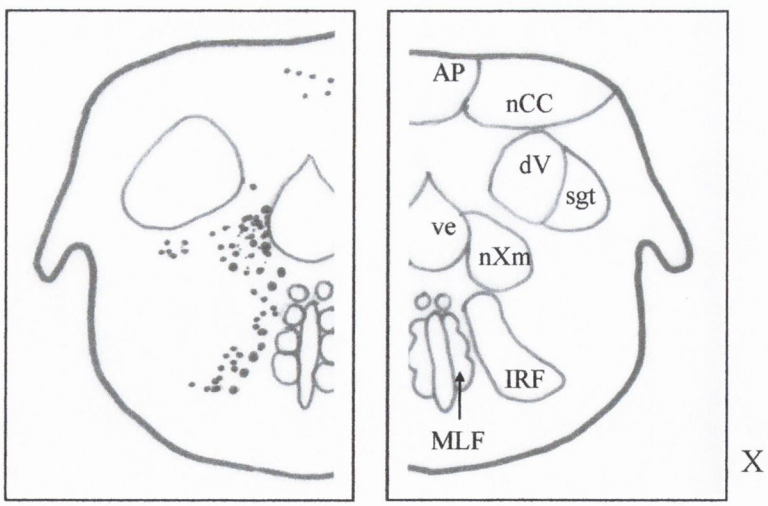
AP	area postrema
cc	cerebellar crest
Cven	ventral commissure of rhombencephalon
DO	descending octavolateral nucleus
dV	descending tract of trigeminal nerve
IRF	inferior reticular formation
LX	vagal lobe
MLF	medial longitudinal fasciculus
nCC	nucleus commissuralis of Cajal
nIXm	glossopharyngeal motor nucleus
nXm	vagal motor nucleus
Rinf	inferior raphe nucleus
sgt	secondary gustatory tract
ve	ventricle


cc
DO
LX
nIXm
Cven
MLF
IRF
Rinf

V


AP
LX
nXm
MLF
IRF
Rinf

W


AP
nCC
dV
sgt
ve
nXm
IRF
MLF

X

3.3.1 Telencephalon

The distribution of BDNF mRNA staining in the telencephalon is summarised in Table 3.1. In the olfactory bulb, some scattered small cells in the internal and external cell layers, and larger cells of the glomeruli were lightly stained (Figs. 3.2A).

The telencephalic hemispheres can be divided into the area ventralis, which consists of a number of periventricularly located nuclei and the area dorsalis, which is made up of large cell masses (Fig. 3.2B-F). In the rostral telencephalic hemispheres (Fig. 3.2B; Fig 3.3), moderate staining was seen mainly in the dorsal (Dd; Fig 3.3 b), medial (Dm), lateral (Dl) and the dorsal part of the central (Dc) zone of the area dorsalis and only the lateral nucleus of the area ventralis (Vl; Fig. 3.3 c). More caudally in the telencephalon (Figs.3.2C), moderate staining was still apparent in the area dorsalis; however, staining was absent in the central zone of the area dorsalis (Dc). Moderate staining of small densely packed cells in the ventral nucleus of the area ventralis (Vv) was also apparent at this level, however the lateral nucleus of the area ventralis (Vl) was not stained (Fig. 3.2C).

At the level of the anterior commissure (Fig. 3.2D), light staining of most cells was seen in dorsal (Vd), supracommissural (Vs) and ventral (Vv) nuclei of the area ventralis, and of scattered cells in the commissural nucleus of the area ventralis (Vc). Moderate staining of most cells was again obvious in the central (Dc), dorsal (Dd) and lateral (Dl) zone of the area dorsalis. Moderate staining of a few scattered cells in the medial (Dm) zone was also seen. Cells in the entopeduncular nucleus (E) were lightly stained and moderate staining of cells of the nucleus taenia (nT) was also observed.

In sections caudal and adjacent to the anterior commissure of the telencephalon (Fig. 3.2E and Fig. 3.4 a-d), densely packed cells in the posterior area of lateral zone of the area dorsalis (Dlp) were moderately stained (Fig. 3.4 b). The medial region of lateral zone of the area dorsalis (Dl) was not stained although more laterally located cells in the lateral zone did show a moderate signal. Most of the cells in the medial (Dm) and central (Dc; Fig.3.4 c) zone of the area dorsalis were moderately stained. A few scattered cells lining the ventricle in the posterior nucleus of the area ventralis (Vp) were lightly stained. As in the more rostral sections, light staining of scattered cells was also seen in the entopeduncular nucleus (E).

In caudalmost sections of the telencephalon (Fig. 3.2F and Fig. 3.5 a-c), the majority of cells in the medial (Dm), lateral (Dl), posterior lateral (Dlp; Fig. 3.5 b) and central (Dc) zone of the area dorsalis were moderately stained. A few scattered cells were lightly stained in the entopeduncular nucleus (E; Fig. 3.5 c) and the posterior nucleus of the area ventralis (Vp).

No corresponding ISH signals were obtained in telencephalic sections hybridised to BDNF sense control probes. Figure 3.6 a, c and e shows an example of BDNF mRNA expression in a horizontal section of the telencephalon alongside a serial section from the same brain region, hybridised to the BDNF sense probe (Fig. 3.6 b, d and f).

Table 3.1. Summary of BDNF mRNA expression in the telencephalon of *A. anguilla.*

Brain region Nucleus	Staining intensity/ cells stained §	Reference for Fig. 3.2
Olfactory bulb		A
Internal cell layer (IC)	+/2	
External cell layer (EC)	+/2	
Glomerulus (GL)	+/2	
Telencephalic nuclei of the telencephalic hemispheres		
Area dorsalis		
Medial zone (Dm; rostral)	++/3	B
Medial zone (Dm; medial)	++/2	C, D
Medial zone (Dm; caudal)	++/3	E, F
Lateral zone (Dl)	++/3	B-F
Lateral zone, posterior area (Dlp)	++/4	E, F
Dorsal zone (Dd)	++/3	B-D
Central zone (Dc; rostral)	-	B, C
Central zone (Dc; caudal)	++/3	D-F
Nucleus taenia (nT)	++/3	D
Entopeduncular nucleus (E)	+/2	D-F
Area ventralis		
Lateral nucleus (Vl; rostral)	++/4	B
Lateral nucleus (Vl; caudal)	-	C
Dorsal nucleus (Vd; rostral)	-	B, C
Dorsal nucleus (Vd; caudal)	+/3	D
Ventral nucleus (Vv; rostral)	-	B
Ventral nucleus (Vv; medial)	++/4	C
Ventral nucleus (Vv; caudal)	+/3	D
Supracommissural nucleus (Vs)	+/3	D
Commissural nucleus (Vc)	+/2	D
Posterior nucleus (Vp)	+/2	E, F

§ Staining intensity: - = absent += light ++ = moderate +++=strong; cells stained: 1= occasional cells, 2= some scattered cells, 3=most cells stained, 4=densely packed cells

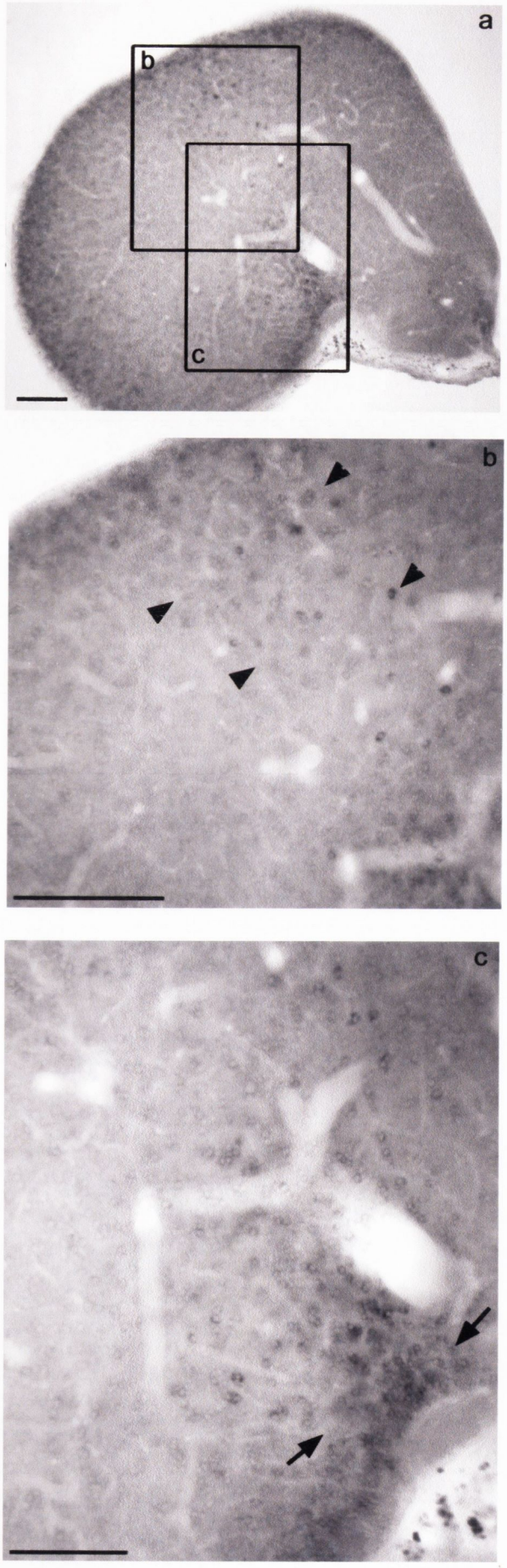


Figure 3.3. BDNF mRNA expression in a transverse section of the the rostral telencephalon. This figure corresponds to Figure 3.2B. a: Low power image of the section. b and c are higher power images of boxes b and c in a respectively. b: Staining of cells in the dorsal zone of the area dorsalis (Dd) indicated by arrowheads. c: Shows BDNF mRNA expression indicated by arrows in the lateral nucleus of the area ventralis (Vl). Scale bars = 200 μm.

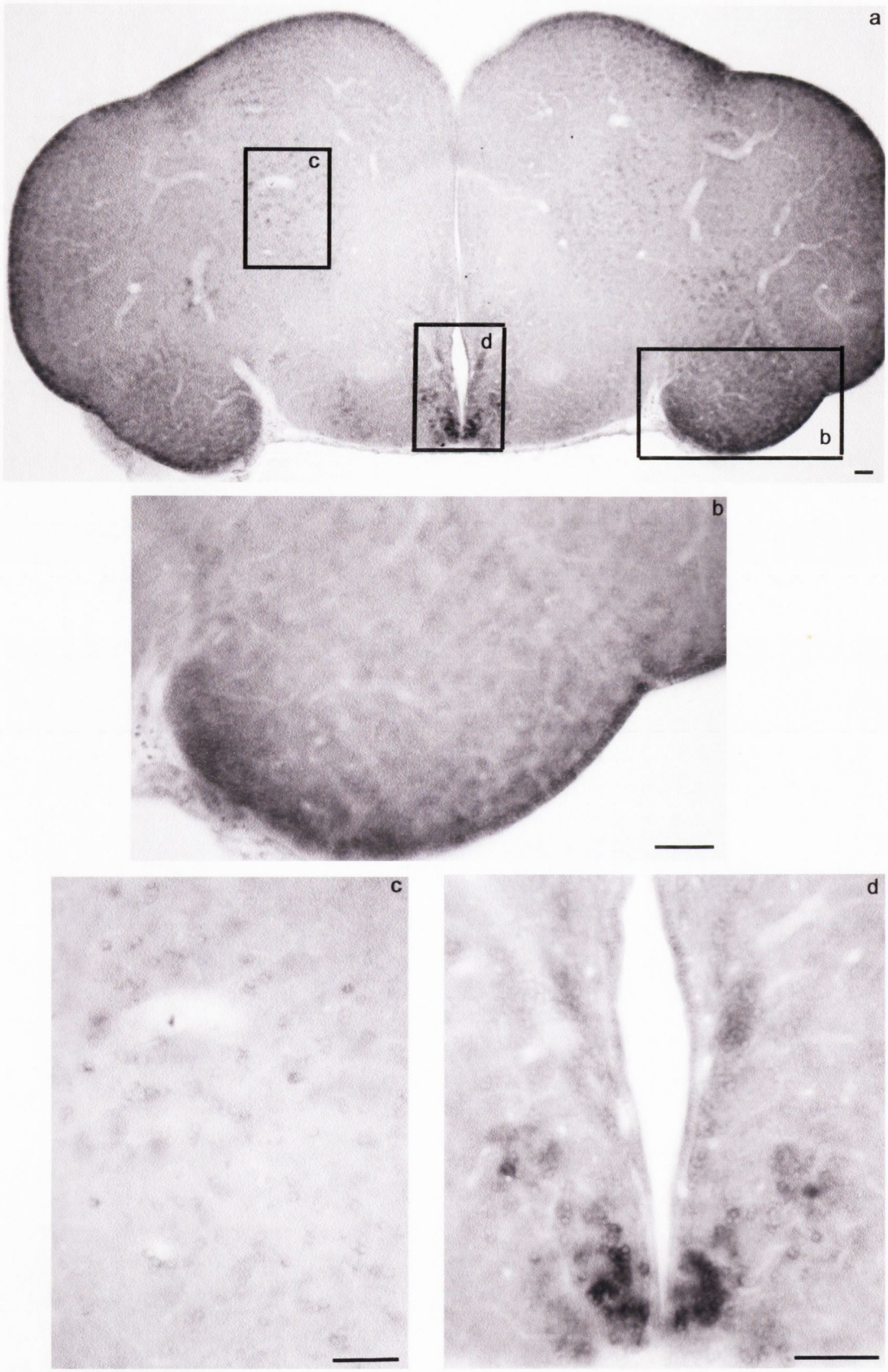


Figure 3.4. BDNF mRNA expression in a transverse section of the caudal telencephalon (b and c) and rostral diencephalon (d). The brain region shown corresponds to the region depicted in Figure 3.2E. a: Low power image of a transverse section showing the overall staining pattern in this region. b, c, d: Higher power photographs of stained cells in the central zone of area dorsalis (box c in a), lateral zone of the area dorsalis (box b in a) and nucleus preopticus magnocellularis, pars parvocellularis (box d in a), respectively. Scale bars = 100 μm

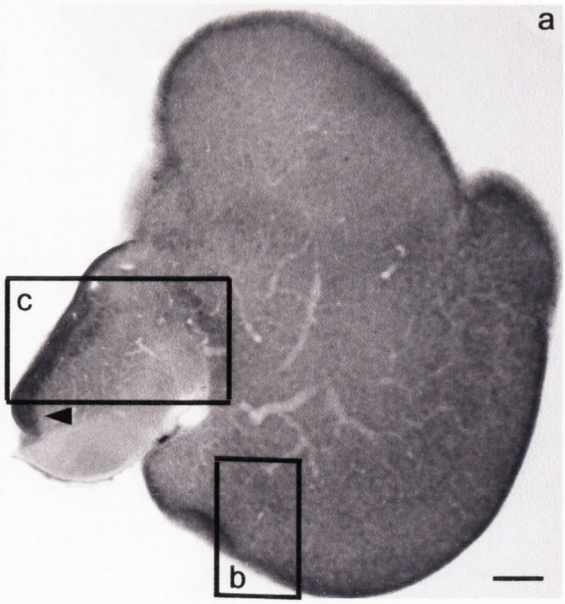
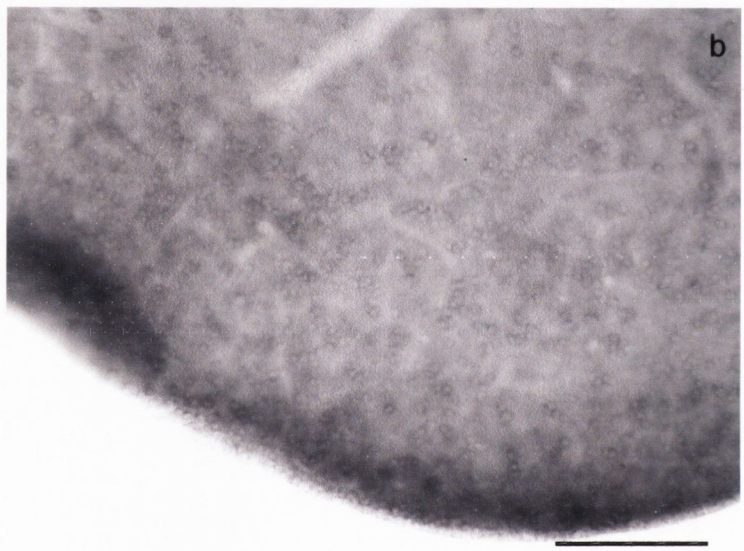
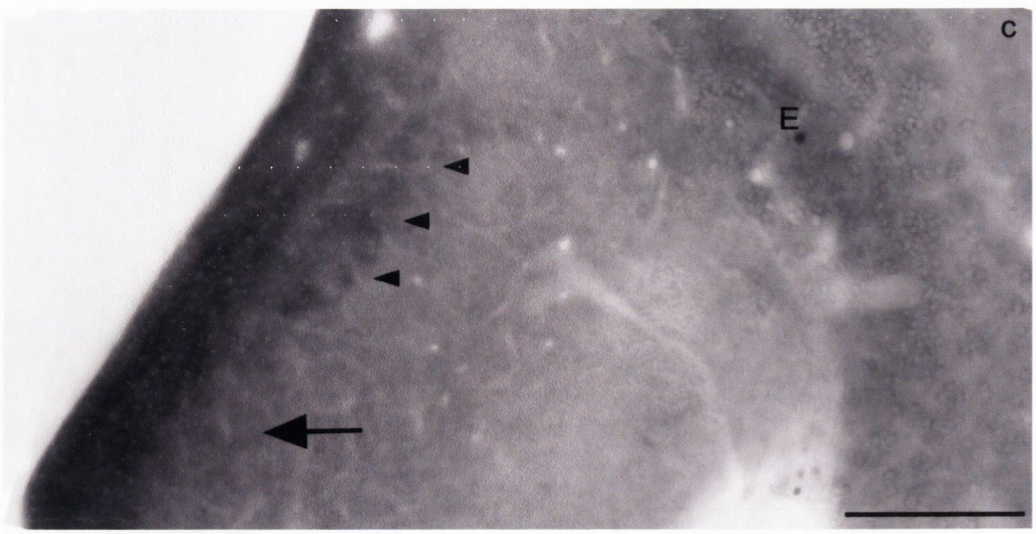


Figure 3.5. Photomicrographs showing BDNF mRNA expression in the nucleus preopticus magnocellualris, pars magnocellualis (PMm), the posterior nucleus preopticus parvocellularis (PPp), suprachiasmatic nucleus (SC) and in regions of the telencephalon including the lateral zone of the area dorsalis. The brain region pictured corresponds to the area illustrated in Figure 3.2F. a: Low power image of the overall expression pattern in the transverse section photographed. The SC is indicated in a by the arrowhead. b: Higher power image of staining in the lateral zone of the area dorsalis (box b in a).c: Higher power image of box c in a. The PMm and PPp are indicated by the arrowheads and arrow respectively. The entopeducular nucleus is located at E. Scale bars = 200 μm.





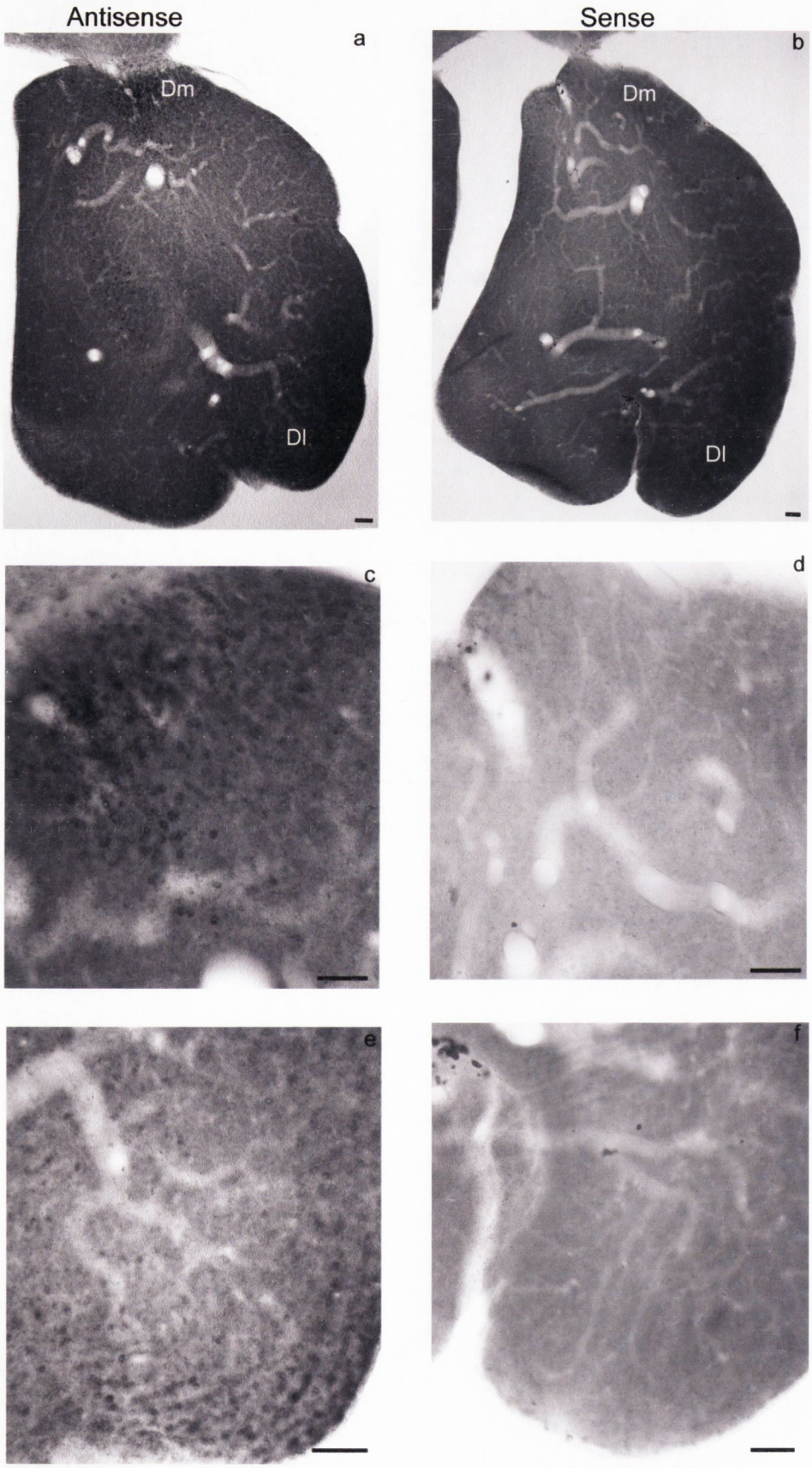


Figure 3.6. Photomicrographs of BDNF mRNA expression in horizontal sections of the telencephalic hemispheres with corresponding sense controls. a: Low power photo of staining in telencephalic hemispheres. c: High power image of stained cells in the medial zone of the area dorsalis (Dm in a). e: High power image of lateral zone of the area dorsalis (Dl in a). b, d and f are the corresponding sense controls for a, c and e respectively. Scale bars= 100 μm.

3.3.2 Diencephalon

BDNF mRNA expression in the nuclei of the diencephalon is summarised in Table 3.2. In the rostral portion of the diencephalon, BDNF mRNA expression was visible in the nucleus preopticus parvocellularis anterior (PPa), which showed moderate staining in many cells (Fig. 3.2D). In the rostral nucleus preopticus parvocellularis posterior (PPp), strong staining of small densely packed cells was observed (Fig. 3.2F and Fig. 3.5 c) and more caudally, densely packed cells were moderately stained in this nucleus (Fig. 3.2G and H). Staining was also obvious in the magnocellular nuclei of the preoptic area (PMp, PMm and PMg; Fig. 3.2E-H). Strong staining of most cells in the nucleus preopticus magnocellularis, pars parvocellularis (PMp; Fig. 3.2E and Fig. 3.4 d) was observed. The majority of cells in the rostral area of the nucleus preopticus magnocellularis, pars magnocellularis (PMm; Fig. 3.2F and Fig. 3.5 c) were heavily stained whereas more caudally, a few scattered cells in this nucleus showed moderate staining (Fig. 3.2G). Small densely packed cells were moderately stained in the nucleus preopticus magnocellularis, pars gigantocellularis (PMg; Fig. 3.2H and Fig. 3.7 b). BDNF mRNA expression was also evident in the suprachiasmatic nucleus (SC), the cells of which were heavily stained in the rostral region of this nucleus (Fig. 3.2F and Fig. 3.5 a) and moderately stained more caudally (Fig. 3.2G and H; Fig. 3.7 a). In the epithalamus, most of the cells of the dorsal and ventral habenular nuclei (HAd and HAv; Fig. 3.2G and H) were moderately stained.

Nuclei of the thalamus, the hypothalamus, the posterior tuberculum and the pretectal area are located in the caudal diencephalon. Moderate to light staining of cells was seen various thalamic nuclei (Fig. 3.2H-J). Scattered cells were moderately stained in the ventrolateral nucleus of the thalamus (VL; Fig. 3.2H and Fig. 3.7 b). Staining was absent in the rostral portion of ventromedial nucleus of the thalamus (VM; Fig. 3.2H and Fig. 3.7 b) however, most cells were lightly stained in the caudal portion of this thalamic nucleus (VM; Fig. 3.2J). A few scattered cells were moderately stained in the anterior (A; Fig. 3.2H) and dorsal posterior (DP; Fig. 3.2I) thalamic nuclei. Most cells were moderately stained in the central posterior thalamic nucleus (CP; Fig. 3.2I and Fig. 3.8 c) and lightly stained in the posterior thalamic nucleus (PT; Fig. 3.2J).

Strong staining was apparent in densely packed hypothalamic cells with a periventricular location such as the nucleus of the lateralis tuberus (nTL; Fig. 3.2I and J; Fig. 3.8 b), the caudal (HC; Fig. 3.9 d) and ventral (HV; Fig. 3.9 e) zones of the periventricular hypothalamus (Fig. 3.2J-N and see also Fig. 3.10 d and e for cresyl violet staining of HC and HV). Moderate staining was also observed in some scattered cells in

the magnocellular hypothalamic nucleus (Mgh; Fig. 3.2I and Fig. 3.8 c) and in the majority of the cells in the diffuse nucleus of the inferior lobe (ndil; Fig. 3.2I, K-N; Fig. 3.8 d).

Pretectal nuclei that showed light staining included the subcommissural organ (SCO; Fig. 3.2I) in occasional cells and the nucleus pretectalis superficialis, pars parvocellularis (PSp; Fig. 3.2I) in scattered cells. Moderate staining was seen in the majority of cells in the nucleus pretectalis periventricularis, pars dorsalis (PPd; Fig. 3.2I and Fig. 3.8 c) and the nucleus pretectalis periventricularis, pars ventralis (PPv; Fig. 3.2I and Fig. 3.8 c).

Nuclei of the posterior tuberculum that showed light staining were the preglomerular nuclear complex in scattered cells (PGm; Fig. 3.2H-J, L; Fig. 3.8 d) and the periventricular nucleus of the posterior tuberculum in the majority of cells (TPp; Fig. 3.2I and Fig. 3.8 c) Moderate staining of most of the cells was seen in the paraventricular organ (PVO; Fig. 3.2I) and strong staining of densely packed cells in the nucleus posterior tuberis (TP; Fig. 3.2L; Fig. 3.10 d shows cresyl violet staining of this nucleus) was observed.

Corresponding staining of diencephalic nuclei was not seen in sections hybridised to BDNF sense control probes. See Figure 2.12A and G for an example of BDNF mRNA expression in a horizontal sections showing staining in the caudal zone of the periventricular hypothalamus (HC) alongside a serial section from the same brain region, hybridised to the BDNF sense probe (Fig. 2.12B and H).

Table 3.2. Summary of BDNF mRNA expression in the diencephalon of *A. anguilla.*

Brain region Nucleus	Staining intensity/ cells stained §	Reference for Fig. 3.2
Preoptic area		
Nucleus preopticus		
parvocellularis anterior (PPa)	++/3	D
magnocellularis, pars parvocellularis (PMp)	+++/3	E
magnocellularis, pars magnocellularis (PMm; rostral)	+++/3	F
magnocellularis, pars magnocellularis (PMm; caudal)	++/2	G
parvocellularis posterior (PPp; rostral)	+++/4	F
parvocellularis posterior (PPp; caudal)	++/4	G, H
magnocellularis, pars gigantocellularis (PMg)	++/4	H
Suprachiasmatic nucleus (SC; rostral)	+++/4	F
Suprachiasmatic nucleus (SC; caudal)	++/3	G, H
Epithalamus		
Dorsal habenular nucleus (HAb)	++/3	G, H
Ventral habenular nucleus (HAv)	++/3	G, H
Thalamus		
Anterior nucleus (A)	++/2	H
Ventrolateral nucleus (VL)	++/3	H
Ventromedial nucleus (VM; rostral)	-	H
Ventromedial nucleus (VM; caudal)	+/3	J
Dorsal posterior (DP)	++/2	I
Central Posterior (CP)	++/3	I
Posterior (PT)	+/3	J
Hypothalamus		
Magnocellular hypothalamic nucleus (Mgh)	++/3	I
Nucleus lateralis tuberis (nTL)	+++/4	I, J
Diffuse nucleus of inferior lobe (ndil)	+++/3	I, K-N
Caudal zone of periventricular hypothalamus (HC)	+++/4	J-L
Ventral zone of periventricular hypothalamus (HV)	+++/4	J-N
Pretectum		
Subcommissural organ (SCO)	+/1	I
Nucleus pretectalis		
periventricularis, pars dorsalis (PPd)	++/3	I
periventricularis, pars ventralis (PPv)	++/3	I
superficialis, pars parvocellularis (PSp)	+/2	I
Posterior tuberculum		
Preglomerular nuclear complex (PGm)	+/2	H-J, L
Periventricular nucleus of posterior tuberculum (TPp)	+/3	I
Paraventricular organ (PVO)	++/3	I
Nucleus posterior tuberis (TP)	+++/4	L

§ Staining intensity: - = absent += light ++ = moderate +++=strong; cells stained: 1= occasional cells, 2= some scattered cells, 3=most cells stained, 4=densely packed cells

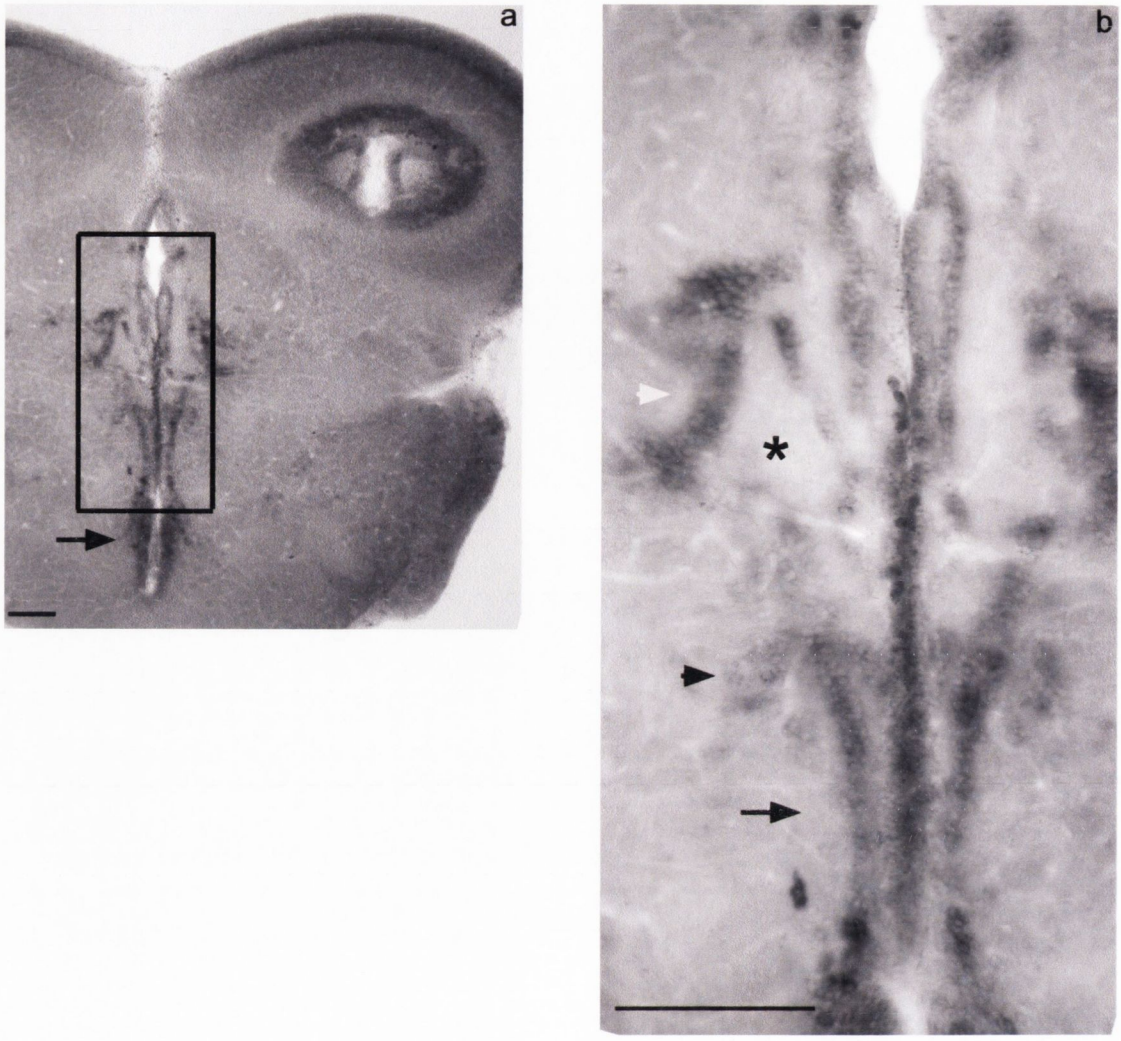


Figure 3.7. BDNF mRNA expression rostrally located nuclei of the diencephalon. a: Low power photograph of a transverse section from this brain region. Staining in the suprachiasmatic nucleus (SC) is indicated by the arrow. b: Higher power image of the boxed region in a. Staining in the ventrolateral nucleus of the thalamus (VL) is indicated by the white arrowhead. BDNF mRNA expression in the nucleus preopticus magnocellularis, pars gigantocellularis (PMg) is indicated by the black arrowhead. Staining in the nucleus preopticus parvocellularis, pars posterior (PPp) is denoted by the black arrow. Note lack of staining in the rostral portion of the ventromedial nucleus of the thalamus (VM) which is the region indicated by *. This figure corresponds to Figure 3.2H. Scale bars = 200 μm.

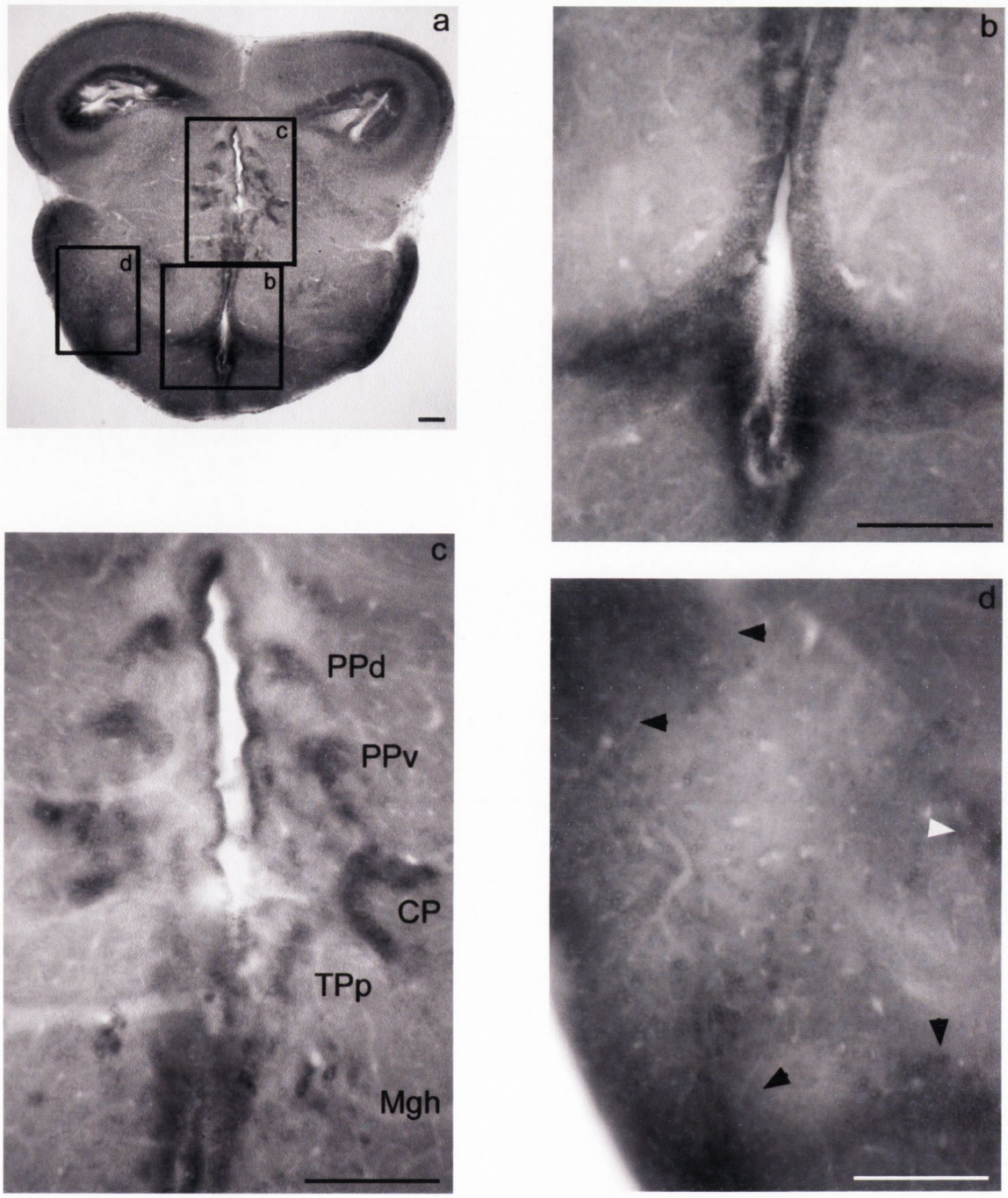


Figure 3.8. BDNF mRNA expression in a transverse section of the diencephalon at the level of the rostral portion of the nucleus lateralis tuberis (nTL). This figure corresponds to Figure 3.2I. a: Low power image of the section. b: Higher power image of box b in a showing staining in the nTL. c: Higher power photograph of box c in a showing staining in various diencephalic nuclei. Abbreviations: PPd: nucleus preopticus parvocellularis posterior; PPv: nucleus pretectalis periventricularis, pars ventralis; CP: central posterior thalamic nucleus; TPp: periventricular nucleus of posterior tuberculum; Mgh: magnocellular hypothalamic nucleus. d: Higher power image of box d in a showing BDNF mRNA expression in the diffuse nucleus of the inferior lobe (ndil) indicated by black arrowheads and in the preglomerular nuclear complex (PGm) indicated by white arrowhead. Scale bars = 200 μm.

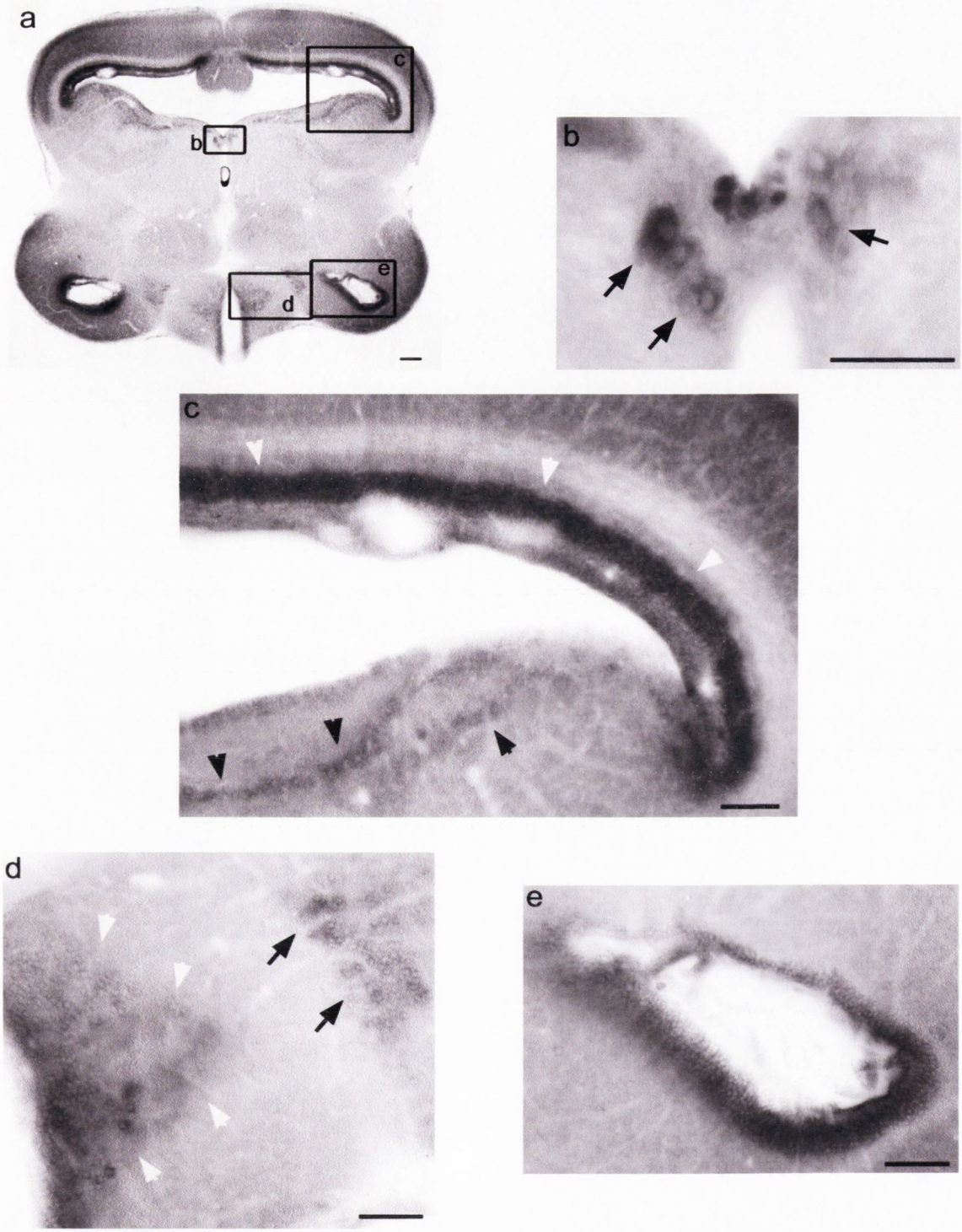


Figure 3.9. BDNF mRNA expression in a transverse section of the midbrain at the level of the nucleus of the medial longitudnal fasciculus (nMLF). This section corresponds to Figure 3.2L and see also Figure 3.10. a: Low power image of the section. b, c, d and e are higher photomicrographs of boxes b, c, d and e in a respectively. b: Staining in the cells of the nMLF is indicated by the black arrows. c: Shows staining in the stratum periventricular of the tectum indicated by the white arrowheads and in the cells of the torus semicircularis (TS) denoted by the black arrowheads. d: Staining of cells in the caudal zone of the periventricular hypothalamus (Hc) indicated by the white arrowheads and in the diffuse nucleus of the inferior lobe (ndil) indicated by the black arrows. e: Shows staining in the ventral zone of periventricular hypothalamus. This section is caudal to the nucleus posterior tuberis (TP) and therefore it is not featured in this photograph. Scale bars = 200 μm for a and 100 μm for b, c, d and e.

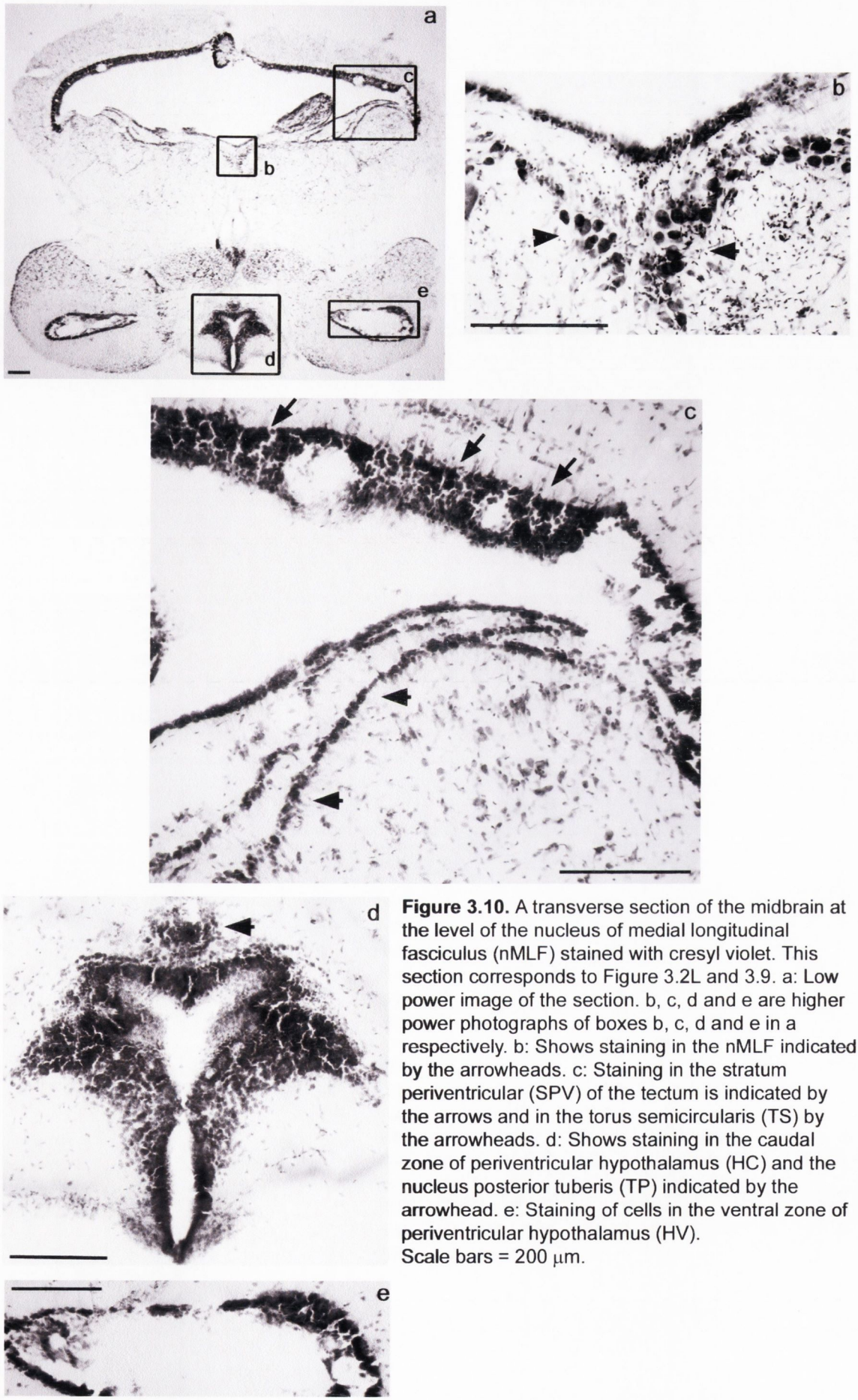


Figure 3.10. A transverse section of the midbrain at the level of the nucleus of medial longitudinal fasciculus (nMLF) stained with cresyl violet. This section corresponds to Figure 3.2L and 3.9. a: Low power image of the section. b, c, d and e are higher power photographs of boxes b, c, d and e in a respectively. b: Shows staining in the nMLF indicated by the arrowheads. c: Staining in the stratum periventricular (SPV) of the tectum is indicated by the arrows and in the torus semicircularis (TS) by the arrowheads. d: Shows staining in the caudal zone of periventricular hypothalamus (HC) and the nucleus posterior tuberis (TP) indicated by the arrowhead. e: Staining of cells in the ventral zone of periventricular hypothalamus (HV).
Scale bars = 200 μm.

3.3.3 Mesencephalon

Mesencephalic cells stained for BDNF mRNA after ISH are summarised in Table 3.3. The mesencephalon or midbrain consists of the tectum, the torus semicircularis (TS) and the tegmentum (Fig. 3.2H-O). The teleost tectum can be divided into seven layers (Meek, 1983, Nieuwenhuys and Pouwels, 1983, Meek, 1990, Molist *et al.,* 1993). The deepest layer known as the stratum periventriculare (SPV), containing numerous small, densely packed neurons, showed strong staining throughout the tectum (Fig. 3.2I-O; Fig. 3.9 c; Fig. 3.10 c shows cresyl violet staining of the SPV). The other layers of the tectum were not stained. Densely packed cells in the torus semicircularis (TS) were moderately stained (Fig. 3.2J-O; Fig. 3.9 c; Fig. 3.11 c; Fig. 3.10 c and 3.11 d show cresyl violet staining of the TS).

In the tegmentum, large cells in the nucleus of the medial longitudinal fasciculus (nMLF) were moderately stained (Fig. 3.2K, L; Fig. 3.9 b; Fig. 3.10 b is a high power image of cresyl violet staining in the nMLF) and moderate staining was also apparent in the superior reticular nucleus (RS, Fig. 3.2O and Fig. 3.11 c). Densely packed cells were moderately stained in the oculomotor nucleus (III; Fig. 3.2M) and nucleus of trochlear nerve (IV; Fig. 3.2N and O; Fig. 3.11 e; see Fig. 3.11 f for cresyl violet staining of IV) and heavily stained in the nucleus lateralis valvulae (LV; Fig. 3.2N and O; Fig. 3.11 a; see Fig. 3.11 d for cresyl violet staining of LV). Moderate staining of most cells was also seen in the secondary gustatory nucleus (GS; Fig. 3.2N and O; Fig. 3.11 e; Fig. 3.11 f shows cresyl violet staining in GS) and the nucleus isthmi (nI; Fig. 3.2N and O). Mesencephalic nuclei in the tegmentum that were not stained included the red nucleus (R; Fig. 3.2K), the superior raphe nucleus, the tegmental lateral group, the lateral mesencephalic profundus nucleus and the interpeduncular nucleus (data not shown).

Table 3.3. Summary of BDNF mRNA expression in the mesencephalon of *A. anguilla.*

Brain region Nucleus	Staining intensity/ cells stained §	Reference for Fig. 3.2
Torus semicircularis	++/4	J-O
Tectum		
Stratum periventriculare (SPV)	+++/4	I-O
Tegmentum		
Nucleus of the medial longitudinal fasciculus (nMLF)	++/2	K, L
Red nucleus (R)	-	K
Oculomotor nucleus (nIII)	++/4	M
Nucleus of trochlear nerve (nIV)	++/4	N, O
Nucleus lateralis valvulae (LV)	+++/4	N, O
Secondary gustatory nucleus (GS)	++/3	N, O
Nucleus isthmi (nI)	++/3	N, O
Superior reticular nucleus (RS)	++/3	O

§ Staining intensity: - = absent += light ++ = moderate +++=strong; cells stained: 1= occasional cells, 2= some scattered cells, 3=most cells stained, 4=densely packed cells

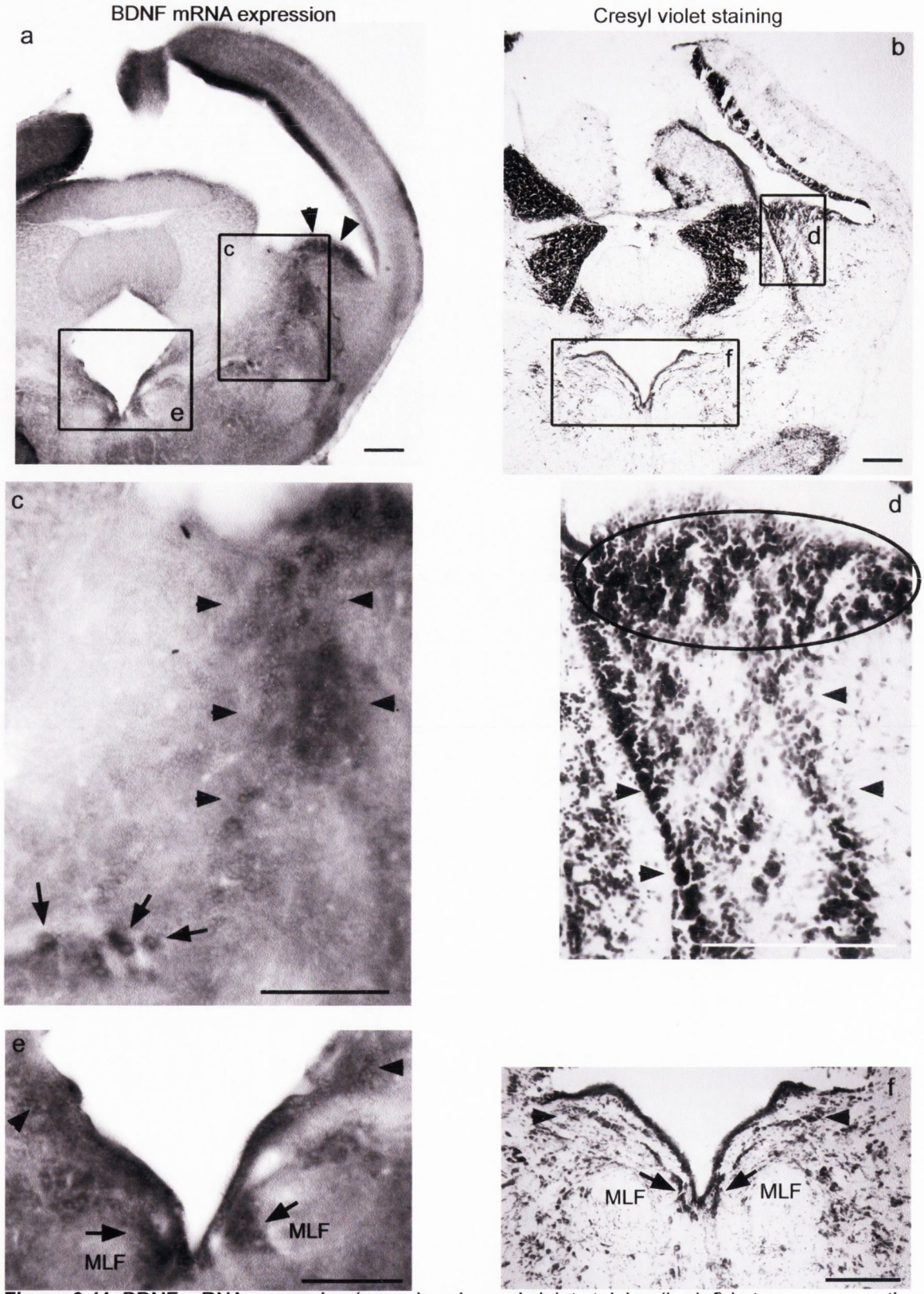


Figure 3.11. BDNF mRNA expression (a, c, e) and cresyl violet staining (b, d, f) in transverse sections of the caudal mesencephalon. This figure corresponds to Figure 3.2O. a and b are low power images of the sections. The position of the lateral valvula (LV) is indicated by the arrowheads in a. c and e are higher power images of boxes c and e in a respectively. d and f are higher power images of boxes d and f in b respectively. c: BDNF mRNA expression in the torus semicircularis (TS) is indicated by arrowheads and in the superior reticular nucleus (RS) by arrows. d: cresyl violet staining in the TS (arrowheads) and LV (circled region). e and f: Staining in the nucleus of trochlear nerve (nIV) is indicated by arrows and in the secondary gustatory nucleus (GS) by arrowheads. MLF= medial longitudnal fasciculus. Scale bars = 200 μm.

3.3.4 Rhombencephalon

The expression pattern of BDNF mRNA in the rhombencephalon is summarised in Table 3.4. The rhombencephalon is made up of the cerebellum and the medulla oblongata (Fig. 3.2P-X). The tract of the medial longitudinal fasciculus can be seen running through the ventromedial region of each section. The descending tract of the trigeminal nerve (dV) and the secondary gustatory tract (sgt) are also located in the dorsolateral region of most rhombencephalic sections.

Little staining was seen in the cerebellum although a few scattered cells were lightly stained in the Purkinje cell layer, which lies at the border of the outer molecular layers (CbSm) and the granular layer (CbSg; Fig. 3.2Q).

Large cells in the superior (SRF; Fig. 3.2P and Fig. 3.12 c), medial (MRF; Fig. 3.2Q-T; Fig. 3.13 c and Fig. 3.14 c; see Fig. 3.13 d for cresyl violet staining of the MRF) and inferior (IRF; Fig. 3.2U-X; Fig. 3.15 e and f and Fig. 3.16 c) divisions of the reticular formation were moderately to strongly stained. No corresponding staining was seen in the cells of the superior (SRF; Fig. 3.12 d and Fig. 3.17 b), medial (MRF; Fig. 3.14 d and 3.17 d) and inferior (IRF; 3.16 d) reticular formation when sections were hybridised to sense probes for BDNF. The Maunther cells (M) were moderately stained (Fig. 3.2Q and Fig. 3.13 c and e; see Fig. 3.13 d for cresyl violet staining of the M cell). Some cells in the superior olive (SO; Fig. 3.2P) and the locus coeruleus (LC) were also stained (Fig. 3.2P and Fig. 3.12 c; note lack of staining in the LC after hybridisation to a sense probe for BDNF in Fig. 3.12 a). In the most caudal brain sections, signal was seen in the inferior raphe nucleus (Rinf; Fig. 3.2U-W; Fig. 3.15 e and f). A few cells in the area postrema (AP) were lightly stained (Fig. 3.2W and X).

In the octavolateral system, moderate staining was seen in a few scattered cells in the rostral portion only of the nucleus medialis (nMed; Fig. 3.2P and Fig. 3.12 c), in the magnocellular octavolateral nucleus (MO, Fig. 3.2Q, R, S; Fig. 3.13 c and Fig. 3.14 a; Fig. 3.13 d shows cresyl violet staining in the MO) and octavolateral efferent nucleus (OEN; Fig. 3.2T and U; Fig. 3.15 d), and in most of the cells of the tangential octavolateral nucleus (TO, Fig. 3.2S; Fig. 3.14 a). Light staining of occasional cells in the descending octavolateral nucleus (DO; Fig. 3.2V) was observed. Staining was absent in the anterior octavolateral nucleus (data not shown). The nuclei of the octavolateral system were not stained when sections were hybridised to the sense control probe for BDNF. For example, no staining was seen in the nucleus medialis (nMed; Fig. 3.12 a) or in the magnocellular (MO) and tangential (TO) octavolateral nuclei (Fig. 3.14 b).

Moderate staining was seen in the majority of cells in the motor nuclei of the following cranial nerves- the facial nerve (nVIIm; Fig. 3.2T and U; Fig. 3.15 c), the glossopharyngeal nerve (nIXm; Fig. 3.2V; Fig. 3.15 d) and the vagal nerve (nXm; Fig. 3.2W and X; 3.16 c), and in a few scattered cells in the trigeminal nerve (nVm; Fig. 3.2P; Fig. 3.12 c and Fig. 3.17 e). Moderate staining was observed in the majority of cells in the sensory nucleus of the trigeminal nerve (nVs; Fig. 3.2P) and the rostral portion of the vagal lobe only (LX; Fig. 3.2V and W). Occasional cells in the facial lobe (LVII; Fig. 3.2R-T) were lightly stained. The commissural nucleus of Cajal (nCC; Fig. 3.2X) was not stained. No staining was seen in the motor nuclei of the cranial nerves such as the trigeminal nerve (nVm; Fig. 3. 12 d and Fig. 3.17 f) and the vagal nerve (nXm; Fig. 3.16 d) after sections were hybridised to the BDNF sense probe.

Table 3.4. Summary of BDNF mRNA expression in the rhombencephalon of *A. anguilla.*

Brain region Nucleus	Staining intensity/ cells stained §	Reference for Fig. 3.2
Cerebellum		
Purkinje cell layer	+/2	Q
Medulla oblongata		
Locus coeruleus (LC)	+/2	P
Superior olive (SO)	++/3	P
Inferior raphe nucleus (Rinf)	++/2	U-W
Area postrema	+/2	W, X
Reticular formation:		
Superior reticular formation (SRF)	++/2	P
Mauthner cell (M)	++	Q
Medial reticular formation (MRF)	++/2	Q-T
Inferior reticular formation (IRF)	++/3	U-X
Octavolateral nuclei:		
Nucleus medialis (nMed; rostral only)	++/2	P
Magnocellular octavolateral nucleus (MO)	++/2	Q, R
Tangential octavolateral nucleus (TO)	++/3	S
Octavolateral efferent nucleus (OEN)	++/2	T, U
Descending octavolateral nucleus (DO)	+/1	V
Cranial nerves:		
Sensory nucleus of the trigeminal nerve (nVs)	++/3	P
Motor nucleus of trigeminal nerve (nVm)	++/2	P
Facial lobe (LVII)	+/2	R-T
Facial motor nucleus (nVIIm)	++/3	T, U
Glossopharyngeal motor nucleus (nIXm)	++/3	V
Vagal lobe (LX; rostral)	-	V
Vagal lobe (LX; caudal)	++/3	W
Vagal motor nucleus (nXm)	++/3	W,X
Commissural nucleus of Cajal (nCC)	-	X

§ Staining intensity: - = absent += light ++ = moderate +++=strong; cells stained: 1= occasional cells, 2= some scattered cells, 3=most cells stained, 4=densely packed cells

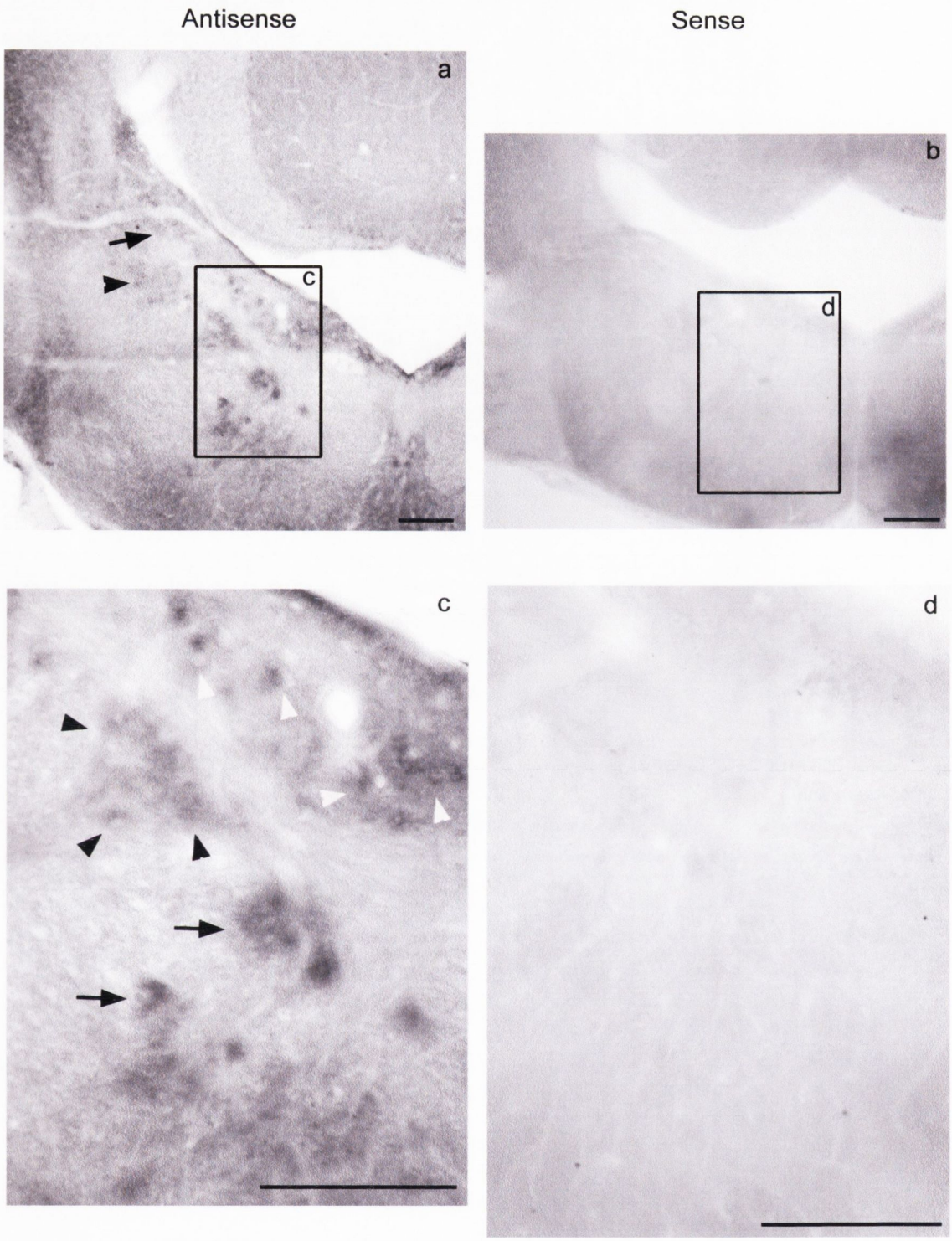


Figure 3.12. Transverse sections of the rostral rhombencephalon hybridised to the BDNF antisense (a and c) and sense probe (b and d). This figure corresponds to Figure 3.2P. a and b are low power photographs of the sections. a: Staining in the locus coeruleus (LC) is indicated by the arrowhead and in the trigeminal sensory nucleus (nVs) by the arrow. c: Higher power image of box c in a. Staining in the superior reticular formation (SRF) is indicated by the arrows, in the nucleus medialis (nMed) by the black arrowheads and in the trigeminal motor nucleus (nVm) by the white arrowheads. d: Higher power image of box d in b. Scale bars = 200 μm.

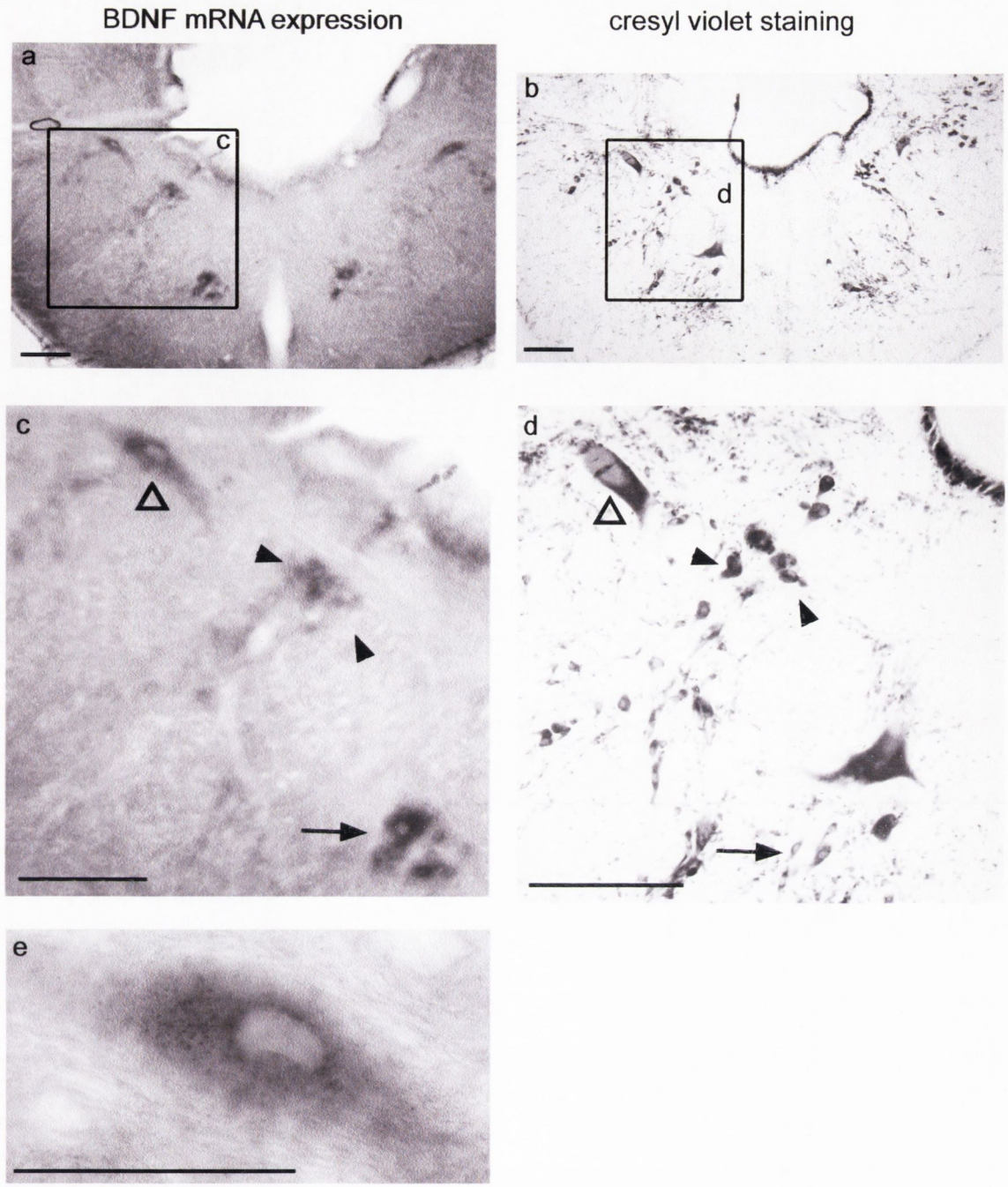


Figure 3.13. Transverse sections of the rhombencephalon at the level of the Mauthner cell showing BDNF mRNA expression (a, c and e) or stained with cresyl violet (b and d). a and b are low power photographs of staining in this brain region. c: Higher power image of box c in a. d: Higher power image of box d in b. c and d: Staining in the Mauthner cell (M) is indicated by the open arrowhead, in the magnocellular octavolateral nucleus (MO) by the closed arrowheads and in the medial reticular formation (MRF) by the arrow. e: High power photograph of M indicated in c by the open arrowhead. This figure corresponds to Figure 3.2Q. Scale bars = 200 μm in a, b, c and d and 100 μm in e.

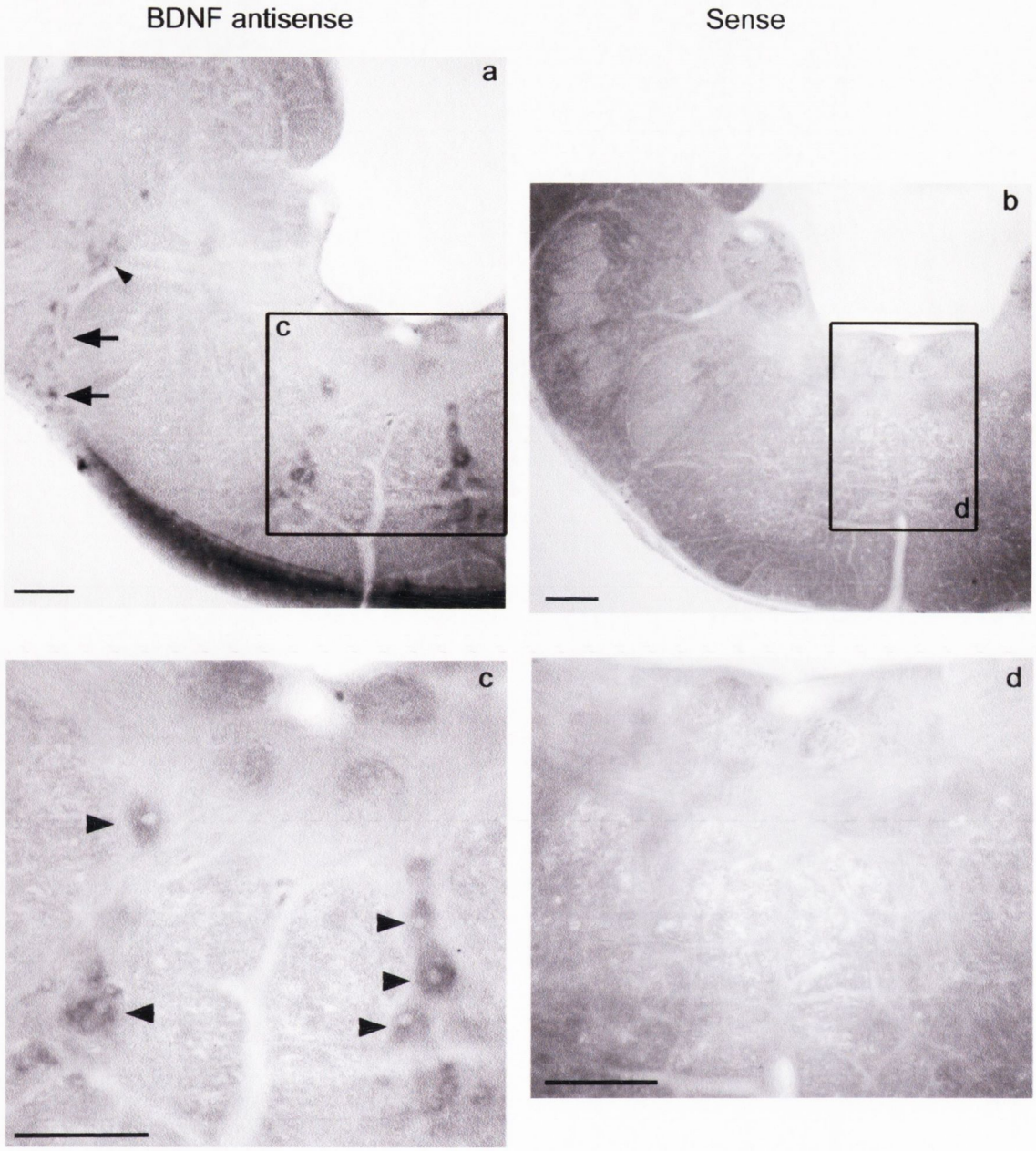


Figure 3.14. Transverse sections of the rhombencephlon at the level of the tangential octavolateral nucleus (TO) hybridised to BDNF antisense (a and c) and sense probes (b and d). This figure corresponds to Figure 3.2S. a and b are low power photographs of the sections. a: Staining in TO is indicated by arrows and in the magnocellular octavolateral nucleus (MO) by the arrowhead. c: Higher power image of box c in a. Staining in the medial reticular formation (MRF) is denoted by the arrowheads. d: Higher power image of box d in b. Scale bars = 200 μm.

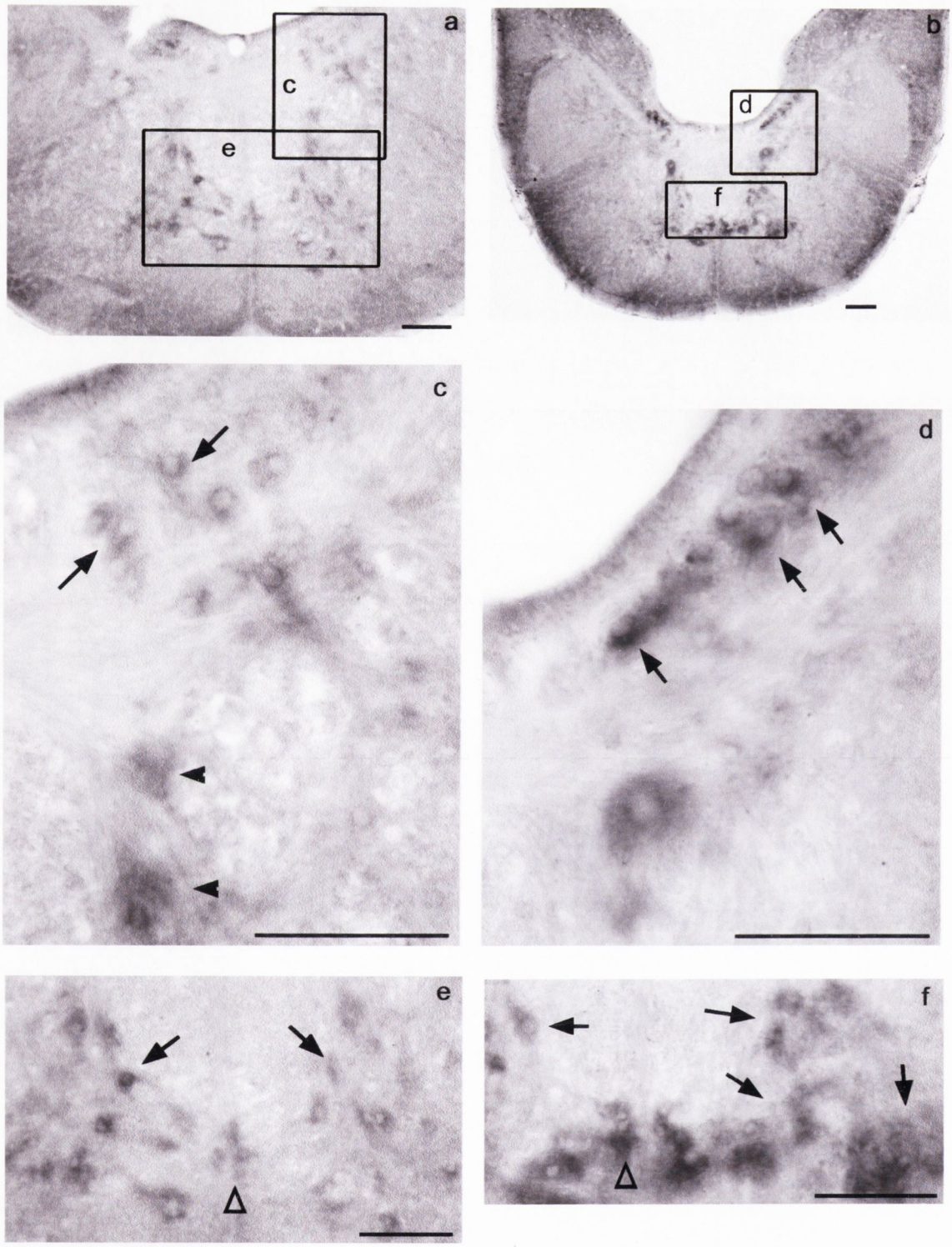


Figure 3.15. BDNF mRNA expression in transverse sections of the caudal rhombencephalon. a: Low power photograph of a section which corresponds to Figure 3.2U. c: Higher power image of box c in a. Staining in the facial motor nucleus (nVIIm) is indicated by the arrows and in the octavolateral efferent nucleus (OEN) by the arrowheads. e: Higher power photograph of box e in a showing staining in the inferior reticular formation (IRF) denoted by the arrows and in the inferior raphe nucleus (Rinf) indicated by the open arrowhead. b: Low power image of a section which corresponds to section 3.2V. d: Higher power image of box d in b. Staining in the glossopharyngeal motor nucleus (nIXm) is denoted by the arrows. f: Higher power image of box f in b showing staining in the IRF denoted by the arrows and in the Rinf indicated by the open arrowhead. Scale bars = 200 μm.

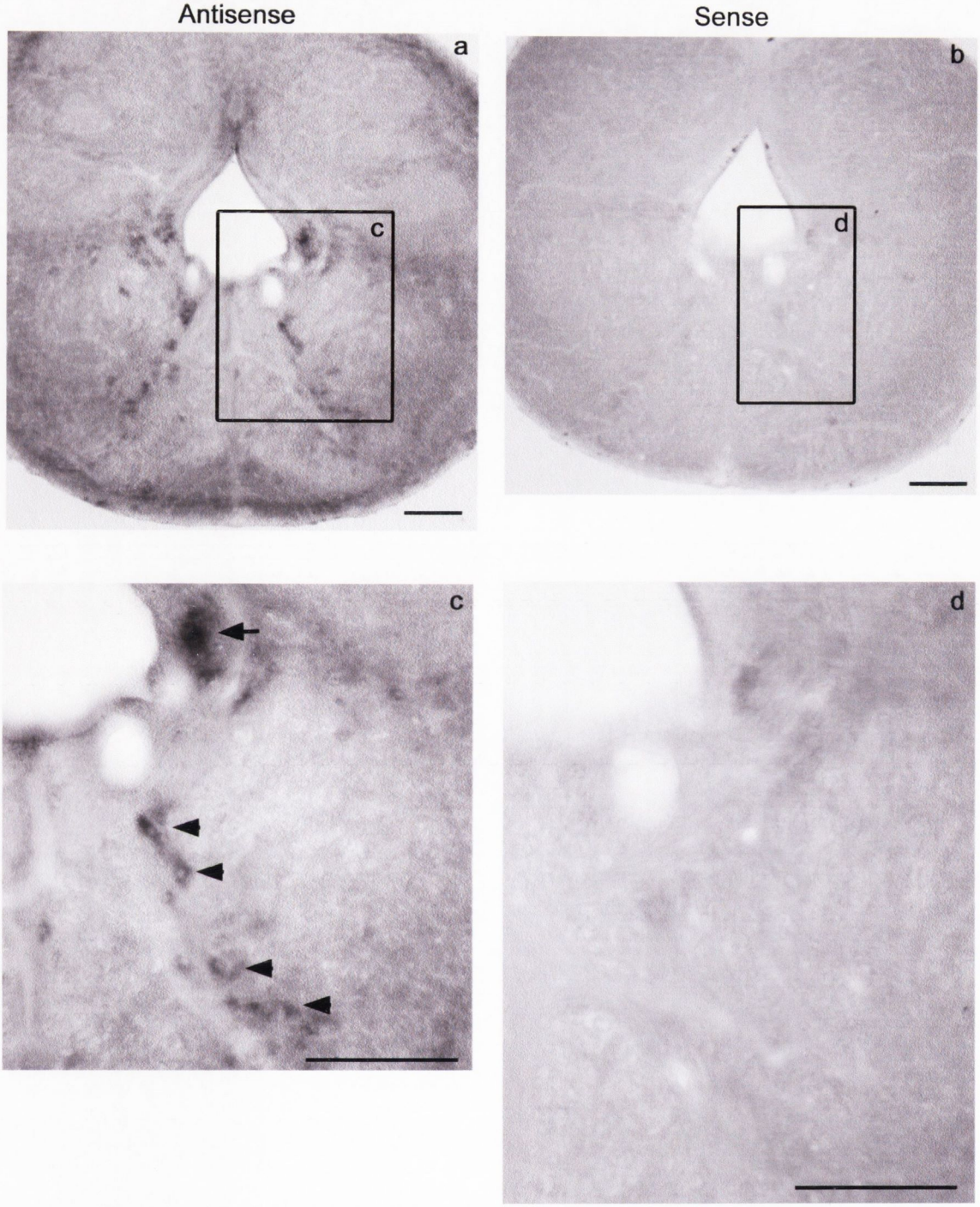


Figure 3.16. Transverse sections of the caudal rhombencephalon hybridised to BDNF antisense (a and c) and sense probes (b and d). This figure corresponds to Figure 3.2X. a: Low power image of BDNF mRNA expression in the caudal rhombencephalon. c: Higher power image of box c in a. BDNF mRNA expression in the vagal motor nucleus (nXm) is indicated by the arrow and in the inferior reticular formation (IRF) by the arrowheads. b: Low power photograph of a section from the brain same region as a, hybridised to the BDNF sense probe. d: Higher power image of box d in b. Scale bars = 200 μm.

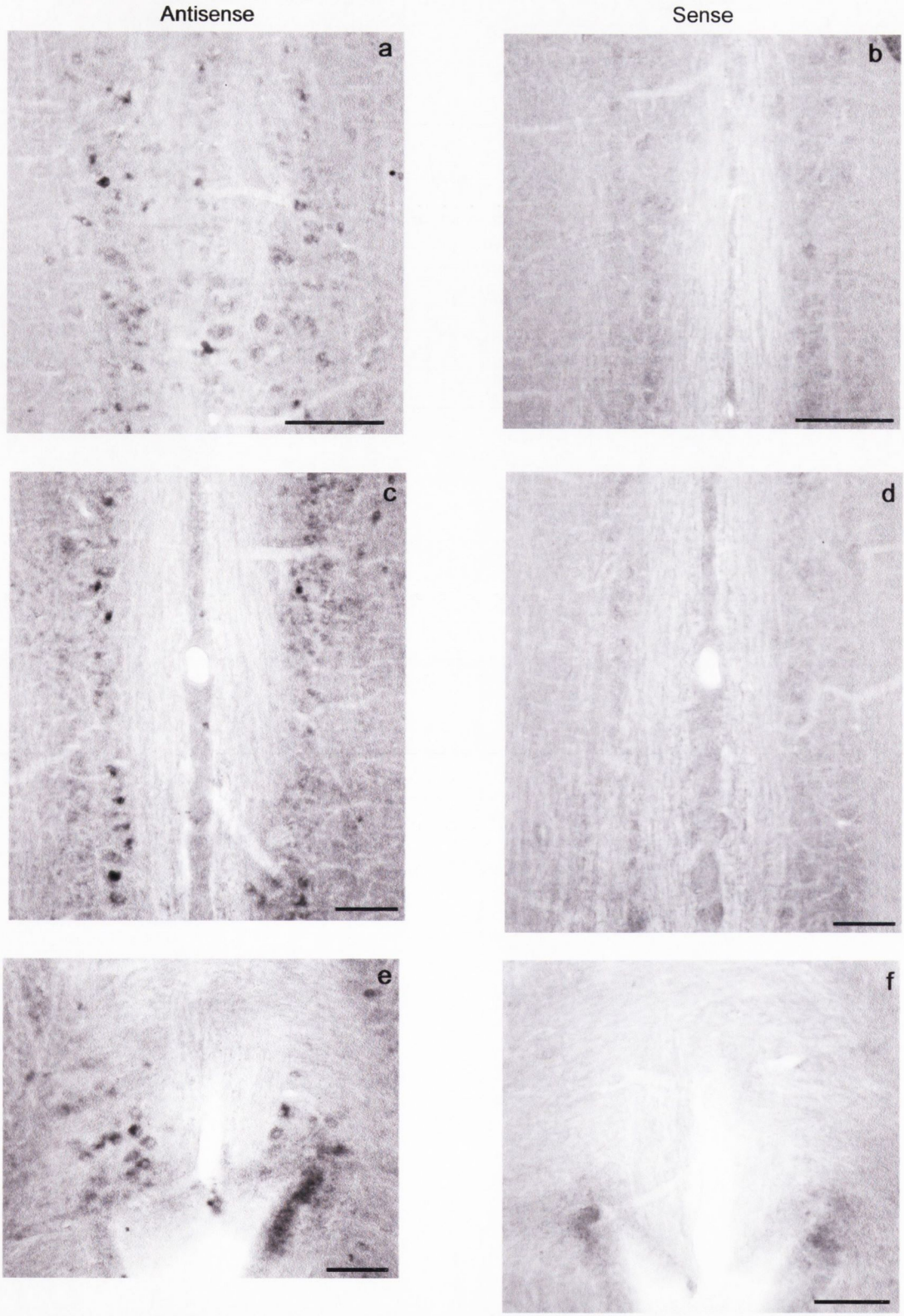


Figure 3.17. Horizontal sections of the rhombencephalon hybridised to BDNF antisense (a, c and e) and sense (b, d and f) probes. a: BDNF mRNA expression in the superior reticular formation (SRF). b: Corresponding sense control for a. c: BDNF mRNA expression in the medial reticular formation (MRF). d: Corresponding sense control for c. e: BDNF mRNA expression in the trigeminal motor nucleus (nVm). f: Corresponding sense control for e. Scale bars = 200 μm.

3.4 Discussion

This survey of BDNF mRNA expression in the eel brain shows parallels with that reported for mammalian (Castrén *et al.*, 1995; Conner *et al.*, 1997; Liang *et al.*, 1998a; Allen and Earnest, 2005), avian (Herzog and von Bartheld, 1998) and amphibian species (Duprey-Diaz *et al.*, 2002; Wang *et al.* 2005). As in these animal groups, in the eel, expression was seen in the telencephalon, hypothalamus, tectum, many primary and secondary sensory centres, and cranial motor nuclei. ISH staining was particularly strong in the preoptic area (Table 3.2), the hypothalamus (Table 3.2), the periventricular nucleus of the posterior tuberis (Table 3.2) and the nucleus lateralis valvulae (Table 3.3). However, in contrast to the adult mammal, BDNF mRNA expression was also seen in the reticular formation and other brain stem nuclei (Table 3.4) involved in the control of movement, some of which project down the spinal cord.

The structure of this discussion follows the rostral to caudal organisation of the brain wherever possible. However, the primary sensory centres such as the olfactory bulb (Table 3.1), the tectum of the mesencephalon (Table 3.3), the pretectum of the diencephalon (Table 3.2) and the octavolateral system of the brain stem (Table 3.4), and the sensory related multimodal relay stations, e.g. the torus semicircularis of the mesencephalon (Table 3.3) and the thalamus and the preglomerular complex of the diencephalon (Table 3.2), are located throughout the brain and are dealt with in a separate section (3.4.3). Some regions of the eel brain have multiple functions such as the nuclei of the octavolateral system (Table 3.3; see Meredith and Roberts (1987) and Meredith *et al.* (1987) for more information on the functions of the octavolateral system in the eel) and therefore appear in more than one section of the discussion, e.g. the octavolateral system arises in sections 3.4.3 and 3.4.5. In addition, in some brain regions, anatomically identical nuclei, e.g. the nuclei of the thalamus (Ito *et al.*, 1986), do not exist in fish and mammals and when this occurs, homologous nuclei are compared below.

Inevitably, much of the discussion that follows will be speculative since little is yet known about the function of BDNF in the fish brain. These ideas, nevertheless, may facilitate the development of future experiments.

3.4.1 BDNF mRNA expression in the telencephalon.

The teleostean telencephalic hemispheres can be divided the area ventralis, which is thought to correspond to the subpallial region in other vertebrates (Northcutt and Braford, 1980; Nieuwenhuys and Meek, 1990; Kapsimali *et al.*, 2000; Wullimann and Rink, 2002; Rink and Wullimann, 2004) and the area dorsalis, thought to represent the pallium (Northcutt and Braford, 1980; Nieuwenhuys and Meek, 1990; Kapsimali *et al.*,

2000; Northcutt, 2006). Although attempts have been made to homologise parts of the area dorsalis with the pallium of other vertebrate groups, it is not clear whether an equivalent of the highly specialised isocortex of the mammalian brain is present in teleost fish (Nieuwenhuys and Meek, 1990; Kapsimali *et al.*, 2000).

The subpallial area ventralis, in the eel, can be divided into various regions, which include the commissural (Vc), dorsal (Vd), lateral (Vl), posterior (Vp), supracommissural (Vs) and ventral (Vv) nuclei (see Table 3.1). The nuclei of the dorsal and commissural area ventralis in teleost fish have been proposed as being the equivalent of the striatum (Kapsimali *et al.*, 2000; Wullimann and Rink, 2002). This homology is based on the fact that these nuclei share many characteristics with the striatal region of the basal ganglia of non-teleost ray finned fish (Reiner and Northcutt, 1987; Reiner and Northcutt, 1992) and tetrapods (Reiner *et al.*, 1998). In teleosts, this area of the brain has been identified, for example, as containing substance P cell bodies and fibres (Reiner and Northcutt, 1992), GABAergic neurons (Medina *et al.*, 1994) and dopaminergic cell bodies and fibres (Roberts *et al.*, 1989; Ekström *et al.*, 1990), and dopamine receptors (Kapsimali *et al.*, 2000). These markers correspond with those in the striatum of amphibians, birds and mammals (Reiner *et al.*, 1998). The nuclei of posterior and supracommissural area ventralis in the posterior telencephalon share a similar immunocytochemical profile with the dorsal nucleus of the area ventralis (Roberts *et al.*, 1989; Ekström *et al.*, 1990; Medina *et al.*, 1994). Dopamine receptor expression in these two nuclei has also been described in the eel (Kapsimali, 2000). It may be the case based on this evidence that the nuclei of the posterior and supracommissural area ventralis also form part of the striatum in teleosts (Kapsimali, 2000).

In the eel, cells in the rostral part of the dorsal nucleus of the area ventralis were not stained (Vd; Fig. 3.2B-C; Table 3.1) however, cells were lightly stained in the caudal part of this nucleus (Fig. 3.2D; Table 3.1) and in portions of the commissural (Vc), posterior (Vp) and supracommissural (Vs) nuclei of the area ventralis (Fig. 3.2D-F; Table 3.1). In mammals, BDNF mRNA is not expressed in the striatum (Altar *et al.*, 1997; Conner *et al.*, 1997). BDNF protein is present in nerve terminals in the striatum however and appears to be anterogradely derived from neurons in the cortex and substantia nigra (Altar *et al.*, 1997; Altar and DiStefano, 1998). It is possible that the portions of the dorsal (Fig. 3.2B and C) and commissural (Fig. 3.2D) nuclei of the area ventralis that did not stain for BDNF in the eel brain are in fact the regions that constitute the teleostean equivalent of the mammalian striatum. On the other hand, perhaps fish, unlike mammals, synthesise BDNF in some of the cell groups that constitute the teleostean equivalent of the

mammalian striatum. The purpose of BDNF activity in the mammalian striatum may be to promote neuronal survival and plasticity (Altar *et al.*, 1997). BDNF may perform a similar function in the dorsal nucleus of the area ventralis in the eel.

The nuclei of the ventral and lateral area ventralis have been suggested to constitute the septal formation in teleosts (Northcutt and Braford, 1980; Nieuwenhuys and Meek, 1990). The topological position of neurons in this region and the cholinergic nature of their cell bodies agree with findings in the septal formation in mammals and amphibians (Nieuwenhuys and Meek, 1990; Marin *et al.*, 1997; Wullimann and Rink, 2002). In the eel, dopamine receptor expression patterns in the nuclei of the ventral and lateral area ventralis mirror those in the mammalian septal formation (Kapsimali *et al.*, 2000). Similarly to the amniote septal formation, the ventral nucleus of the area ventralis in fish such as the trout (Folguiera *et al.*, 2004a) and zebrafish (Wullimann and Rink, 2002; Rink and Wullimann, 2004), also has strong efferent and afferent connections with the hypothalamus. In the eel brain, the cells in the nuclei of the ventral (Vv; Fig. 3.2C and D; Table 3.1) and lateral (Vl; Fig. 3.2B and Fig. 3.3 c; Table 3.1) area ventralis showed weak to moderate BDNF mRNA staining. This correlates with expression in the septal formation of the rat brain where BDNF may act as an autocrine survival factor for cholinergic neurons (Alderson *et al.*, 1990; Castrén *et al.*, 1995; Conner *et al.*, 1997; Grosse *et al.*, 2005).

In teleosts, the pallial region of the area dorsalis is divided into a number of different nuclei known as the central (Dc), dorsal (Dd), lateral (Dl) and medial (Dm) zones of the area dorsalis (see Table 3.1). The lateral zone of the area dorsalis (Dl; Fig. 3.2B-F) has been suggested to be the teleostean equivalent to the mammalian hippocampus and the medial zone has been proposed as the teleost amygdala (Braford, 1995, Wullimann and Rink, 2002). The entopeduncular nucleus may also form part of the teleostean equivalent of the mammalian amygdala (Braford and Northcutt, 1974; Reiner and Northcutt, 1992). Connectivity with other brain regions (Wullimann and Rink, 2002; Folguiera *et al.*, 2004b, Northcutt, 2006), electrophysiological studies (Prechtl *et al.*, 1998), dopamine receptor mRNA expression in these areas (Kapsimali *et al.*, 2000), developmental studies (Wullimann and Rink, 2002) and behavioural studies (Rodriguez *et al.*, 2002; Broglio *et al.*, 2005) strongly support this interpretation.

In agreement with ISH studies in the rat (Wetmore *et al.*, 1990; Kokaia *et al.*, 1993; Conner *et al.*, 1997) and pig (Wetmore *et al.*, 1990) hippocampus, in the eel, BDNF mRNA expression was seen in the lateral zone of the area dorsalis (Dl; Fig. 3.2B-F; Fig. 3.4 b and Fig. 3.5 b; Table 3.1). In the mammalian hippocampus, regions of strong staining in the pyramidal cell layer of the CA 2 and 3 regions and the granule cell layer of the

dentate gyrus (Conner *et al.*, 1997). In contrast, in the lateral zone of the area dorsalis in the eel brain, only moderate staining of cells was seen throughout.

As mentioned in section 3.1.3, BDNF and other neurotrophins have been implicated in hippocampal synaptic plasticity in mammals (Korte *et al.*, 1995; Figurov *et al.*, 1996; Patterson *et al.*, 1996; Kang *et al.*, 1997; Chen *et al.*, 1999). It has been shown that fish have to ability to learn and form memories (Rodriguez *et al.*, 2002; Williams *et al.*, 2002; Broglio *et al.*, 2005; Colwill *et al.*, 2005; Schuster *et al.*, 2006) and ablation of the lateral pallium in goldfish hinders their performance in tasks designed to test hippocampal learning (Rodriguez *et al.*, 2002; Broglio *et al.*, 2005). Similarly to its role in the mammalian hippocampus, BDNF may function therefore in the process of synaptic plasticity in the lateral zone of the area dorsalis of the eel telencephalon. In addition, BDNF may act as a survival factor for the neurons in the lateral zone of the area dorsalis since BDNF supports the survival of hippocampal neurons *in vitro* and *in vivo*, has been implicated in the pathology of Alzheimer's disease (Connor and Dragunow, 1998) and is important for the survival of particular neuronal populations during hippocampal development (Grosse *et al.*, 2005). It is also possible that BDNF may be involved in the control of neuropeptide synthesis and release based on evidence from mammalian hippocampal cultures (Rage *et al.*, 1999) in the lateral zone of the area dorsalis.

Light to strong BDNF mRNA expression has been described in the amygdala of rats (Conner *et al.*, 1997). In addition, BDNF protein has been observed in the amygdala of rats (Yan *et al.*, 1997a) and humans (Murer *et al.*, 1999) at baseline conditions. In concurrence with mammalian studies, moderate BDNF mRNA expression in the eel brain was seen in regions of medial zone of the area dorsalis (Dm; Fig. 3.2B-F; Table 3.1) and light staining was seen in the entopeduncular nucleus (E; Fig. 3.2D-F and Fig. 3.5 c; Table 3.1). The function of BDNF activity in the mammalian amygdala is as yet unclear but evidence suggests that it may be important in synaptic plasticity in this brain region (Rattiner *et al.*, 2004). For example, BDNF and Trk B have recently been implicated in amygdala-dependent fear learning in the rat, which is a plastic event (Rattiner *et al.*, 2004). Rattiner *et al.* (2004) showed that acquisition of fear conditioning could be disrupted by treatment with the Trk receptor inhibitor, K252a and blocked by treatment with a lentiviral vector expressing a dominant-negative Trk B isoform (Rattiner *et al.*, 2004). It is possible therefore that BDNF expressed in the medial zone of the area dorsalis and the entopeduncular nucleus in the eel performs similar functions to BDNF in the mammalian amygdala (Rattiner *et al.*, 2004; Broglio *et al.*, 2005).

3.4.2 BDNF mRNA expression in the diencephalon

In the present study, moderate to strong BDNF mRNA expression was seen in the suprachiasmatic nucleus (SC) of the eel brain (Fig.3.2F-H; Fig. 3.5 a and Fig. 3.7 a; Table 3.2). The suprachiasmatic nucleus in fish is thought to be similar to that in mammals and may be involved in circadian rhythm generation (Schellart, 1990; Hastings, 1997; Tamai *et al.*, 2003). In the rat, the suprachiasmatic nucleus shows BDNF protein and mRNA expression as well as Trk B receptor presence (Liang *et al.*, 1998a,b; Allen and Earnest, 2005). An endogenous cyclical change is seen in BDNF in the suprachiasmatic nucleus in the rat over a 24 hour period whereby BDNF levels are elevated during the subjective night when light shifts the phase of circadian rhythms (Liang *et al.*, 1998b). Application of BDNF in the rat suprachiasmatic nucleus alters circadian responses to light allowing the photic induction of large phase shifts in activity rhythm during the mid-subjective day, a time when the clock mechanism is normally insensitive to the effects of light (Liang *et al.*, 2000). In BDNF heterozygous mutant mice, or rats treated with the Trk inhibitor K252a, the usual phase-shifting effect of light during the subjective night is greatly decreased (Liang *et al.*, 2000). Hence, the function of BDNF in circadian rhythm generation may be to regulate the sensitivity of the circadian clock to light. The increased levels of BDNF seen during the subjective night may potentiate neuronal signalling by enhancing neurotransmitter release (Liang *et al.*, 1998b; Allen and Earnest, 2005) as described for the cortex and hippocampus (Lessmann and Heumann, 1998; Jovanovic *et al.*, 2000; Schinder and Poo, 2000; Allen and Earnest, 2005). Circadian rhythms in fish, as in mammals, are light dependent (Liang *et al.*, 2000; Kaneko and Cahill, 2005) and the suprachiasmatic nucleus in teleosts is known to be retinorecipient (Schellart, 1990). BDNF expressed in the suprachiasmatic nucleus of the eel, therefore, may perform a similar function to that of BDNF in the mammalian suprachiasmatic nucleus.

The preoptic and hypothalamic nuclei of the teleostean diencephalon (Table 3.2) form part of the hypothalmo-hypophysial system (Cumming *et al.*, 1982; Olivereau *et al.*, 1990; Duarte *et al.*, 2001) and like the mammalian hypothalmo-hypophysial system, produce important peptides and hormones such as vasotocin, isotocin, corticotropin-releasing factor, somatostatin, growth hormone releasing factor and gonadotropin-releasing hormone (Cumming *et al.*, 1982; Olivereau *et al.*, 1988, 1990; Montero *et al.*, 1994; Duarte *et al.*, 2001; Steven *et al.*, 2003; Liu *et al.*, 2006b). Vasotocin and isotocin, the teleostean equivalents of mammalian vasopressin and oxytocin, are produced in the nucleus preopticus magnocellularis in the goldfish brain (Cumming *et al.*, 1982; Venkatesh and Brenner, 1995). The nucleus preopticus magnocellularis and the nucleus preopticus

parvocellularis anterior have been localised as the site of production for corticotrophin releasing factor in the eel (Olivereau *et al.*, 1988) and growth hormone releasing factor in various teleosts such as the eel, goldfish, carp and salmonids (Olivereau *et al.*, 1990). Gonadotropin-releasing hormone is produced in the nucleus preopticus parvocellularis anterior in the eel (Montero *et al.*, 1994) and its mRNA has been described in the preoptic area of the hypothalamus in the zebrafish (Steven *et al.*, 2003).

BDNF mRNA expression ranging from light to strong has been described in most of the nuclei that make up the hypothalo-hypophysial system in mammals such as the medial preoptic nucleus, the medial preoptic area, the magnocellular preoptic nucleus, the paraventricular, ventromedial and dorsomedial hypothalamic nuclei (Castrén *et al.*, 1995; Conner *et al.*, 1997; Aliaga *et al.*, 2002; Marmigère *et al.*, 1998; Rage *et al.*, 2002). BDNF appears to be involved in modifying the activity in the hypothalo-hypophysial system in mammals (Naert *et al.*, 2006) and amphibians (Wang *et al.*, 2005) and has been shown to alter synthesis and release of the neuropeptides somatostatin, corticotropin releasing hormone and vasopressin (Rage *et al.*, 1999; Marmigère *et al.*, 2001; Givalois *et al.*, 2006; Naert *et al.*, 2006). In the eel, moderate to strong BDNF expression was seen in many nuclei within the hypothalmo-hypophysial system (Fig. 3.2D-N; Table 3.2) including the nucleus preopticus magnocellularis (PMp (Fig. 3.4 d) and PMm (Fig. 3.5 c); Fig. 3.2E-G; Table 3.2) and the nucleus preopticus parvocellularis anterior (PPa; Fig. 3.2D; Table 3.2), which have been implicated in the production of several hormones and peptides as described above. BDNF activity in the nuclei of the teleostean hypothalmo-hypophysial system may therefore regulate the synthesis and release of endocrine substances such as somatostatin, corticotrophin releasing factor and vasotocin. In support of this, Trk B immunoreactivity has been described in other parts of the endocrine system in teleosts such as gastric endocrine cells also immunoreactive for somatostatin (de Girolamo *et al.*, 1999) and in the endocrine pancreas (Hannestad *et al.*, 2000; Lucini *et al.*, 2001).

In teleosts, the inferior hypothalamic lobe is likely to be involved in the control of feeding behaviour since it receives gustatory input and projects to the feeding control centres of the vagal and facial lobes (Rink and Wullimann, 1998). In the eel, the nuclei of the inferior hypothalamic lobe (Table 3.2), which include the diffuse nucleus of inferior lobe (ndil; Fig. 3.2I, K-N; Fig. 3.8 d), and the caudal (HC; Fig. 3.2J-L; Fig. 3.9 d) and ventral (HV; Fig. 3.2J-N; Fig. 3.9 e) zones of periventricular hypothalamus, showed moderate to strong staining for BDNF mRNA in most cells that were in some cases densely packed. In mammals, homologous hypothalamic areas that are associated with weight control and feeding behaviours (Tapia-Arancibia *et al.*, 2004; King, 2006; Komori

et al., 2006) and that express BDNF mRNA as mentioned above (Conner *et al.*, 1997) include the paraventricular, dorsomedial and ventromedial hypothalamic nuclei. It has been reported that central infusion of BDNF reduces food intake and leads to weight loss in rats (Lapchak *et al.*, 1992; Pelleymounter *et al.*, 1995). Kernie *et al.* (2000) studied heterozygous mutant mice for BDNF. Significant weight gain compared to the wild type was seen in approximately 50 % of these animals. Infusion of BDNF into the third ventricle reversed this phenotype. Results of ISH experiments showed a decrease in BDNF mRNA expression in the hypothalamic nuclei related to eating behaviour particularly in the ventromedial hypothalamic nucleus (Kernie *et al.*, 2000). Trk B mRNA is expressed in the hypothalamus and BDNF appears to exert its effects on feeding behaviour through interaction with this receptor (Kernie *et al.*, 2000). It has been shown, for example, that a trk B hypomorph expressing the full-length Trk B receptor at only a quarter of the normal levels becomes severely obese at approximately five weeks of age (Xu *et al.*, 2003). It is possible that BDNF may affect feeding behaviour and weight control by modulating the synthesis and release of neuropeptides involved in feeding (Lawrence *et al.*, 1999), in the same way as it does for other hypothalamic neuropeptides such as somatostatin (Rage *et al.*, 1999; Marmigère *et al.*, 2001; Givalois *et al.*, 2006), corticotropin releasing hormone and vasopression (Naert *et al.*, 2006). It is possible therefore that BDNF expression in the inferior lobe hypothalamic lobe of the eel brain may regulate body weight and feeding behaviour as it does in mammals (Lapchak *et al.*, 1992; Pelleymounter *et al.*, 1995; Kernie *et al.*, 2000).

Interestingly, the eels used in this study did not feed well in the aquarium and it was often noted that on the day after feeding, food was left uneaten. As mentioned above, an increase in BDNF levels in the hypothalamic feeding control centres in rats, suppresses feeding behaviour (Lapchak *et al.*, 1992; Pelleymounter *et al.*, 1995). The strong expression of BDNF mRNA in the inferior lobe of the eel may have been a contributing factor in the low level of food intake observed.

The dorsal (HAb; Fig. 3.2G and H) and ventral (HAv; Fig. 3.2G and H) habenular nuclei of the epithalamus (Table 3.2) showed moderate staining for BDNF mRNA in most of the cells that constitute these nuclei. In the rat epithalamus, a light haze of BDNF mRNA labelling in the lateral habenular nucleus was seen. No mRNA labelling was observed in the medial habenular nucleus but extremely heavily stained BDNF immunoreactive fibres were reported in this nucleus in the rat (Conner *et al.*, 1997).

In the region of the posterior tuberculum (Table 3.2), light staining for BDNF mRNA was seen in the preglomerular complex (PGm; Fig. 3.2H-J, L; Fig. 3.8 d).

Moderate to heavy staining was seen in the periventricular nucleus of the posterior tuberculum (TPp; Fig. 3.2I and Fig. 3.8 c), the paraventricular organ (PVO; Fig. 3.2I) and the nucleus posterior tuberis (TP; Fig. 3.2L). The preglomerular complex (PGm) of the posterior tuberculum functions as a multimodal relay centre (Zupanc, 1997; Folgueira *et al.*, 2005) and the potential significance of BDNF mRNA expression in this nucleus is discussed below in section 3.4.3.

The posterior tuberculum in teleosts possesses dopaminergic cells (Rink and Wulliman, 2001) and has reciprocal connections with the dorsal and ventral telencephalon (Folgueira *et al.*, 2004a, b; Rink and Wulliman, 2004). It has been proposed as the origin of the ascending dopaminergic system in the teleostean brain (Rink and Wulliman, 2001; Wullimann and Rink, 2002). In amniotes, the origin of the ascending dopaminergic system is located in the mesencephalic tegmentum (Wullimann and Rink, 2002) and it has been shown that mesencephalic dopaminergic neurons in the brain, including those of the substantia nigra, synthesize BDNF (Seroogy *et al.*, 1994; Conner *et al.*, 1997). It is thought that BDNF contributes to the maintenance of normal functioning of dopaminergic midbrain neurons (Seroogy *et al.*, 1994) but also plays survival promoting and neuroprotective roles in these neurons (Seroogy *et al.*, 1994; Aliaga *et al.*, 1999; Venero *et al.*, 2000). BDNF may have similar functions in the dopaminergic neurons of the posterior tuberculum of the eel.

The diencephalic regions of the thalamus, pretectum and preglomerular complex (Table 3.2) are discussed below in section 3.4.3.

3.4.3 BDNF mRNA expression in primary and secondary sensory centres.

In the eel, strong BDNF mRNA expression was seen in the mesencephalic tectum (Fig. 3.2I-O; Fig. 3.9 c; Table 3.3). This is in agreement with studies in mammals where BDNF expression is seen in the superior colliculus, the mammalian homologue of the tectum, in the adult rat (Conner *et al.*, 1997) and adult hamster (Frost *et al.*, 2001). In addition, as mentioned in section 3.1.2, BDNF mRNA and protein have also been observed the optic tectum in amphibians (Duprez-Díaz *et al.*, 2002) and birds (Herzog and von Bartheld, 1998; Theiss and Güntürkün, 2001).

The tectum is the primary visual centre in fish (Meek, 1990). During development, evidence suggests that BDNF synthesised in the tectum functions as a retrograde survival promoting factor for retinal ganglion cells in the chick (Herzog and von Bartheld, 1998) and rat (Friedman *et al.*, 1991; Hirsch *et al.*, 2000; Spalding *et al.*, 2004). It is possible that BDNF continues to perform this role in adulthood.

As well as being as the primary visual centre, the tectum functions as a multimodal relay centre (Meek, 1990). Not only does it receive input from the retina but also sends and receives multimodal projections to and from various brain regions such as the pretectum, thalamus, nucleus isthmi, torus semicircularis and brain stem (Grover and Sharma, 1981; Meek, 1983; Nieuwenuys and Pouwels, 1983; Northmore, 1991; Molist *et al.*, 1993; Herrero *et al.*, 1998; Xue *et al.*, 2001; Angeles Luque et *al.*, 2005). BDNF expressed in the tectum may act as a survival promoting factor for efferent and afferent neuronal populations (Davies and Wright, 1995; Altar *et al.*, 1997; Herzog and von Bartheld, 1998). In addition, BDNF may be involved in the modulation of synaptic transmission (Lessmann and Heumann, 1998; McAllister *et al.*, 1999; Jovanovic *et al.*, 2000; Schinder and Poo, 2000; Blum and Konnerth, 2005; Bramham and Messaoudi, 2005) and plasticity (Snider, 1988; Cohen-Cory *et al.*, 1991; Zhang *et al.*, 1994; McAllister *et al.*, 1995; Inoue and Sanes, 1997; Schwarz *et al.*, 1997; Morrison and Mason, 1998; Lom and Cohen-Cory, 1999; Xu *et al.*, 2000a; Lom *et al.*, 2002; Vicario-Abejón *et al.*, 2002; Cohen-Cory and Lom, 2004) during the numerous electrophysiological responses to stimuli seen in this complex structure (Guthrie, 1990).

Moderate to light BDNF mRNA expression in the eel was seen in the nuclei of the octavolateral system in the rhombencephalon (Table 3.4), e.g. the octavolateral efferent nucleus (OEN; Fig. 3.2T and U; Fig. 3.15 c), magnocellular (MO; Fig. 3.2Q and R; Fig. 3.13 c and 3.14 a), tangential (TO; Fig. 3.2S and Fig. 3.14 a) and descending (DO; Fig. 3.2V) octavolateral nuclei, and the rostral region of the nucleus medialis (Fig. 3.2P and Fig. 3.12 c). The octavolateral efferent nucleus innervates the end organs responsible for auditory, vestibular and lateral line sensation (Meredith and Roberts, 1987, Meredith *et al.*, 1987). Afferent projections from the auditory, vestibular and lateral line systems in the eel project to the various nuclei of the octavolateral system including those listed above (Meredith and Roberts, 1987; Meredith *et al.*, 1987). Moderate BDNF mRNA expression was also seen in the superior olive (SO; Fig. 3.2P; Table 3.4), which is another structure associated with audition in the eel brain (Meredith and Roberts, 1986). There is no lateral line system in amniotes but vestibular and auditory projections remain, and the nuclei can be homologised with mammalian counterparts (Meredith and Roberts, 1986; Meredith, 1988). For example, the vestibular and auditory efferent supply, provided in the eel by the octavolateral efferent nucleus, arises in mammals from the dorsal and ventral vestibular efferent nuclei, the olivocochlear nucleus and the superior olive (Roberts and Meredith, 1990).

BDNF protein has been detected in mammalian auditory brain stem nuclei including the ventral cochlear nucleus, trapezoid body and lateral and medial superior olive (Furukawa *et al.*, 1998; Hafidi *et al.*, 1999; Tierney *et al.*, 2001; Suneja *et al.*, 2005) and in vestibular brain stem nuclei such as the spinal vestibular nucleus (Conner *et al.*, 1997; Furukawa *et al.*, 1998). Despite this presence of the protein, BDNF mRNA expression in auditory and vestibular nuclei has not been detected by ISH in the rat brain. However, BDNF mRNA has been detected by RT-PCR in RNA extracted from cochlear nuclei, which constitute part of the adult mammalian auditory brain stem nuclei (Lefebvre *et al.*, 1994). It may be the case that BDNF mRNA expression is below the level of detection for ISH in mammalian auditory and possibly vestibular nuclei.

As suggested for the visual system, BDNF may function as a retrogradely-transported or autocrine-derived survival and plasticity promoting factor for the octavolateral systems in the eel supporting primary and second order neurons, and possibly sensory hair cells (Sobreviela *et al.*, 1996; Herzog and von Bartheld, 1998; Mufson *et al.*, 1999; Tierney *et al.*, 2001; Germana *et al.*, 2002). In agreement with this idea, retrograde transport of BDNF has been suggested in the mammalian auditory system by the results of studies by Sobreviela *et al.* (1996) and Tierney *et al.* (2001). Furthermore, Trk B receptors have recently been described in the teleost lateral line system (Germana *et al.*, 2002).

Strong BDNF mRNA expression was also seen in the torus semicircularis of the mesencephalon (Fig. 3.2J-O; 3.9 c and 3.11 c; Table 3.3), which receives multimodal input from the octavolateral system and the tectum, and is considered to be a homologue of the mammalian inferior colliculus (Schellart, 1990). In agreement with these findings in the eel, BDNF mRNA is found in the nucleus brachium and external cortex of the inferior colliculus in the adult rat brain (Conner *et al.*, 1997). BDNF mRNA was also expressed in other multimodal relay centres in the eel brain diencephalon (Table 3.2), such as the thalamus (Fig. 3.2H-J; Fig. 3.7 b and Fig. 3.8 c; see Ito *et al.*, 1986 for more information on the role of the thalamus as a multimodal relay centre in teleosts) and the preglomerular complex (Fig. 3.2H-J, L; Fig. 3.8 d; see Zupanc, 1997 and Folgueira *et al.*, 2005 for more information on the functions of the preglomerular complex). In rats, the thalamus also functions as a multimodal relay station and BDNF mRNA expression has been described in many of its nuclei (Conner *et al.*, 1997) however, a homologous region to the preglomerular complex of the teleost brain has not been found in other amniote vertebrates (Butler, 1994). Strong BDNF mRNA expression was also seen in the nucleus lateralis valvulae, a precerebellar relay station that appears to be concerned mainly with visual information in fish (Meek, 1990; Schellart, 1990; Wullimann and Rink, 1996). A nucleus

homologous to the nucleus lateralis valvulae has not been described in the mammalian brain (Wullimann and Rink, 1996). It is possible that the functions of BDNF in these multimodal relay centres may be similar to those proposed above for BDNF expressed in the tectum, i.e. to promote the survival of efferent and afferent neuronal populations, and to modulate synaptic transmission and plasticity.

In *A. anguilla,* BDNF expression was also seen other in regions that receive primary sensory input from the periphery such as the olfactory bulb (Fig. 3.2A; Table 3.1) and visual centres in the diencephalon including the pretectum (Fig. 3.2I and Fig. 3.8 c; Table 3.2) and preoptic area (Fig. 3.2D-H; Fig. 3.4 d, Fig 3.5 a and c and Fig. 3.7; Table 3.2). BDNF mRNA expression has been reported in the homologous brain regions in the rat (Conner *et al.,* 1997). In the eel, BDNF mRNA was also seen in the vagal lobe (LX) of the rhombencephalon (Fig. 3.2W; Table 3.4), which is involved in gustatory sensation, and the secondary gustatory nucleus (GS) in the mesencephalon (Fig. 3.2N and O; Fig. 3.11 e; Table 3.3) to which the vagal lobe projects (see Morita *et al.,* 1983 for more information on the innervation of the gustatory system in fish). In the mammalian brain, BDNF mRNA expression has not been reported in the dorsal motor nucleus or the nucleus ambiguus (Wetmore *et al.,* 1990; Conner *et al.,* 1997) both of which are nuclei of the vagus nerve but BDNF protein has been observed in the nucleus ambiguus (Yan *et al.,* 1997). As for the tectum and octavolateral system, BDNF may promote the survival of efferent and afferent neurons in these sensory centres.

3.4.4 BDNF mRNA expression in the brain stem nuclei involved in respiration.

In the eel, BDNF mRNA expression was seen in the motor nuclei of the trigeminal (nVm; Fig. 3.2P; Fig. 3. 12 c and Fig. 3.17 e), facial (nVIIm; Fig. 3.2T and U; Fig. 3.15 c), glossopharyngeal (nIXm; Fig. 3.2V and Fig. 3.15 d) and vagal (nXm; Fig. 3.2W and X; Fig. 3.16 c) nerves (Table 3.4). In teleosts, these cranial nerves are important in the control of breathing (Taylor *et al.,* 1999; Sundin *et al.,* 2003). The rostral areas of the trigeminal and facial motor nuclei, for example, innervate the muscles in the head involved in the contraction phase of respiration whereas the caudal portion of these motor nuclei innervate muscles that expand the buccal and opercular cavities during inspiration (Luiten, 1976).

In developing and adult mammals, BDNF mRNA and protein expression is seen in the spinal trigeminal tract nucleus, the vagal motor nucleus and the gracile and cuneate nuclei (Conner *et al.,* 1997; Yan *et al.,* 1997a; Peiris *et al.,* 2004). These mammalian brain stem nuclei have similar functions to the trigeminal, facial, glossopharyngeal and vagal motor nuclei in fish. In mammals, BDNF is important in the development, maintenance and plasticity of neurons in the respiratory system (Brady *et al.,* 1999; Johnson *et al.,* 2000;

Katz *et al.*, 2003; Thoby-Brisson *et al.*, 2003; Borday *et al.*, 2004; Peiris *et al.*, 2004). For example, losses in peripheral chemoafferent neurons and, deficits in chemoreflex function are seen in BDNF knockout mice (Katz *et al.*, 2003; Borday *et al.*, 2004). In addition, exposure to hypercapnic hypoxia alters BDNF mRNA and protein levels in piglet brain stem respiratory nuclei suggesting that BDNF may play a neuroprotective role in response to hypoxic insult (Peiris *et al.*, 2004). It is possible that BDNF may perform similar roles in the motor nuclei of the trigeminal, facial, glossopharyngeal and vagal nerves in the eel.

3.4.5 BDNF in the motor brain stem

The brain stem is important in the control of movement and provides an interface between higher brain processing centres and neurons in the spinal cord (Whelan, 1996; Deliagina *et al.*, 2000; Bosch and Roberts, 2001; Deliagina *et al.*, 2002; Drew *et al.*, 2004; Matsuyama *et al.*, 2004). In fish, all information is channelled through this route as, in contrast to mammals, there is no direct corticospinal pathway. Little BDNF expression is seen in the brain stem nuclei that are involved in motor control and project down the spinal cord in the adult, mammalian brain. Immunoreactive cells for BDNF are seen in the spinal vestibular, dorsal raphe nucleus and gigantocellular reticular nucleus (Conner *et al,* 1997, Furukawa *et al.*, 1998; Murer *et al.*, 1999). However, these nuclei, along with the other cell groups that make up the reticular formation which include the magnocellular, oral pontine and caudal pontine reticular nuclei (Conner *et al.*, 1997; Matsuyama *et al.*, 2004), the raphe nucleus (Galter *et al.*, 2000), red nucleus (Harvey *et al.*, 2005) and nucleus of the medial longitudinal fasciculus (Conner *et al.*, 1997) are lacking in BDNF mRNA expression in the adult mammal (Ceccatelli *et al.*, 1991; Friedman *et al.*, 1991; Conner *et al.*, 1997). During development in the mammal, BDNF mRNA expression appears to move in a caudal to rostral gradient in the brain. It is expressed transiently in regions that mature early on such as the brain stem but is expressed in a more persistent manner in forebrain regions that mature later, e.g. the hippocampus (Friedman *et al.*, 1991). Although BDNF mRNA is transiently expressed in mammalian brain stem nuclei such as the gigantocellular reticular nucleus and raphe magnus nucleus, this expression decreases two weeks after birth (Friedman *et al.*, 1991).

In teleosts, groups of neurons in the brain stem such as the nuclei of the superior, medial and inferior reticular formation, inferior raphe nucleus, red nucleus, the anterior, descending, magnocellular and tangential octavolateral nuclei, nucleus of the medial longitudinal fasciculus and ventromedial nucleus of the thalamus are important in the control of movement (Prasada Rao *et al.*, 1987; Prasada Rao *et al.*, 1993; Bosch and Roberts, 1994; Becker *et al.*, 1997; Bosch and Roberts, 2001; Doyle *et al.*, 2001). The

reticular formation in fish is homologous to the reticular formation in mammals (Grillner and Wallén, 1985; Prasada Rao *et al.*, 1987; Noga *et al.*, 1988; Lee *et al.*, 1993; Grillner *et al.*, 1995; Guertin and Dubuc, 1997; Deliagina *et al.*, 2000, 2002). Both the rubrospinal (red) and raphespinal cells groups in fish and mammals may also be considered as homologous brain stem regions (Prasada Rao *et al.*, 1987; Lee *et al.*, 1993). In mammals, the vestibulospinal neurons are important in the control of balance and posture. The vestibular spinal brain stem nuclei in mammals perform similar functions to the anterior, magnocellular, tangential and descending octavolateral nuclei in the teleost brain stem, which are important in balance control in fish (Meredith *et al.*, 1987; Meredith, 1988). The nucleus of the medial longitudinal fasciculus in mammals (Yeomans and Frankland, 1995; Goto and Hoshino, 2001) and fish (Gahtan *et al.*, 2002) also appear to be homologous regions. The ventromedial nucleus of the thalamus in teleosts is thought to be homologous to regions of the mammalian thalamus such as the intralaminar nuclei, the ventrolateral thalamic nucleus and the ventral portion of the lateral geniculate nucleus (Ito *et al.*, 1986).

No BDNF mRNA expression was seen in the red nucleus in the eel (Fig. 3.2K; Table 3.3) but in contrast to adult mammals, moderate staining for BDNF mRNA was seen in the inferior raphe nucleus (Rinf; Fig. 3.2U-W; Fig. 3.15 e and f; Table 3.4), the Maunther cell (M; Fig. 3.2Q and Fig. 3.13 c and e) and in the superior (SRF; Fig. 3.12 c), medial (MRF; Fig. 3.13 c; Fig. 3.14 c; Fig. 3.17 a) and inferior (IRF; Fig. 3.15 e and f; Fig. 3.16 c; Fig. 3.17 c) divisions of the reticular formation (Fig. 3.2P-X; Table 3.4). BDNF mRNA expression was also seen in the magnocellular (MO; Fig.3.2Q and R; Fig. 3.13 c and 3.14 a), tangential (TO; Fig. 3.2S and Fig. 3.14 a) and descending (DO; Fig. 3.2V) octavolateral nuclei (Table 3.4), nucleus of the medial longitudinal fasciculus (nMLF; Fig. 3.2K, L; Fig. 3.9 b; Table 3.3). In the rat, BDNF mRNA staining was seen only in the posterior intralaminar nucleus and not in the ventrolateral thalamic nucleus and the ventral portion of the lateral geniculate nucleus (Conner *et al.*, 1997). In the eel, BDNF mRNA expression was seen in the ventromedial nucleus of the thalamus (VM; Fig. 3.2J; Table 3.2).

The superior reticular nucleus (RS; Fig. 3.2O and Fig. 3.11 c; Table 3.3) and the locus coeruleus (LC; Fig.3.2P and Fig 3.12 a; Table 3.4) also are involved in the control of movement (Molist *et al.*, 1993; Wullimann *et al.*, 1996) but do not project down the spinal cord in the eel (Molist *et al.*, 1993; Bosch and Roberts, 2001). The superior reticular nucleus showed moderate and the locus coeruleus showed light BDNF mRNA expression in the eel. The counterpart of the superior reticular nucleus in mammals has been proposed as the pedunculopontine nucleus (Molist *et al.*, 1993) and the locus coeruleus in fish and

mammals are homologous (Wullimann *et al.,* 1996). In the rat, the pedunculopontine nucleus cells were moderately labelled (Conner *et al.,* 1997) and the cells in the locus coeruleus lightly to moderately labelled (Castrén *et al.,* 1995; Conner *et al.,* 1997) for BDNF mRNA. *In vitro,* BDNF does not promote survival of cultured locus coeruleus neurons and it has been suggested that BDNF does not provide autocrine support for neurons in this nucleus (Castrén *et al.,* 1995). BDNF synthesised in the locus coeruleus may instead just provide paracrine support for neuronal populations that project to this nucleus and to which this nucleus projects (Castrén *et al.,* 1995).

In teleosts, the oculomotor, the trochlear and abducens nuclei control eye movements and are located in the mesencephalic motor brain stem (Wullimann *et al.,* 1996; Deguchi *et al.,* 2005). In the eel, BDNF mRNA expression was seen in the oculomotor nucleus (nIII; Fig. 3.2M; Table 3.3) and nucleus of the trochlear nerve (nIV; Fig. 3.2N and O; Fig. 3.11 e; Table 3.3). BDNF mRNA expression has been reported in the medial accessory oculomotor nucleus in the rat (Conner *et al.,* 1997) but not in the trochlear nucleus. BDNF mRNA was not seen in the avian oculomotor and trochlear nuclei (Steljes *et al.,* 1999). The function of BDNF in the oculomotor nucleus and nucleus of the trochlear nerve in the eel may be to provide support for efferent and afferent neuronal groups (Davies and Wright, 1995; Altar *et al.,* 1997; Mufson *et al.,* 1999).

As mentioned in section 2.4.4, eels undergo a metamorphosis from "yellow", fresh water dwelling to "silver", salt water dwelling animals in the course of their life cycle (Rossi and Palombi, 1976; Yamoto and Hirano, 1978; Ellerby *et al.,* 2001; Aroua *et al.,* 2005) during which time dramatic changes in eye morphology including changes in eye size take place (Egginton, 1986; Braekevelt, 1988; Muller *et al.,* 2003). It is also possible that BDNF may promote any plastic changes that may be required in the trochlear and abducens nuclei due to changes in eye morphology during metamorphosis.

3.4.6 Concluding remarks

In conclusion, BDNF mRNA is expressed in regions of the eel brain homologous to those in other vertebrates. BDNF, therefore, may perform similar functions in the postnatal eel brain to those in the brains of other species such as mammals (Wetmore *et al.,* 1990; Conner *et al.,* 1997; Murer *et al.,* 2001), birds (Herzog and von Bartheld, 1998) and amphibians (Duprey-Diaz *et al.*, 2002; Wang *et al.* 2005). For instance, in agreement with mammalian studies (Wetmore *et al.,* 1990; Conner *et al.,* 1997) and as discussed in section 3.4.1, BDNF mRNA expression was seen in the lateral region of the area dorsalis of the eel brain, which has been proposed as the teleostean equivalent of the mammalian hippocampus (Nieuwenhuys and Meek, 1990).

The fact that BDNF appears to be expressed in the cells that project down the spinal cord in the eel brain may be important for neuronal regeneration in this animal. BDNF application to the cell bodies of injured neurons in the CNS promotes their survival and regeneration (Sawai *et al.,* 1996; Kobayashi *et al.,* 1997; Salie and Steeves, 2005). BDNF expression has also been shown to be transiently upregulated for a period of approximately two to three days in the cell bodies of injured neurons in the mammalian CNS after which time expression begins to decline (Gao *et al.,* 1997; Hirsch *et al.*, 2000; Chidlow *et al.,* 2005). The apparent constant basal expression of BDNF mRNA in the eel brain, in the cell bodies of supraspinal neurons, may be a factor that aids in their regeneration after axotomy in the spinal cord. The changes in total brain mRNA expression after spinal cord injury will be examined in the next chapter.

Chapter 4

Messenger RNA expression of neurotrophins before and after complete spinal cord transection in the CNS of the brain stem-spinal cord regenerating model, *Anguilla anguilla.*

4.1 Introduction

As previously stated in chapter 1, numerous studies show that neurotrophins, such as BDNF and NGF, are upregulated after injury in the mammalian CNS (Dougherty *et al.*, 2000; Hirsch *et al.*, 2000; Ikeda *et al.*, 2000; Widenfalk *et al.*, 2001; Brown *et al.*, 2004) and PNS (Raivich and Kreutzberg, 1993; Kobayashi *et al.*, 1996; Lee *et al.*, 2001a; Obata *et al.*, 2005). Not only are they upregulated by cells in the vicinity of the injury (Ikeda *et al.*, 2000; Widenfalk *et al.*, 2001; Brown *et al.*, 2004; Omura *et al.*, 2005) but also in cell bodies of the injured neurons themselves (Sebert and Shooter, 1993; Hirsch *et al.*, 2000; Chidlow *et al.*, 2005). In some cases neurotrophin receptors, e.g. Trk B, are also upregulated at the site of lesion (Lee *et al.*, 2001a) and in the cell somata (Hirsch *et al.*, 2000; Cui *et al.*, 2002) after neuronal insult. Upregulated neurotrophins appear to contribute to any subsequent regeneration seen in the PNS (Zhang *et al.*, 2000; Lee *et al.*, 2001a; Omura *et al.*, 2005) but the neurotrophic response in the mammalian CNS may be too brief and/or insufficient.

4.1.1 Neurotrophin expression at the site of injury in the PNS and CNS

Studies of the regenerating PNS suggest that the cells at the site of lesion may be important source of neurotrophins after injury and during the course of regeneration (Meyer *et al.*, 1992; Funakoshi *et al.*, 1993; Raivich and Kreutzberg, 1993; Zhang *et al.*, 2000; Lee *et al.*, 2001a; Omura *et al.*, 2005). Sciatic nerve lesion in the rat, for example, leads to a rapid increase in non-neuronal cell expression of NGF mRNA six hours after lesion and a slower but more marked increase (10 times greater than NGF) in BDNF mRNA expression three days post lesion reaching maximum levels three to four weeks after axotomy (Meyer *et al.*, 1992; Omura *et al.*, 2005). During this time, protein levels for BDNF are continuously increasing (Omura *et al.*, 2005). Furthermore, after sciatic nerve injury, deprivation of endogenous BDNF by treatment of rats with an anti-BDNF antibody impairs neuronal regeneration (Zhang *et al.*, 2000). An increase in neurotrophin production may also be accompanied by an upregulation in neurotrophin receptor expression (Lee *et al.*, 2001a). In response to vagus nerve axotomy, the mRNA expression levels of the neurotrophin receptors trk A, trk B, and trk C are increased at the site of injury along with their ligands, NGF, BDNF, NT-4 and NT-3. Increases were seen as early as one day after injury, peaked after 14 days and had declined on axonal reconnection with the target, 45 days after injury (Lee *et al.*, 2001a).

The purpose of an upregulation in neurotrophins and their receptors at the site of injury may be to promote neuronal survival, pathfinding and regrowth (Levi-Montalcini, 1987; Henderson, 1996; Lewin and Barde, 1996; McAllister *et al.*, 1999; Zhang *et al.*,

2000; Ginty and Segal, 2002, Markus *et al.*, 2002, Gillespie, 2003, Omura *et al.*, 2005; Zweifel *et al.*, 2005). As in the developing nervous system, neurotrophins may activate receptors expressed on the injured neurons and initiate survival and growth promoting signalling cascades at the site of injury and/or in the cell body after retrograde transport (Miller and Kaplan, 2001; Ginty and Segal, 2002; Heersen and Segal, 2002; Markus *et al.*, 2002; Howe and Mobley, 2005; Zweifel *et al.*, 2005). Neuronal regeneration is impaired in animals with reduced potential for neurotrophin activity after injury, i.e. trk B heterozygous knockout mice (Boyd and Gordon, 2001; Irintchev *et al.*, 2005; Eberhardt *et al.*, 2006). For example, after femoral nerve transection, reinnervation of the quadriceps muscle and functional recovery after injury is reduced in trk B heterozygote mice compared to wild-type littermates (Irintchev *et al.*, 2005; Eberhardt *et al.*, 2006).

Neurotrophins may also play an important role in promoting the remyelination of neurons after injury (Zhang *et al.*, 2000; Chan *et al.*, 2001; Notterpek, 2003). During postnatal development, increasing BDNF levels in the leg of one-day old mouse pups by subcutaneous injection of exogenous BDNF, enhances myelin formation in the sciatic nerve whereas removal of endogenous BDNF with a BDNF scavenger in the form of a Trk B-Fc fusion protein, inhibits myelination (Chan *et al.*, 2001). After injury in the PNS, as well as impairing regeneration as previously stated, deprivation of endogenous BDNF by treatment of rats with an anti-BDNF antibody impairs remyelination of the sciatic nerve (Zhang *et al.*, 2000).

Neurotrophins have also been implicated in the processes of dendritic and axonal arborisation, and synapse stabilisation in the nervous system (Snider, 1988; Cohen-Cory *et al.*, 1991; Zhang *et al.*, 1994; McAllister *et al.*, 1995; Inoue and Sanes, 1997; Schwarz *et al.*, 1997; Morrison and Mason, 1998; Lom and Cohen-Cory, 1999; Xu *et al.*, 2000a; Lom *et al.*, 2002; Vicario-Abejón *et al.*, 2002; Cohen-Cory and Lom, 2004). Recently, the direct involvement of BDNF in axonal arborisation and synapse stabilisation in the tectum of *Xenopus* (Alsina *et al.*, 2001; Hu *et al.*, 2005) and its receptor, Trk B, in dendritic spine maintenance in the mouse visual cortex (Chadravarthy *et al.*, 2006) have been visualized using *in vivo* imaging techniques. It is likely therefore that neurotrophins expressed in the vicinity of the injury may also promote synapse formation between regenerating neurons and their targets (McAllister *et al.*, 1999; Alsina *et al.*, 2001; Hu *et al.*, 2005).

In the non-regenerating mammalian CNS, increases in neurotrophin expression levels also take place in neuronal and non-neuronal cells such as astrocytes at the site of injury after spinal cord lesion (Johnson *et al.*, 2000; Krenz and Weaver, 2000; Ikeda *et al.*, 2001; Nakamura and Bregman, 2001; Uchida *et al.*, 2003; Brown *et al.*, 2004), probably in

an attempt to promote neuronal survival and regrowth similarly to the PNS as described above. These changes in expression however may not be sufficient to promote neuronal regeneration for a number of possible reasons.

Firstly, in contrast to the PNS, increases in neurotrophin expression at the site of injury in the spinal cord appear to be short-lived (Krenz and Weaver, 2000; Ikeda *et al.,* 2001; Nakamura and Bregman, 2001; Widenfalk *et al.,* 2001; Brown *et al.,* 2004) and protein expression may not be sustained for a long enough period to promote axonal pathfinding and regrowth. The results of studies carried out in mammals, whereby neurons have been induced to regenerate by exogenous neurotrophin application after a cord lesion, suggest that it takes at least one month for regrowing neurons to transverse the injury site and to reach the caudal cord (Menei *et al.,* 1998; Liu *et al.,* 1999; Jin *et al.,* 2002). In the rodent spinal cord, BDNF mRNA expression after injury is increased in motor and sensory neurons, and in glia at a timepoint of 24 hours after injury (Ikeda *et al.,* 2001; Nakamura and Bregman, 2001; Widenfalk *et al.,* 2001) but returns to control levels within three days (Ikeda *et al.,* 2001; Nakamura and Bregman, 2001). Ikeda *et al.,* (2001) reported a later phase of BDNF mRNA expression by macrophages and/or microglia seven days after injury using ISH which they did not detect using RT-PCR suggesting that it may have had a relatively small impact on overall BDNF mRNA levels. Furthermore, this later phase of expression was not detected by Nakamura and Bregman (2001) at seven days or by Widenfalk *et al.* (2001) six weeks after injury using ribonuclease protection assays. To the best of my knowledge, a study monitoring overall levels in BDNF protein expression at different timepoints after spinal cord injury has not been carried out. However, Buck *et al.,* (2000) showed a correspondence of BDNF protein and mRNA expression in spinal cord motoneurons using immunohistochemistry and ISH respectively. Dreyfus *et al.,* (1999) showed, using immunohistochemistry, that BDNF protein is present in astrocytic cells in the normal spinal cord which is in agreement with Ikeda *et al.,* (2001) who reported BDNF mRNA expression in astrocytes before spinal cord lesion. The apparent correspondence of mRNA and protein in the normal spinal cord suggests that an increase in mRNA after injury may reflect an increase in the protein expression profile. A decline in BDNF mRNA expression in the spinal cord three days after injury (Ikeda *et al.,* 2001; Nakamura and Bregman, 2001) therefore, may indicate a subsequent decline in protein expression and thus a loss of BDNF associated neurotrophic support for injured neurons before reconnection with their targets.

Increases in NGF mRNA expression in the mammalian spinal cord persist for longer than BDNF appearing one day after lesion and lasting for up to seven days

(Nakamura and Bregman, 2001; Widenfalk *et al.*, 2001; Brown *et al.*, 2004) but not six weeks after injury (Widenfalk *et al.*, 2001). NGF protein is elevated one week after spinal cord injury (Krenz and Weaver, 2000; Brown *et al.*, 2004) and has returned to control levels after two weeks (Krenz and Weaver, 2000). Similarly to BDNF, the decline in NGF protein between one and two weeks after injury indicates that the expression period for NGF is probably not long enough to support neuronal regrowth and target reconnection.

It may also be the case that endogenous levels of neurotrophins, even though upregulated after injury, are not sufficient to stimulate neuronal regeneration particularly in combination with the inhibitory influences present in the adult mammalian CNS after injury detailed in section 1.1. This is suggested by the fact that neurotrophin application at the site of injury, especially that accompanied by the implantation of a permissive cell graft, greatly improves neuronal survival and regrowth (Schnell *et al.*, 1994; Bregman *et al.*, 1997; Ye and Houle, 1997).

In addition, any survival and growth-promoting effects that an upregulation in neurotrophins may have, even though short lived, could be hindered due to a deficiency in the biologically active, growth promoting Trk A, B and C receptors at the site of injury and also, by the presence of the biologically inactive truncated Trk B receptor (Fryer *et al.*, 1997; King *et al.*, 2000; Liebl *et al.*, 2001). In rats, mRNA expression for trk A, B and C in the lesion site was downregulated one day (Liebl *et al.*, 2001) and remained so for four weeks (King *et al.*, 2000) after spinal cord injury. Furthermore, mRNA expression for the truncated trk B receptor was increased in the injured cord after three days, peaked at two weeks and remained at this level for at least four weeks post lesion (King *et al.*, 2000; Liebl *et al.*, 2001). This receptor is thought to be biologically inactive and its increased presence may sequester endogenous BDNF thus reducing its availability for injured neurons (Fryer *et al.*, 1997; King *et al.*, 2000; Liebl *et al.*, 2001).

4.1.2 Neurotrophin expression in the neuronal cell body after axotomy in the PNS and CNS

As well as behaving as target derived, retrogradely acting molecules, in the developing and adult nervous system, neurotrophins are also synthesised in the neuronal cell body where they act in an autocrine manner (Ghosh *et al.*, 1994; Acheson *et al.*, 1995; Davies and Wright, 1995; Wetmore and Olson, 1995) or are anterogradely transported along the axon (Altar *et al.*, 1997; Heymach and Barres, 1997; Conner *et al.*, 1997; Caleo *et al.*, 2000). It follows therefore that as well being altered in the target region, neurotrophin and neurotrophin receptor expression is often increased in the cell bodies of the injured neurons themselves in the CNS (Kokaia *et al.*, 1993; Kobayashi *et al.*, 1996;

Hirsch *et al.*, 2000; Cui *et al.*, 2002) and PNS (Sebert and Shooter, 1993; Tonra *et al.*, 1998; Michael *et al.*, 1999). In the PNS, sciatic nerve axotomy in the rat, for example, leads to a marked increase in NGF mRNA (Sebert and Shooter, 1993) and, BDNF mRNA and protein expression in the DRG (Sebert and Shooter, 1993; Tonra *et al.*, 1998; Michael *et al.*, 1999) and anterograde transport of BDNF protein to the site of injury (Tonra *et al.*, 1998).

Neurotrophins and their receptors may be upregulated in the cell bodies of injured neurons in an attempt to promote neuronal survival by the autocrine activation of receptors colocalised on the somata (Kokaia *et al.*, 1993; Miranda *et al.*, 1993; Davies and Wright, 1995; Shen *et al.*, 1999; Hammarberg *et al.*, 2000). For example, mRNAs coding for BDNF and its receptor, trk B, are coexpressed in the cell bodies of hippocampal and cortical neurons in rats. After a brain insult in the form of seizure induction, BDNF and trk B mRNA expression are increased in hippocampal and cortical neuronal cell bodies, which suggests that BDNF may act in an autocrine manner to promote cell survival after injury (Kokaia *et al.*, 1993). The autocrine actions of neurotrophins during regeneration also have the potential to promote neuronal regrowth since application of neurotrophins, such as BDNF and NT 4/5, to the cell bodies of injured mammalian (Kobayashi *et al.*, 1997) and chick (Salie and Steeves, 2005) spinal cord neurons induces axonal sprouting.

Cell body-derived, anterogradely transported neurotrophins also have the potential to promote survival of postsynaptic neurons in target fields after injury (Tonra *et al.*, 1998). During development in the rodent for example, BDNF acts as a survival factor for neurons of retinal ganglion cell targets, the superior colliculus and the lateral geniculate nucleus (Caleo *et al.*, 2000; Spalding *et al.*, 2002). In addition, anterogradely transported, cell body-derived neutrotrophins could promote axonal regrowth after injury. In the developing rat visual system for instance, an intraocular injection of antisense oligonucleotides to suppress BDNF synthesis in the cell bodies of retinal ganglion cells results in the retraction of their axons from their target, the lateral geniculate nucleus (Menna *et al.*, 2003). This effect could not be induced by the retinal application of the Trk inhibitor, K252a, suggesting that the axonal retraction was due to a lack cell body-derived, anterogradely-transported BDNF and not due to the autocrine or paracrine actions of BDNF at the retinal ganglion cell bodies (Menna *et al.*, 2003).

The activated trk B receptor may exert its neuronal survival and growth promoting actions by inducing the expression of regeneration associated genes such as GAP-43, Tα1 tubulin and synapsin I. A study, already mentioned above, by Kobayshi *et al.* (1997) examined the expression of trk B receptors in the cell bodies of spinally axotomised

rubrospinal neurons in the rat brain. Expression of trk B started to decline seven days after injury however infusion of the trk B receptor ligands BDNF and NT 4/5 in the vicinity of the rubrospinal neuron cell bodies fully prevented their atrophy, maintained trk B receptor levels and stimulated expression of GAP-43 and Tα1 tubulin. BDNF infusion at the cell bodies was also found to increase the number of injured neurons that regenerated into a peripheral nerve graft.

Further insight into the mechanism by which trk receptor activity promotes regeneration may be gained from the results of a study by Molteni *et al.* (2004). They showed that sensory neurons from exercise-conditioned rats showed increased regeneration potential both *in vivo* and *in vitro* compared to those from sedentary rats. An examination of mRNA expression in the cell bodies of the sensory neurons from exercised animals showed higher levels of BDNF, NT-3, synapsin I and GAP-43 expression compared to sedentary animals. The regeneration-promoting effects of exercise were decreased post injection of the trk receptor inhibitor, K252a, into dorsal root ganglia before exercise (Molteni *et al.*, 2004).

Like retrogradely transported neurotrophins, anterogradely-derived neurotrophins may also be important in neuronal remyelination (Zhang *et al.*, 2000; Chan *et al.*, 2001; Notterpek, 2003). Furthermore, they may be involved synapse formation between regenerating neurons and their targets (Altar *et al.* 1997; Heymach and Barres, 1997).

There are various examples of neuronal cell bodies in the adult mammal, that upregulate neurotrophins and their receptors in response to CNS injury (Kokaia *et al.*, 1993; Kobayashi *et al.*, 1996; Gao *et al.*, 1997; Hirsch *et al.*, 2000; Cui *et al.*, 2002; Chidlow *et al.*, 2005; Suneja *et al.*, 2005; Zhang *et al.*, 2006). In the visual system, the retina and optic nerve lie within the CNS. The optic nerve comprises the axons of retinal ganglion cells whose cells bodies are located in the retinal ganglion cell layer of the retina. BDNF mRNA levels are enhanced in the rat retina for a period of approximately two to three days following optic nerve lesion but have declined two weeks after injury (Gao *et al.*, 1997; Hirsch *et al.*, 2000; Chidlow *et al.*, 2005). BDNF protein levels are elevated after two days also and remain so in surviving neurons 14 days after injury (Hirsch *et al.*, 2000). Protein levels of the Trk A, B and C neurotrophin receptors have also been seen to increase following optic nerve transection but start to decline approximately one week after injury (Cui *et al.*, 2002). Furthermore, in the auditory system, Suneja *et al.* (2005) examined the effect of cochlear ablation on neurotrophin expression in the cell bodies of some auditory brain stem nuclei in the guinea pig that project to the cochlea. They reported an increase in BDNF and NT-3 protein levels after injury.

To the best of my knowledge, no studies have examined the effect of a spinal cord lesion on neurotrophin expression in the cell bodies of supraspinal neurons that project down the cord. However, Trk receptors are found on the cell bodies rubrospinal, corticospinal (Liebl *et al.,* 2001), vestibular, raphe and reticular (Yan *et al.,* 1997b) neurons. As mentioned above, BDNF application to cell bodies of spinally axotomised neurons has been shown to improve the survival and regrowth of rubrospinal in the rat (Kobayahsi *et al.,* 1997) and vestibulospinal and spinal projection neurons from the raphe nuclei in the chick (Salie and Steeves, 2005). BDNF application to the cell bodies of corticospinal neurons in the rodent, also promotes their survival after axonal injury (Lu *et al.,* 2001; Hiebert *et al.,* 2002) and in combination with NT-3 application in the spinal cord, their regeneration (Zhou and Shine, 2003). Despite increases in neurotrophin expression in CNS cell bodies the visual system for example (Gao *et al.*, 1997; Hirsch *et al.*, 2000; Chidlow *et al.*, 2005), neuronal regeneration is extremely limited in the CNS of mature amniotes. Similarly to studies conducted in the spinal cord mentioned above (Schnell *et al.*, 1994; Bregman *et al.*, 1997; Ye and Houle, 1997), application of neurotrophins and upregulation of their receptors in CNS cell bodies after injury improves neuronal regeneration (Sawai *et al.*, 1996; Kobayashi *et al.,* 1997; Cheng *et al.,* 2002; Mo *et al.*, 2002; Zhou and Shine, 2003; Salie and Steeves, 2005) again suggesting a lack of adequate endogenous neurotrophic support after injury in the CNS.

4.1.3 The focus of this study

Teleost fish recover well after CNS lesion with a regain of function often seen soon after injury (Bernhardt *et al.*, 1989; Becker *et al.,* 1997; Hanna *et al.,* 1998; Nona, 1998; Caminos *et al.*, 1999; Doyle *et al.*, 2001; Matsukawa *et al.,* 2004). Neurotrophins and their receptors are expressed in the fish CNS (Benowitz and Shashoua, 1979; Martin *et al.,* 1995; Hashimoto and Heinrich, 1997; Caminos *et al.,* 1999; Hannestad *et al.*, 2000; Heinrich and Lum, 2000; Dethleffsen *et al.*, 2003) and it is possible that they may be involved in neuronal regeneration (Benowitz and Shashoua, 1979; Turner *et al.,* 1982; Yip and Grafstein, 1982; Caminos *et al.*, 1999; Doyle and Roberts, 2006).

As mentioned in chapter 1, some indirect evidence for the involvement of neurotrophins in spinal cord regeneration in the experimental model used in this investigation, the European eel, comes from the results of studies examining the effect of neuronal activity after injury (Doyle and Roberts, 2004a, b; Doyle and Roberts, 2006). An increase in physical activity after spinal cord transection, by L-DOPA treatment (Doyle and Roberts, 2004a) or an exercise regime (Doyle and Roberts, 2006) improved some aspects of regeneration and functional recovery. On the other hand, a disruption of

neuronal activity by the NMDA receptor inhibitor, AP5, application in the target region of the spinal cord after injury results in a slowing of regeneration by up to 60 days (Doyle and Roberts, 2004b). In other studies, neurotrophin expression has been shown to be regulated by neuroelectric activity (Castrén *et al.*, 1992; Herzog *et al.*, 1994; Bozzi *et al.*, 1995; Al-Majed *et al.*, 2000; Hartmann *et al.*, 2001). Exercise in mammals, for example, has been shown to cause an increase in BDNF mRNA (Neeper *et al.*, 1996; Oliff *et al.*, 1998; Gomez-Pinilla *et al.*, 2002; Ying *et al.*, 2005) and protein (Gomez-Pinilla *et al.*, 2002; Skup *et al.*, 2002; Griesbach *et al.*, 2004), Trk B receptor mRNA (Gomez-Pinilla *et al.*, 2002) and protein (Skup *et al.*, 2002), and NGF mRNA (Neeper *et al.*, 1996) expression levels. In contrast, a lack of activity leads to a decline in BDNF and trk B mRNA expression (Widenfalk *et al.*, 1999), and a decrease in BDNF and NGF protein levels (Radak *et al.*, 2006). The improvement in regeneration seen in the eel therefore after an increase in physical activity may be associated with an exercise-induced increase in neurotrophin activity (Doyle and Roberts, 2004a, b; Vaynman and Gomez-Pinilla, 2005; Doyle and Roberts, 2006). On the other hand, the retardation of recovery after blockade of neuronal activity may have been related to a decline of neurotrophin synthesis in the target region (Doyle and Roberts, 2004b).

Based on the results of studies mentioned above into the expression and involvement of neurotrophins after neuronal injury in mammals and birds, and the findings that exercise improves regeneration in the eel, the hypothesis was formulated that neurotrophins may be upregulated in the fish CNS after spinal cord transection and may aid the regeneration process. Changes in the mRNA expression of the neurotrophins BDNF, an NGF-like factor and the high affinity receptor for BDNF, trk B, in the eel CNS before and after complete spinal cord transection were chosen as the focus of this study for the following reasons:

- Roles are supported, by literature pertaining to neuronal regeneration, for BDNF and NGF activity in the target region (Meyer *et al.*, 1992; Schnell *et al.*, 1994; Bregman *et al.*, 1997; Ye and Houle, 1997; Krenz and Weaver, 2000; Lee *et al.*, 2001a; Nakamura and Bregman, 2001; Brown *et al.*, 2004) and cell bodies of lesioned neurons (Kokaia *et al.*, 1993; Sebert and Shooter, 1993; Kobayashi *et al.*, 1996; Kobayahsi *et al.*, 1997; Tonra *et al.*, 1998; Hirsch *et al.*, 2000; Cui *et al.*, 2002; Chidlow *et al.*, 2005; Salie and Steeves, 2005; Suneja *et al.*, 2005) after injury.
- Several studies suggest the involvement of these neurotrophins in neuronal regeneration in fish, such as the study in the goldfish which showed that NGF

application by intraocular injection or by local application to the site of injury improves regeneration after optic nerve crush (Yip and Grafstein, 1982), and the research suggesting that the improvement in regeneration seen in the eel after an increase in physical activity may be neurotrophin-activity related (Doyle and Roberts, 2004a; Doyle and Roberts, 2006).

- Due to DNA sequence availability for fish BDNF (e.g. NCBI GenBank accession numbers NM_131595, L27171 and X59942), *A. anguilla* NGF-like factor (accession number AY205346) and *A. anguilla* trk B receptor (accession number DQ396402).

Using RT-PCR, mRNA levels for BDNF, an NGF-like factor and trk B were examined before and after injury in the target region of the spinal cord and in the eel brain, which contains the cell bodies of the spinally axotomised neurons. The spatial expression patterns for BDNF mRNA in the brain were also investigated before and after injury using *in situ* hybridisation. Timepoints of one, 10, 15 and 20 days post spinal cord transection (DPT) were chosen for examination to correlate with stages in regeneration where neurotrophins and their receptors may have important roles in promoting recovery. One day after injury, neurotrophin activity may be important in neuronal survival and initiating axonal regrowth (Götz *et al.*, 1992; Meyer *et al.*, 1992; Acheson *et al.*, 1995; Davies and Wright, 1995; Tonra *et al.*, 1998; Shen *et al.*, 1999; Ikeda *et al.*, 2001; Lee *et al.*, 2001a). Ten DPT was examined since regenerating neurons have begun to regrow and regenerating axons are seen to bridge the gap created by the transection however functional connections have not been formed at this timepoint (Doyle *et al.*, 2001; Doyle, 2002). At 10 DPT, neurotrophins may be important in promoting axonal pathfinding and elongation (Turner *et al.*, 1982; Yip and Grafstein, 1982; Raivich and Kreutzberg, 1993; Kobayashi *et al.*, 1996; Tonra *et al.*, 1998; Song and Poo, 1999; Lee *et al.*, 2001a; Markus *et al.*, 2002; Gillespie, 2003; Menna *et al.*, 2003). At 15 and 20 DPT, most regenerating neurons are still regrowing. Others have bridged the gap completely and synapses have started to form between axonal terminals and their targets in the distal cord (Doyle *et al.*, 2001; Doyle, 2002). At this stage, as well as promoting axonal elongation, neurotrophins may also play a role in synaptogenesis (Altar *et al.*, 1997; Heymach and Barres, 1997; McAllister *et al.*, 1999; Schinder and Poo, 2000; Blum and Konnerth, 2005; Bramham and Messaoudi, 2005).

4.1.4 Establishing RT-PCR protocols in the eel

Study design- estimation of animal number

In the design of experiments involving animals and expensive reagents, it is important to use the minimal number of animals without reducing or compromising scientific output (Festing *et al.*, 2002; Ruxton and Colegrave, 2003). First, the size of the effect to be detected by the experimental system should be decided. The effect size is usually based on the magnitude of the biological change to be detected that would indicate a result of scientific interest. The greater the variation between sample units, the larger the sample size needs to be in order to ensure the study will have sufficient power to reveal a significant difference between experimental groups, particularly if the effect size to be detected is small (Festing *et al.*, 2002; Ruxton and Colegrave, 2003).

A method known as power analysis can be used to determine appropriate sample size for a study and depends on the relationship of six variables:

1) the effect size of biological interest
2) the standard deviation
3) the desired power of the experiment
4) the sample size
5) the significance level
6) the alternative hypothesis (i.e. a one- of two-sided test).

When five of these variables are fixed, a mathematical relationship can be used to estimate the sixth (Festing *et al.*, 2002). Power analysis tables and methods are described by Cohen (1988). Power analysis can also be performed using computer packages such as MINITAB, SAS (SAS, USA), nQuery Advisor (Statistical Solutions Ltd, Ireland), PASS 2000 (NCSS, USA) and Power and Precision (USA) (Festing *et al.*, 2002).

Prior to this study, a semiquantitative RT-PCR approach to investigate changes in gene expression in the eel during spinal cord regeneration had not been carried out in our laboratory therefore the approximate estimates of the standard deviation with which to carry out formal power analysis for RT-PCR experiments were unavailable (Festing *et al.*, 2002). In this situation and indeed when planning complicated experiments where the power analysis method may not be practical or appropriate, it is also possible to estimate sample size based on similar experiments carried out by other groups (Ruxton and Colegrave, 2003) and by using the Mead's (1988) resource equation which depends on the law of diminishing returns, i.e. increasing the sample size beyond a certain point yields little extra information (Festing *et al.*, 2002).

Statistically significant changes in gene expression determined by semiquantitative RT-PCR are reported for experiments carried out with sample sizes that range from three upwards (Kobayashi *et al* 1996, n = 4; Hirsch *et al.,* 2000, n = 4; Hashimoto *et al,* 2004, n = 4-6; Pierce *et al.,* 2004, n = 4-6; Al-Bader and Al-Sarraf, 2005, n = 4; Huang *et al.,* 2005; Omura *et al.,* 2005, n = 5; Valter *et al.,* 2005, n = 10). The sample size ranged from five to seven in an RT-PCR investigation carried out by Weltzien *et al.* (2005) to compare expression levels of tyrosine hydroxylase in six different regions in the brain of *A. anguilla*. Degani *et al.* (2003) used RT-PCR to compare mRNA levels of follicle stimulating hormone and lutenising hormone in the pituitary glands of male and female *A. anguilla* and had a sample size of 14 (Degani *et al.,* 2003). In examples of semiquantitative RT-PCR carried out with tissue from *A. japonica,* the Japanese eel, which has a close genetic relationship to *A. anguilla* (Mank and Avise, 2003; Minegishi *et al.,* 2005), sample sizes range from three to eight (Takei *et al.,* 2001, n = 3-8; Li *et al.,* 2003, n = 5-6; Yuge *et al.,* 2003, n = 5-7). For RT-PCR studies carried out in mammals to quantify neurotrophin mRNA expression changes after events such as CNS insult and after exercise, sample sizes were between four and five (Kobayahsi *et al.,* 1996, n = 4; Hirsch *et al.,* 2000, n = 4; Huang *et al.,* 2005, n = 4; Harvey *et al.,* 2005, n = 4; Omura *et al.,* 2005, n = 5). In other studies carried out in rodents in order to measure changes in gene expression in the CNS after insult, sample sizes range from three to six (Van Beek *et al.,* 2000, n = 3; Briscini *et al.,* 2002, n = 6; Tachibana *et al.,* 2002, n = 3; Benani *et al.,* 2003, n = 4; Hashimoto *et al.,* 2004, n = 4-6; Wu *et al.*, 2005, n = 4-5). Sample sizes ranged from four to 11 for RT-PCR analyses of gene expression changes in the mammal brains related to other biological events such as memory formation (Huang *et al.,* 1997, n = 4-11; Chen *et al.,* 1999, n = 8; Hager *et al.,* 1999, n = 6; Al-Bader and Al-Sarraf, 2005, n = 4).

Based on results of other RT-PCR studies (e.g. Kobayashi *et al* 1996; Benani *et al.,* 2003; Pierce *et al.,* 2004; Al-Bader and Al-Sarraf, 2005; Wu *et al.*, 2005) and with an aim to conserving animal numbers and other resources but without compromising scientific output, a sample size of four was chosen for semiquantitative RT-PCR experiments investigating gene expression in untransected animals and animals one, 15 and 20 DPT.

Mead's (1988) resource equation for experiments not using blocking states:

$$E = N - T$$

where E = degrees of freedom of error, N = total number of experimental units and T = number of treatment combinations. In general, E should lie between 10 and 20 since increasing the degrees of freedom of error from one to 10 leads to a substantial reduction in the critical level of Student's *t* that would be declared statistically significant but

increasing the degrees of freedom of error above 20 hardly alters the critical value of Student's *t* and therefore may be wasteful of resources (Mead, 1988). According to Mead's (1988) resource equation,

$$E = 16\text{-}4 = 12$$

for semiquantitative RT-PCR experiments investigating gene expression in untransected animals and animals one, 15 and 20 DPT in this study. A sample size of four therefore should in theory be adequate to detect a statistically significant change in mean band intensity for BDNF, the NGF-like factor or trk B.

A separate set of RT-PCR experiments was carried out with brain tissue from *A. rostrata* (the reasons for this are stated below in section 4.2.1) to compare gene expression in four untransected animals with that in four animals 10 days after spinal cord transection. Although Mead's equation gives a return of six for these experiments, a significant difference has been detected between two treatment groups in other RT-PCR studies with sample sizes of three to four (Hirsch *et al.*, 2000; Tachibana *et al.*, 2002; Hashimoto *et al.*, 2004; Huang *et al.*, 2005).

For studies carried out to examine levels of the genes of interest before and after injury in the target region of the spinal cord, three cDNA samples were synthesised from three pools of two untransected cords and three were synthesised from three pools of two cords removed from eels 15 DPT. Del Signore *et al.* (2006) carried out a study with a similar design to examine gene expression levels before and after CNS injury in the rat. They found significant differences in mRNA expression levels of genes including vimentin and decorin when cDNA samples prepared from three pools of three cervical ganglia from control animals were compared with cDNA samples prepared from three pools of three cervical ganglia from injured animals using RT-PCR.

Selection of housekeeping genes for semiquantitative analysis of RT-PCR results

Variations between RT-PCR samples may occur at a number of stages during the protocol such as RNA extraction, reverse transcription and due to differences in PCR efficiency. In order to control for these errors between samples when measuring RNA expression, RNA levels can be normalised to a housekeeping gene such as β-actin, β-tubulin, cyclophilin and hypoxantine phosphoribosyltransferase (Thellin *et al.*, 1999). Ideally, a housekeeping gene should be ubiquitously expressed in the tissue of interest and its expression should not be affected by the experimental treatment being carried out (Bustin, 2000, 2002; Dheda *et al.*, 2004). Nevertheless, the expression levels of some housekeeping genes do appear to vary in certain biological situations (Freeman *et al.*, 1999; Thellin *et al.*, 1999; Bustin, 2000, 2002; Bustin and Nolan, 2004; Dheda *et al.*, 2004;

Yadav *et al.*, 2004; Al-Bader and Al-Sarraf, 2005; Huggett *et al.*, 2005; Wu and Storey, 2005; Zupanc *et al.*, 2005) and it is important therefore to choose a housekeeping gene according to the nature of the study and experimental model under examination (Thellin *et al.*, 1999; Medhurst *et al.*, 2000; Giulietti *et al.*, 2001; Bustin and Nolan, 2004; Dheda *et al.*, 2004; 2005; Al-Bader and Al-Sarraf, 2005). For example, the housekeeping genes β-actin and β-tubulin are essential for the structure and kinetics of the cytoskeleton (Thellin *et al.*, 1999). However, alterations in the expression of cytoskeletal proteins occur after neuronal injury (Skene, 1989; Tetzlaff *et al.*, 1991; McKerracher *et al.*, 1993) and changes in β-actin and β-tubulin protein levels have been shown to take place in the regenerating fish brain (Zupanc *et al.*, 2005) therefore β-actin and β-tubulin were deemed unsuitable for use as internal standards in this study.

The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acidic ribosomal phosphoprotein (ARP) were chosen as internal controls for semiquantitative analysis of RT-PCR results in this study. GAPDH is commonly used as a housekeeping gene and is an important enzyme in the glycolytic pathway (Thellin *et al.*, 1999; Winer *et al.*, 1999, Dheda *et al.*, 2004; Wu *et al.*, 2005). Its expression has been shown to fluctuate for example during development (Puissant *et al.*, 1994; Calvo *et al.*, 1997; Al-Bader and Al-Sarraf, 2005), during the cell cycle (Mansur *et al.*, 1993) and in human whole blood samples and blood cell cultures (Dheda *et al.*, 2004). On the other hand, it has been used successfully as a housekeeping gene standard in studies examining changes in gene expression in CNS tissues after injury in rats (Hirsch *et al.*, 2000; Tachibana *et al.*, 2002; Chidlow *et al.*, 2005; Wu *et al.*, 2005; Yang *et al.*, 2005; Ying *et al.*, 2005), and the frog (Duprey-Díaz *et al.*, 2002). After CNS ischemic insult, GAPDH has been used for normalisation in semiquantitative RT-PCR analysis of glial acidic fibrillary protein mRNA expression in the mouse brain (Van Beek *et al.*, 2000) and protease-activated receptors in the rat brain (Rohatgi *et al.*, 2004). It has also been employed as a RT-PCR standard to normalise neurotrophin mRNA expression changes in T cells and B cells in humans and mice after immunisation with an encephalitogen (Edling *et al.*, 2004). It has also been used as a housekeeping gene for semiquantitative RT-PCR investigation of changes in gonadtropin-releasing hormone mRNA expression in the eel brain before and after testosterone treatment (Okubo *et al.*, 2002) and for comparison of C-type natriuretic peptide mRNA levels in fresh and salt water adapted eels (Takei *et al.*, 2001). Furthermore, GAPDH has been used for mRNA normalisation in studies examining levels of guanylin family member mRNA expression in the eel intestine after sea water adaptation (Yuge *et al.*, 2003), and to monitor the effects of adrenocorticotropin injection

and seawater transfer on steroidgenic acute regulatory protein mRNA expression in the eel head kidney (Li *et al.*, 2003).

ARP is a component of the 60S ribosomal subunit and plays a role in the elongation step of protein synthesis (Rich and Steitz, 1987; Krowczynska *et al.*, 1989; Shimizu *et al.*, 2002; Yadav *et al.*, 2004). As with all housekeeping genes, there are some biological situations in which ARP expression has been shown to change (Barnard *et al.*, 1992; Gorzelniak *et al.*, 2001; Yadav *et al.*, 2004; Wu and Storey, 2005). In the wood frog for example, ARP is upregulated in response to freezing or anoxia (Wu and Storey, 2005). To the best of my knowledge, ARP has not been used before as a housekeeping gene for studies monitoring changes in gene expression during spinal cord regeneration. However, it has been used successfully as a housekeeping gene for RT-PCR experiments in the European eel brain (Weltzien *et al.*, 2005), growth hormone regulation of insulin-like growth factor-I mRNA expression in salmon hepatocyte cultures (Pierce *et al.*, 2004), circadian regulation of gene expression in rat fibroblasts (Grundschober *et al.*, 2001), levels of vascular endothelial growth factor in the mouse retina after hypoxic insult (Simpson *et al.*, 2000) and changes in gene expression in humans in various disease states (de Cremoux *et al.*, 2004; Dheda *et al.*, 2004) and after stroke (Hafer-Macko *et al.*, 2005). It is also one of the housekeeping genes recommended for use with the Taqman RT-PCR kit from ABI (http://docs.appliedbiosystems.com/pebiodocs/04308134.pdf) and by QIAGEN for quantitative analysis of real-time RT-PCR results (http://www1.qiagen.com/products/pcr/quantitect/HouseKeepingGenes.aspx).

4.2 Materials and methods

4.2.1 Animals for RT-PCR

This study is based on European eels, *A. anguilla* L., (n=28) obtained from the wild in Co. Mayo and Co. Westmeath, and *Anguilla rostrata* (n=8) obtained from the Delaware Valley Fish Company, Pennsylvania, USA of unknown sex in a non-migratory, immature stage of the life cycle. Animals ranged in length from 360 to 505 mm. They were kept in large aquaria with recirculating water at temperatures ranging from 21 - 24°C. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were approved by local ethics committees.

Due to animal shortages, some RT-PCR experiments were carried out on RNA extracted from brain tissue from *A. rostrata* which regenerates its spinal cord in the same fashion as *A. anguilla* (Prof. B. L. Roberts, unpublished observations). *A. anguilla* and *A. rostrata* have a close phylogenetic relationship (Minegishi *et al.*, 2005) and since neurotrophins are highly conserved between species (Heinrich and Lum, 2000; Dethleffsen *et al.*, 2003), species differences are unlikely to have an impact on the results. A total of four control, untransected and four cord-transected *A. rostrata*, 10 days after surgery, were used. All other RT-PCR experiments were carried out using RNA extracted from brain and spinal cord tissue from *A. anguilla*. RT-PCR experiments were carried out on brain total RNA from four untransected animals and cord-transected animals one, 15 or 20 DPT (n = 4 for each timepoint after injury). RT-PCR experiments were carried out on RNA extracted from three pools of two spinal cord tissue samples from untransected animals (total n = 6) and three pools of two spinal cord samples from injured animals 15 DPT (total n = 6).

Spinal cord transection surgeries were carried out as described in section 2.2.1. After surgery, eels were returned to individual holding tanks for a one, 10, 15 or 20 day recovery period.

Eels were anaesthetised with MS 222 and alphaxalone (0.4 ml of 9 mg/ml solution, sc; "Saffan", Schering-Plough Animal Health Ireland). Fresh brain tissue from injured animals was then dissected out, snap frozen in liquid nitrogen and stored at –80 °C, stored in RNAlater (QIAGEN) between 4 and -20 °C or processed immediately for RNA extraction. Cord regeneration was confirmed postmortem in all cord-transected animals by examining bridge formation in the spinal cord. Brain tissue from untransected, control animals was prepared in the same fashion. For spinal cord samples, tissue was exposed at the level of 13 segments caudal to the anus (the site of the transection in injured animals) and below, and a piece of tissue approximately 8-10 mm in length was removed from 5 mm caudal to the transection position, snap frozen in liquid nitrogen and stored at -80°C.

4.2.2 Total RNA isolation

Total RNA was extracted from whole brain tissue samples using Tri reagent (MRC) as instructed by the manufacturer. Isolated brain RNA was resuspended in 50µl RNase free water. Each spinal cord RNA preparation was derived from two pooled spinal cord tissue samples taken from animals receiving the same treatment. Isolated spinal cord RNA was resuspended in 15µl RNase free water.

Spectrophotometer readings were obtained to determine RNA concentration and purity for each sample. The purity of RNA samples was estimated by calculating the ratio of the absorbance values at 260 and 280 nm in 10 mM Tris-Cl, pH 7.5. RNA integrity was confirmed by agarose gel electrophoresis. The intensities for the 28S and 18S ribosomal RNA (rRNA) band were measured densitometrically using the AlphaImager™3300 gel documentation and analysis system, and ratios of the 28S to 18S rRNA band intensities were then determined for each sample. Prior to RT-PCR, RNA samples were DNase treated using RQ1 RNase free DNase (Promega) according to the manufacturer's instructions. The enzyme was then removed by phenol-chloroform extraction (Sambrook and Russell, 2001) and RNA was resuspended in 15-30 µl RNase free water.

4.2.3 Agarose gel electrophoresis

RNA and DNA were resolved on agarose/ tris borate EDTA (TBE) gels containing 0.5 µg/ml ethidium bromide with TBE (Sigma) running buffer. RNA samples were denatured with RNA loading buffer (Sigma) diluted 1:1 for 10 min at 65 °C before loading. RNA size standards (Promega) ranged from 0.28 – 6.58 kb. The DNA size standards used were 25 bp (Promega) and 100 bp, 1 kb and 2-log ladders (New England Biolabs).

4.2.4 Semiquantitative RT-PCR, brain

RT-PCR was carried using the QIAGEN® OneStep RT-PCR kit according to the manufacturer's instructions. The QIAGEN® OneStep RT-PCR kit utilizes a hot start *Taq* DNA polymerase which allows the temporal separation of reverse transcription and PCR permitting both processes to be performed sequentially a single tube. The kit is designed to be used with gene-specific primers. In this study, primer sets designed to amplify BDNF, trk B, NGF, ARP and GAPDH were employed to investigate mRNA expression in the eel brain after injury.

Preliminary RT-PCR experiments were conducted as advised in the QIAGEN® OneStep RT-PCR Kit Handbook (QIAGEN) in order to estimate an approximate cycle number and RNA template amount for each primer set. These experiments were typically carried out using 500 pg - 100 ng RNA template from two brain samples, cycle numbers

ranged from 30 to 45, aliquots were taken at five cycle intervals and separated on an agarose gel.

In order to determine cycle number more accurately and to ensure quantification of PCR reactions was not carried out during the plateau phase of amplification for each primer set, two RNA samples from each timepoint were then amplified over a range of four consecutive PCR cycles. The appropriate range was estimated based on the results of preliminary experiments described above. An aliquot amounting to one fifth of each RT-PCR product was removed after each consecutive cycle and stored at 4°C. All aliquots for a particular sample were separated on the same agarose gel in ascending order of cycle number and arbitrary values for the intensity of the resulting bands were determined densitometrically using the AlphaImager™3300 gel documentation and analysis system. The arbitrary value for the intensity of each band was plotted against its cycle number in order to ensure products were accumulating in an exponential fashion over the range of cycles. A dilution series of a DNA size standard of known concentration, separated on an agarose gel, was examined in order to ensure that the band fluorescence for RT-PCR products were proportionally related to the amount of amplification product. Intensities of the resulting bands for the DNA standard in the dilution series were determined densitometrically using the AlphaImager™3300 gel documentation and analysis system, and compared to ensure that the increase in band intensities for the dilution series was proportionally related to the increasing amount of DNA.

Primers used for cloning the 467 bp cDNA fragment of BDNF (see section 2.2.4) were also used for semiquantitative RT-PCR analysis of BDNF mRNA expression. PCR for *A. anguilla* BDNF was carried out for 32 cycles with an annealing temperature of 62 °C.

Primers were designed for the NGF-like factor to give a 277 bp product using MacVector software (Accelerys, Oxford Molecular), based on *A. anguilla* NGF-like sequence (Dethleffsen *et al.*, 2003). Primer sequences for the NGF-like factor were as follows - forward: 5'-AACTGGGTGGGCAACAAGACCAAG-3'and reverse: 5'-ACACACAGGCGGCGTTTATG-3'. RT-PCR for NGF was carried out for 35 cycles with an annealing temperature of 65 °C. It is important to note that primers were designed based on a putative NGF-like sequence described by Dethleffsen *et al.* (2003) who state that they could not reliably decide whether their eel neurotrophin sequence represented an NGF-like or NT-6/7-like neurotrophin.

RT-PCR was performed with primers for *A. anguilla* trk B, designed to yield a 363 bp product by O'Brien (2004) based on a 603 bp cloned cDNA fragment (NCBI GenBank

accession number DQ396402). As mentioned in section 4.1.1, a biologically active form and a truncated, inactive form, which lacks the intracellular domain, of the Trk B receptor exist in mammals (Middlemas *et al.*, 1991) and in zebrafish (Martin *et al.*, 1995). ClustalW alignments (Higgins *et al.*, 1994) were performed with the predicted amino acid sequence for the cloned *A. anguilla* Trk B cDNA fragment (NCBI GenPept accession number ABD62075), the deduced amino acid sequences for zebrafish Trk B (Martin *et al.*, 1995) and rat Trk B (NCBI GenPept accession number NP_036863; Middlemas *et al.*, 1991) in order to determine which form/s of the receptor would be amplified by the primers given above.

Primer sequences for *A. anguilla* trk B were as follows - forward: 5'-GAGACCTCAACAAGTTCCTCAGG-3' and reverse: 5'-GGACGACCCCCAGACTCC-3'. RT-PCR for trk B was carried out for 34 cycles with an annealing temperature of 65 °C.

Primer sequences for *A. anguilla* ARP, amplifying a 107 bp product, were taken from Weltzien *et al.* (2005) and were as follows- forward: 5'-GTGCCAGCTCAGAACACTG-3'and reverse: 5'-ACATCGCTCAAGACTTCAATGG-3'. RT-PCR for ARP was carried out for 35 cycles with an annealing temperature of 60 °C. Primers for *A. anguilla* GAPDH were designed by Dr S. M. Borich to yield a 236 bp product based on a 462 bp cloned cDNA fragment (GenBank accession number AY219884) and sequences were as follows - forward: 5'-TGAAGGGAGGTGCCAAGAGG-3' and reverse: 5'-TCCGTCCACCGTCTTCTGG-3'. RT-PCR for GAPDH was carried out for 32 cycles with an annealing temperature of 57 °C.

Total RNA (500 pg for BDNF, trk B, ARP and GAPDH, 10 ng for NGF) was reverse transcribed at 50 °C for 30 minutes which was followed by a 95 °C PCR activation step for 15 minutes during which time the reverse transcriptase was inactivated and the hot start *Taq* DNA polymerase was activated. PCR cycling conditions were as follows: 94 °C for 30 sec, annealing at the primer-specific temperature for 40 sec, 72 °C for 1 min, (cycle number depending on the primers in use) and a final extension for 10 min at 72 °C. Primers had a final concentration of 0.6 μM in each reaction as advised in the QIAGEN® OneStep RT-PCR Kit Handbook (QIAGEN). RT-PCR experiments with RNA samples from *A. anguilla* as template were replicated three times with BDNF and NGF-like factor primers and twice with trk B and ARP primers. All RT-PCR experiments with *A. rostrata* RNA samples were repeated twice.

A negative, "no DNA" control whereby RNA was replaced with water was included in each RT-PCR experiment for each primer set in order to detect possible contamination of the reaction components. To test for genomic DNA contamination in RNA samples, control reactions were set up for each sample using all components except RNA. The RNA was then added during the 95 °C PCR activation step. The only DNA template available at this stage is contaminating genomic DNA since the reverse transcriptase has been inactivated and formation of PCR products in these reactions would indicate the presence of genomic DNA contamination (QIAGEN® OneStep RT-PCR Kit Handbook, QIAGEN).

One fifth of the RT-PCR products were separated on agarose gels of appropriate concentration. All RT-PCR products from the same experiment were run on the same gel. The resulting bands were photographed, their intensity was determined densitometrically and an arbitrary value for the intensity of each band was assigned using the AlphaImager™3300 gel documentation and analysis system.

4.2.5 Semiquantitative RT-PCR, spinal cord

A two-step RT-PCR approach was employed for semiquantitative RT-PCR analysis of mRNA expression in spinal cord samples. Single stranded complementary DNA (sscDNA) was synthesized by Dr S. M. Borich from 0.5 μg of total RNA using PowerScript™ Reverse Transcriptase (BD Biosciences, UK) in the presence of oligo $(dT)_{12\text{-}18}$ primers (Invitrogen, UK) according to the manufacturer's instructions. Second strand cDNA synthesis was carried out using primers (sequences as described in section 4.2.4), for BDNF (40 cycles, annealing temperature: 62 °C), NGF-like factor (40 cycles, annealing temperature: 65 °C), trk B (40 cycles, annealing temperature: 65 °C) and ARP (28 cycles, annealing temperature: 60 °C). PCR reactions were carried out using 2.5% (BDNF, NGF and trk B) or 0.83% (ARP) of the sscDNA product as template, 0.6 μM of each primer, 2 units *Taq* DNA polymerase, 1x ThermoPol buffer (both New England Biolabs, Inc.) and 0.2 mM dNTPs (Invitrogen, UK) in a 50 μl total volume. Cycling conditions were as follows: 95°C for 1 min, annealing at the primer-specific temperature for 40 sec, 72°C for 1 min for appropriate cycle number for each primer set and finally an extension of 2 min.

In order to ensure the quantification of PCR reactions was carried out during the exponential phase of amplification, experiments were performed as described in section 4.2.4. An additional experiment was also conducted to confirm that RT-PCR analysis was not carried out during the plateau phase of amplification whereby a two-fold dilution series

using 0.2, 0.3, 0.6, 1.3 and 2.5% of sscDNA template from one untransected and one cord transected animal was amplified using ARP primers.

One fifth of the RT-PCR products were separated on agarose gels of appropriate concentration. The resulting bands for BDNF, NGF, trk B and ARP RT-PCR products were photographed and their intensity was determined densitometrically using the AlphaImager™3300 gel documentation and analysis system. For semiquantitative analysis, samples were normalized to ARP expression by determining the relative intensity of the bands (e.g. BDNF / ARP). All RT-PCR experiments were replicated at least twice.

A negative, "no DNA" control whereby RNA was replaced with water was included in each RT-PCR experiment for each primer set in order to detect possible contamination of the reaction components. Samples were tested for genomic DNA contamination by omitting the reverse transcription step and using the equivalent amount of RNA, in the place of sscDNA, as template in the PCR reactions.

4.2.8 Statistical analysis

Unless otherwise stated in section 4.3, for semiquantitative analysis, the average band intensity for each individual sample was calculated from replicate experiments with a particular primer set and normalised to the average ARP value for the same sample from two experiments (e.g. BDNF / ARP) based on the method used in other RT-PCR studies (Briscini *et al.*, 2002; Rohatgi *et al.*, 2004; Chen *et al.*, 2005; Al-Bader and Al-Sarraf, 2005; Omura *et al.*, 2005). Statistical analysis was performed with normalised data using SPSS computer software. Before performing statistical analysis with normalised data, in order to ensure the assumptions for analysis of variance tests and t-tests were met, the normality of each data set was tested using a one-sample Kolmogorov-Smirnov test. Normality was assumed when data sets generated a p value greater than 0.05 (Dytham, 2003). In addition, the homogeneity of variance for each data set was tested using the Levene's test for equality of variances. Equal variance was assumed for data sets generating p values greater than 0.05 (Festing *et al.*, 2002; Dytham, 2003).

BDNF, NGF and trk B mRNA expression levels were compared in the brain of untransected animals and animals one, 15 and 20 DPT using one-way analysis of variance tests (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc tests (Festing *et al.*, 2002). Note that studies carried out on day 10 animals were not included since PCR reactions were not conducted on the same day as those for *A. anguilla,* therefore mRNA expression 10 DPT was compared with expression in untransected *A. rostrata* animals using a two-tailed, Student's *t*-test for independent samples. A two-tailed, Student's *t*-test for independent samples was also used to compare expression levels of the

genes of interest in the spinal cord of untransected animals with those in animals 15 days after injury. Differences were considered significant when $p < 0.05$. Values were expressed as means ± standard error of the mean (SEM).

4.2.9 Animals for in situ hybridisation

This study includes only European eels, *A. anguilla* L., (n=4) obtained from the wild in Co. Mayo of unknown sex in a non-migratory, immature stage of the life cycle. Animals ranged in length from 350 to 530 mm. Spinal cord transection surgeries were carried out on two animals as previously described (section 2.2.1). Animals were allowed to recover for 10 DPT. Brain tissue from cord-transected animals was fixed for ISH as described in section 2.2.1 and 100 µm sections cut in the horizontal plane with a vibrating microtome (VT1000S, Leica) as described in section 2.2.11. Horizontal brain sections prepared from untransected animals (n=2; section 3.2.1) were used in this study for comparison with sections prepared from the eel brain 10 days after spinal cord transection.

4.2.10 In situ hybridisation

BDNF sense and antisense RNA probes were prepared as described in section 2.2.6 from plasmid DNA containing a 467 bp BDNF insert. ISH was carried out as detailed in section 2.2.11. Light microscopic images were collected and reconstructions of the staining patterns in the ventral brain region of cord-transected and untransected fish were compiled from camera lucida drawings as described in section 3.2.4. The more dorsally located brain regions of tectum and cerebellum were not included in the reconstructions.

4.3 Results

4.3.1 Total RNA isolation

In all cord-transected animals used in this study except those 1 DPT, a bridge of regenerating material, connecting caudal and rostral stumps of the transected cord had formed confirming neuronal regeneration had taken place after spinal cord transection. Prior to expression analysis by RT-PCR the quality of the RNA preparations was assessed by calculating the 28S:18S rRNA ratio for each sample. Samples deemed acceptable for use showed sharp bands for 28S and 18S rRNA when separated on an agarose gel and had 28S:18S ratios ranging from 1.0 to 2.0 for brain RNA and 0.9 to 1.7 for spinal cord RNA. Samples that appeared to be degraded and that had a 28S:18S ratio of less than 0.9 were discarded, such as the example in lane 2, Figure 4.1. The ratio of the absorbance values at 260 and 280 nm in 10 mM Tris-Cl, pH 7.5 ranged from 1.7 to 2.1 for *A. rostrata* brain samples and 1.9 to 2.1 for *A. anguilla* brain samples. Spinal cord RNA samples had purity ratios of 1.7 to 1.8. Pure RNA has a ratio of 1.9 to 2.1 in 10 mM Tris-Cl, pH 7.5 (QIAGEN® OneStep RT-PCR Kit Handbook, QIAGEN), indicating that some contaminants such as phenol, chloroform or protein may have been present in spinal cord and *A. rostrata* brain samples. See Appendix A, for a summary of 28S:18S rRNA band ratios and estimations of sample purity for *A. anguilla* brain (Table A1), *A. rostrata* brain (Table A2) and *A. anguilla* spinal cord (Table A3) RNA preparations.

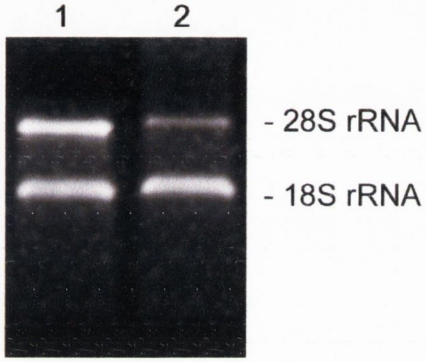


Figure 4.1. Gel electrophoresis separation of total RNA. Lanes 1 and 2: 1.2 μg RNA preparations isolated from the brains of untransected animals. The sample in lane 2 was discarded as it appeared degraded and had a 28S:18S rRNA ratio < 0.9. The 28S:18S rRNA ratio for the sample in lane 1 was 1.3.

4.3.2 Expression levels of mRNA for BDNF, NGF-like factor and trk B in the brain before and after injury.

Semiquantitative RT-PCR was carried out in order to determine changes in brain mRNA expression levels for BDNF, an NGF-like factor and the neurotrophin receptor trk B, relative to a housekeeping gene at different timepoints after spinal cord transection in the eel.

The housekeeping genes GAPDH and ARP were chosen as internal controls for semiquantitative analysis of RT-PCR results in this study. Intersample variation for GAPDH expression was found to be unacceptably high. For example, for *A. anguilla* RT-PCR experiments, the intensity values for the weakest and strongest band differed by a factor of approximately 185 (data not shown). Intersample variation was considerable with coefficients of variation (coefficient of variation (CV) = (standard deviation/mean) X 100) between samples at different timepoints ranging from 40% in animals one DPT to 167% in animals 15 DPT. GAPDH was deemed unsuitable for normalisation of RT-PCR samples and therefore semiquantitative analysis was carried out using ARP as an internal standard only.

ARP primers amplified a discreet band of 109 bp in size. ARP RT-PCR experiments were replicated twice with RNA extracted from *A. anguilla* (i.e. untransected animals and animals 1, 15 and 20 DPT; see Fig. 4.4, photograph shows results for one RT-PCR replicate experiment only) and from *A. rostrata* (i.e. untransected animals and animals 10 DPT; see Fig. 4.5, photograph shows results for one RT-PCR replicate experiment only). Intersample variation was minimal for ARP. In both replicate *A. anguilla* RT-PCR experiments, the intensity values for the weakest and strongest band across all treatments differed only by a factor of 2 (see Fig. 4.4) and for both *A. rostrata* experiments, the intensity values for the weakest and strongest band differed by a factor of 3.5 (see Fig. 4.5). The coefficients of variation between samples at different timepoints ranged from 7% to 25% in experiments carried out with RNA extracted from *A. anguilla* and 39% to 48% for *A. rostrata* experiments. The ARP values for normalisation of each sample were calculated by averaging the values from the two experiments.

No bands were detected after agarose gel electrophoresis of RT-PCR reactions carried out to control for genomic DNA contamination of RNA samples and in negative, "no DNA" controls performed in order to control for DNA contamination of RT-PCR reagents.

In preliminary RT-PCR experiments carried out in order to estimate an approximate cycle number and RNA template amount for each primer set, products of

correct size were seen with primers for BDNF, trk B and ARP and 500 pg RNA template after at least 35 cycles. RT-PCR products for NGF were visible after 35 cycles using 10 ng RNA template (data not shown).

In the experiments that followed, RT-PCR reactions were set up for each primer set with two RNA samples for each timepoint. Aliquots were taken from the RT-PCR reactions over a range of four consecutive cycles. Based on the results of preliminary experiments described above, cycle ranges were as follows: BDNF- 31 to 34, NGF-like factor - 33 to 36, trk B- 33 to 36 and ARP- 34-37. After determination by analysis of gel electrophoresis results (Fig. 4.2 shows results for *A. anguilla* RNA samples; data is not shown *A. rostrata* RNA samples), the arbitrary value for the intensity of each band was plotted against its cycle number in order to ensure products were accumulating in a logarithmic fashion over the range of cycles. Although the plots were not perfectly exponential, a linear relationship was found between cycle number and band intensity for all samples tested indicating that the RT-PCR reaction was in the exponential phase (Freeman *et al.*, 1999; Fig. 4.3 shows plots for one of the two samples at each timepoint for each primer set; data are not shown for untransected *A. rostrata* samples). The final cycle number chosen for semiquantitative RT-PCR analysis for each primer set was one which yielded a band that was clearly visible for both samples at each timepoint examined but at which the reaction was still within the exponential phase of amplification determined by examination of plots as recommended by Al-Bader and Al-Sarraf (2005). For the DNA size standard dilution series, band intensity was found to increase linearly with the DNA starting amount (data not shown) confirming that band intensity measured after RT-PCR was proportionally related to the amount of amplification product.

For statistical analysis of results, the assumptions for ANOVAs and t-tests were met since p values generated for the Kolmogorov-Smirnov test and Levene's test for homogeneity of variances were greater than 0.05 in all cases (Dytham, 2003; data not shown).

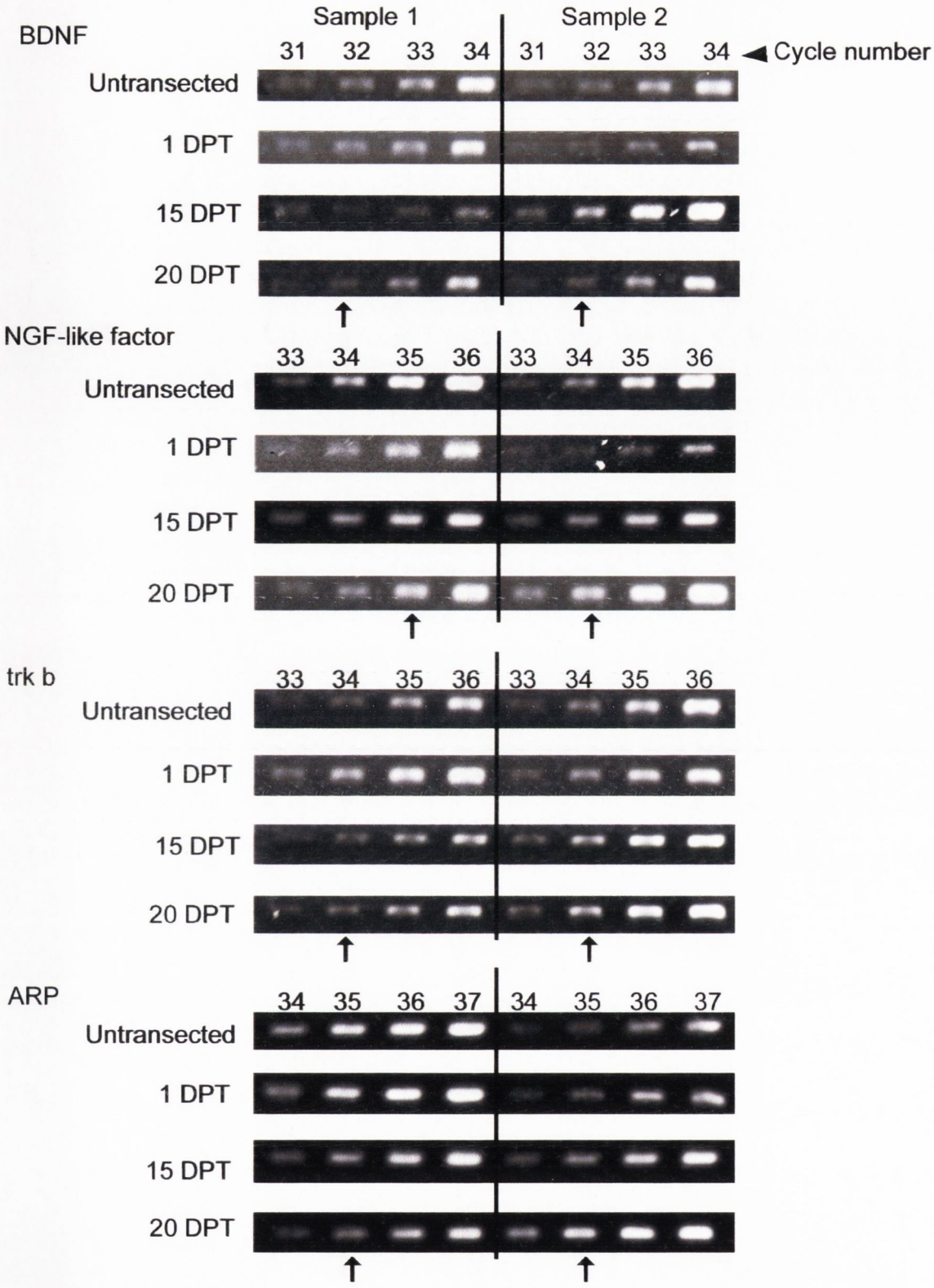


Figure 4.2. Gel electrophoresis of BDNF, NGF-like factor, trk b and ARP RT-PCR products amplified over a range of four consecutive cycles (cycle numbers tested for each gene are given above each group of gel photographs) from two *A. anguilla* brain RNA samples from untransected animals and animals one, 15 and 20 days post transection (DPT).This experiment was carried out in order to determine appropriate RT-PCR cycle number for semiquantitative experiments and ensure that RT-PCR quantification of BDNF expression was performed during the exponential phase of the RT-PCR reaction. The arrow indicates the final cycle number chosen for semiquantitative RT-PCR experiments..

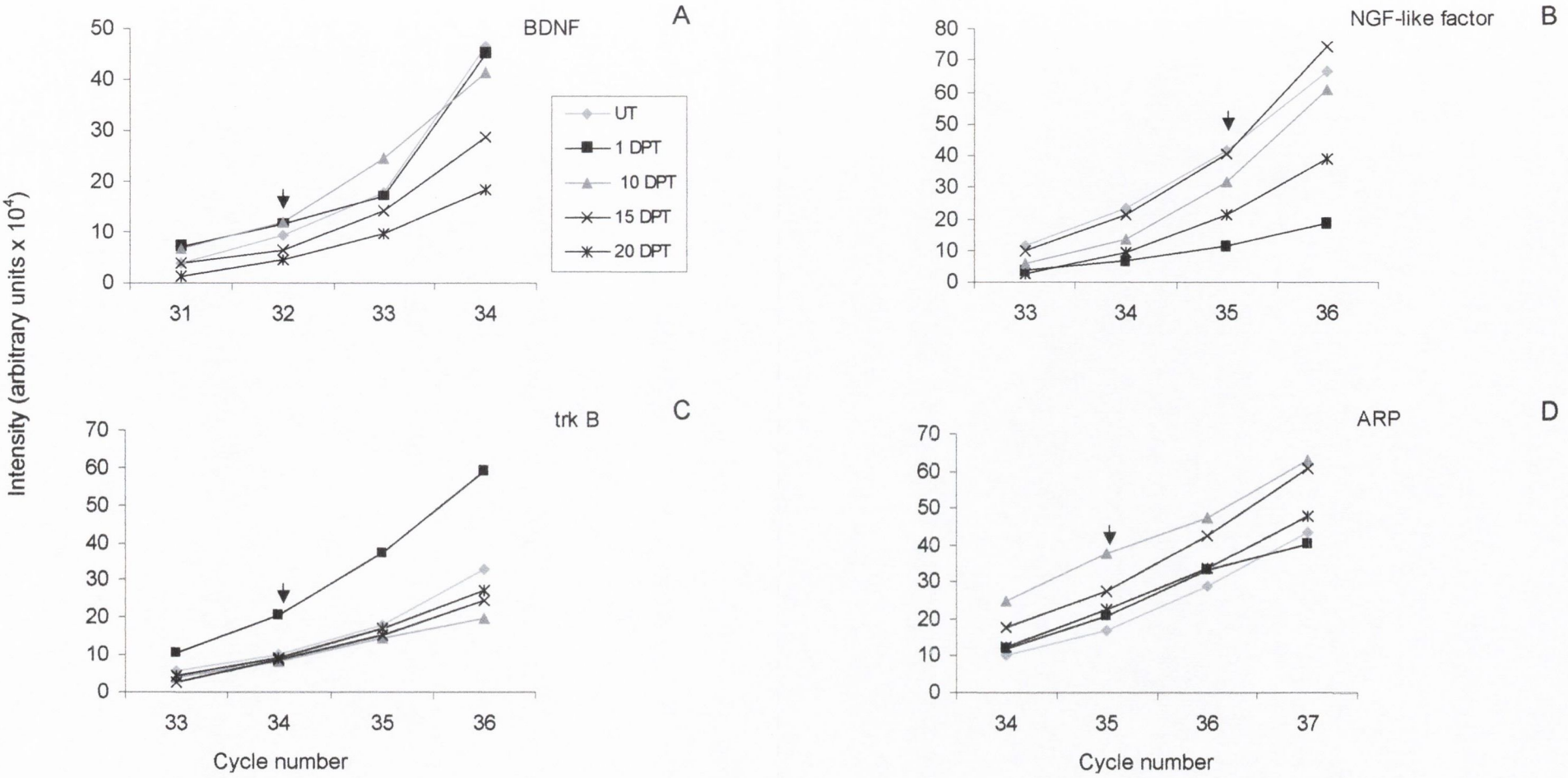


Figure 4.3. Graphs of band intensity (expressed as arbitrary units) against cycle number to verify RT-PCR quantification of mRNA levels in the eel brain during the exponential phase of amplification with BDNF (A), NGF-like factor (B), trk B (C) and ARP (D) primers and RNA template from untransected (UT) animals and animals one, 10, 15 and 20 days post transection (DPT). Arrowheads indicate cycle number used in semiquantitative RT-PCR analysis.

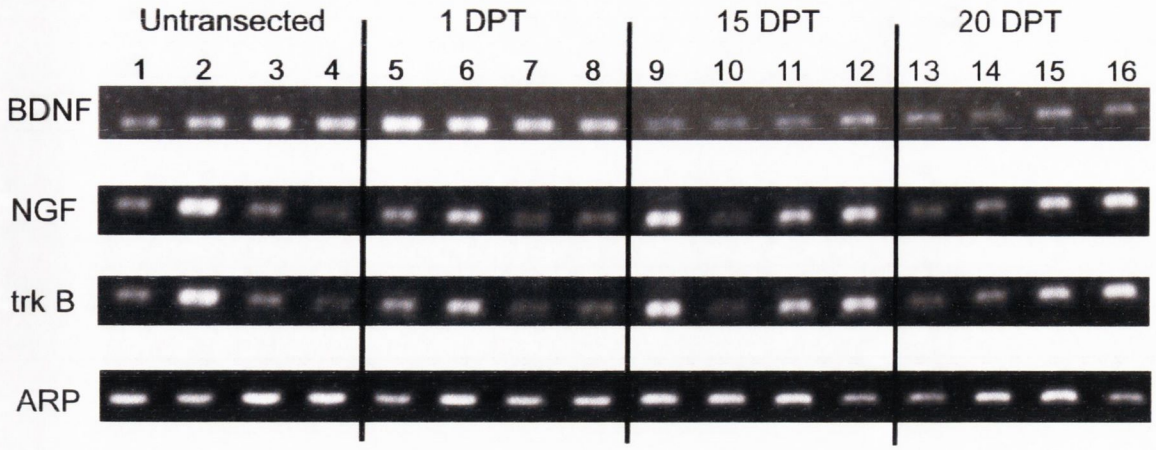


Figure 4.4. RT-PCR analysis of BDNF, NGF-like factor (NGF), trk B, and ARP mRNA expression in the brain of untransected and animals 1, 15 and 20 days post spinal cord transection (DPT). BDNF, NGF, trk B, ARP: Lanes 1-4: untransected, lanes 5-8: 1 DPT, lanes 9-12: 15 DPT, lanes 13-16: 20 DPT. Data are shown for one RT-PCR experiment only with each primer set. No bands were seen in negative, no DNA controls (data not shown).

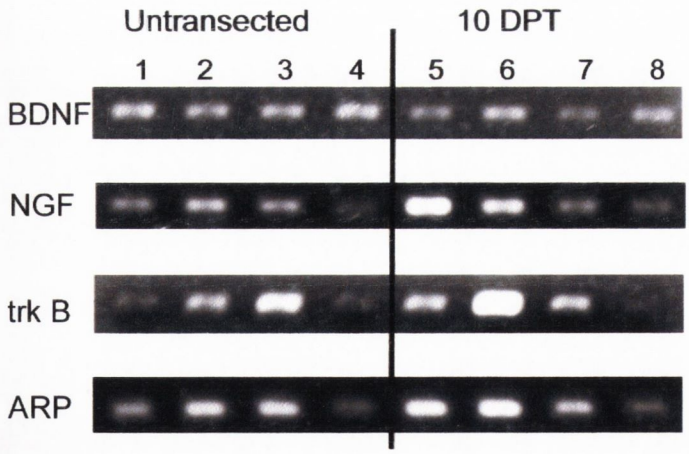


Figure 4.5. RT-PCR analysis of BDNF, NGF-like factor (NGF), trk B, and ARP mRNA expression in the brain of untransected and animals 10 days post spinal cord transection (DPT). BDNF, NGF, trk B, ARP: Lanes 1-4: untransected, lanes 5-8: 10 DPT. Data are shown for one RT-PCR experiment only with each primer set.No bands were seen in negative, no DNA controls (data not shown).

A single band of size 467 bp was amplified by BDNF primers. BDNF RT-PCR experiments with RNA extracted from *A. anguilla* (i.e. untransected animals and animals 1, 15 and 20 DPT) were repeated three times (Fig. 4.4 shows gel electrophoresis results for one replicate RT-PCR experiment). The average band intensity for each individual sample was then calculated from the three experiments and normalised to the average ARP value for the same sample from two experiments. Mean relative band intensities for different timepoints were then compared using one-way ANOVA and Tukey's HSD post hoc tests. Overall, a trend for a slight increase in mean relative band intensity one day after injury, which was not significantly different ($p = 0.387$) from BDNF mRNA levels in untransected animals was observed (Fig. 4.6A shows data for the mean relative band intensity values for BDNF ± SEM). This increase was followed by a decrease in mean relative band intensity at 15 and 20 DPT, which was statistically significant from BDNF mRNA levels in animals one DPT (15 DPT: $p = 0.022$ and 20 DPT: $p = 0.032$) but not from BDNF mRNA levels in untransected animals (15 DPT: $p = 0.325$ and 20 DPT: $p = 0.429$). See Appendix B for values of mean relative band intensities, standard deviations and the CV between samples for this experiment and all the following experiments described in section 4.3.2 with *A. anguilla* RNA brain samples and *A. rostrata* RNA brain samples, and section 4.3.3 with *A. anguilla* spinal cord RNA samples. The results for RT-PCR experiments carried out with BDNF, NGF-like and trk B primers are displayed in Tables B1, B2 and B3 in Appendix B respectively.

BDNF RT-PCR experiments with RNA extracted from *A. rostrata* were replicated twice (Fig. 4.5 shows gel electrophoresis results for one replicate RT-PCR). The BDNF values for each *A. rostrata* sample were calculated by averaging the values from the two experiments and normalising to the average ARP value for the same sample. No statistically significant differences were found in BDNF mRNA expression in untransected animals and animals 10 DPT when data were compared with a Student's *t*-test for independent samples ($p = 0.272$; Fig. 4.6B shows data for the mean relative band intensity values for BDNF ± SEM).

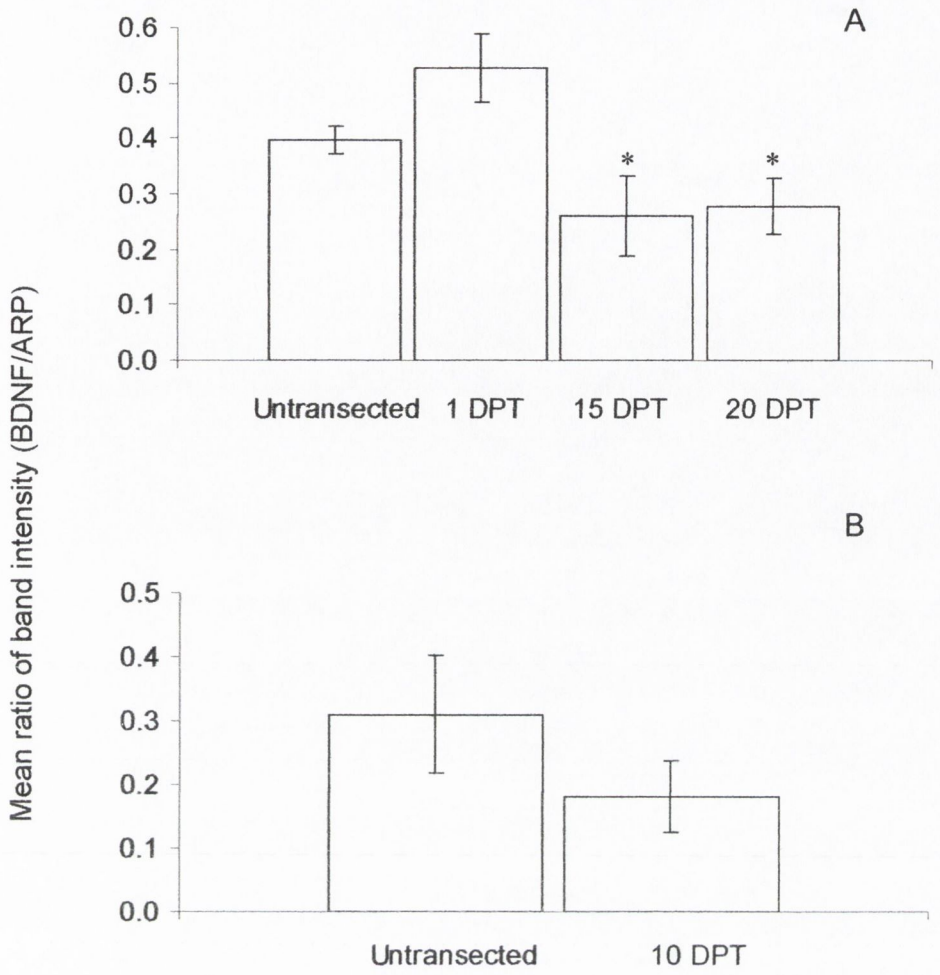


Figure 4.6. Semiquantitative RT-PCR analysis of BDNF mRNA expression in the eel brain before and after spinal cord transection. **A:** Mean relative band intensity values for *A. anguilla* BDNF ± SEM in untransected animals and animals one, 15 and 20 days post transection (DPT; n = 4 for each timepoint). Mean relative band intensity values for different timepoints were compared using one-way analysis of variance tests (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc tests. Mean relative band intensities were calculated for each timepoint from the averaged values for individual samples from three independent experiments that had been normalised to the average ARP value from two replicate experiments for the same sample. * = significant difference ($p < 0.05$, ANOVA followed by Tukey's HSD post hoc tests) from 1 DPT but not from uninjured levels. **B:** Mean relative band intensity values for *A. rostrata* BDNF ± SEM (calculated as described above from the results of two BDNF and two ARP RT-PCR experiments) in untransected animals and animals 10 DPT (n=4 for each timepoint). Differences in mean relative band intensity for untransected animals and animals 10 DPT were not found to be statistically significant when compared using a two-tailed Student's *t*-test for independent samples.

Primers specific for the NGF-like factor yielded a band at the expected size of 277 bp. Three independent RT-PCR experiments to examine mRNA expression of the NGF-like factor with RNA extracted from *A. anguilla* were carried out (see Fig. 4.4 for results for one replicate RT-PCR experiment). The average band intensity for each individual sample was then calculated from the three experiments and normalised to the average ARP value for the same sample. No statistically significant changes in NGF-like factor mRNA expression were detected at any timepoint when data were compared using one-way ANOVA ($p = 0.912$). A high level of intersample variation for NGF-like factor mRNA expression was seen compared with BDNF and ARP experiments, which led to large standard deviations from the mean (see Fig. 4.4 for gel electrophoresis results for one experimental replicate and Fig. 4.7A which shows data for the mean relative band intensity values for NGF-like factor ± SEM).

As for BDNF, NGF-like factor RT-PCR experiments with RNA extracted from *A. rostrata* were replicated twice (Fig. 4.5 gel electrophoresis results are displayed for one RT-PCR replicate), the average values were then calculated for each sample and normalised to the average ARP value for the same sample. No statistically significant differences were found in NGF-like factor mRNA expression in untransected animals and animals 10 DPT when data were assessed with a Student's *t*-test for independent samples ($p = 0.949$; Fig. 4.7B shows data for the mean relative band intensity values for NGF-like factor ± SEM).

Bioinformatics analysis revealed that the 603 bp cDNA fragment (NCBI GenBank accession number DQ396402) cloned by O'Brien (2004) codes for the intracellular domain of the trk B receptor only (data not shown). The *A. anguilla* trk B primers used in this study (see section 4.2.4 for forward and reverse sequences) bind within the 603 bp cDNA fragment for *A. anguilla* trk B and therefore will give an indication of mRNA expression for the full length, biologically active form of the trk B receptor only. In addition, two isoforms of the Trk B receptor exist in the zebrafish (Martin *et al.*, 1995) however, from the sequence data available for the eel, it cannot be determined whether the eel also possesses two Trk B isoforms (O'Brien, 2004).

RT-PCR experiments with trk B primers amplified a single product of 363 bp in size. The trk B values for each *A. anguilla* sample were calculated by averaging the band intensities from the two experiments and normalising to the average ARP value for the same sample (Fig. 4.4 shows gel electrophoresis results for one replicate RT-PCR experiment). No statistically significant changes in trk B mRNA expression were detected

at any timepoint when data were compared using one-way ANOVA ($p = 0.644$). As for the NGF-like factor, a high level of intersample variation of trk B mRNA expression was seen compared with BDNF and ARP experiments, leading to large standard deviations from the mean (see Fig. 4.4 for RT-PCR gel electrophoresis results for one replicate experiment and Fig. 4.8A which shows data for the mean relative band intensity values for trk B ± SEM).

RT-PCR experiments with RNA extracted from *A. rostrata* were replicated twice with trk B primers (Fig. 4.5 shows electrophoresis results for one RT-PCR experiment). The average band intensity for each individual sample was calculated from the two experiments and normalised to the average ARP value for the same sample (Fig. 4.8B shows data for the mean relative band intensity values for trk B ± SEM). No statistically significant differences were found in trk B mRNA expression in untransected animals and animals 10 DPT when data were assessed with a Student's *t*-test for independent samples ($p = 0.581$).

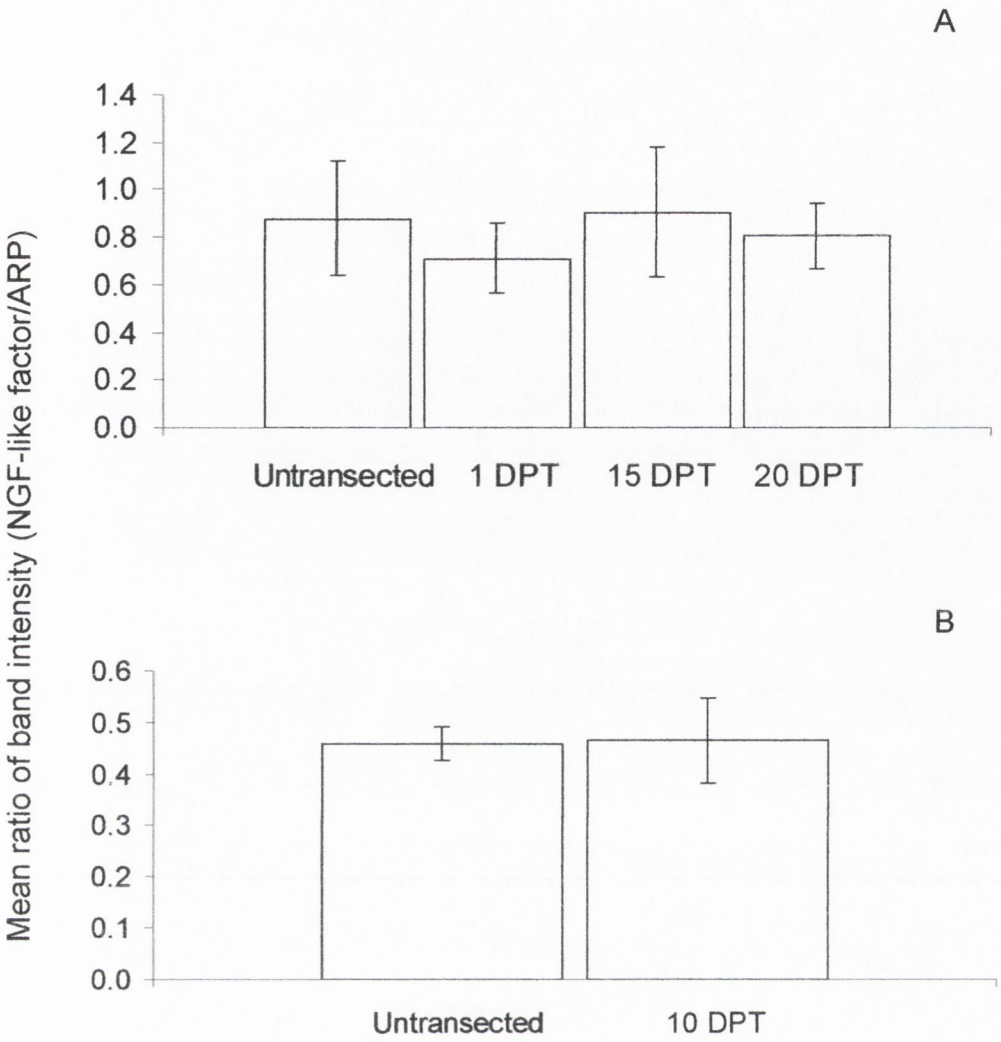


Figure 4.7. Semiquantitative RT-PCR analysis of NGF-like factor mRNA expression in the eel brain before and after spinal cord transection. **A:** Mean relative band intensity values for *A. anguilla* NGF-like factor ± SEM in untransected animals and animals one, 15 and 20 days post transection (DPT; n = 4 for each timepoint). No statistically significant differences in mean relative band intensity values for NGF-like factor were detected at any timepoint when compared using one-way analysis of variance tests (ANOVA). Mean relative band intensities were calculated for each timepoint from the averaged values for individual samples from three independent experiments that had been normalised to the average ARP value from two replicate experiments for the same sample. **B:** Mean relative band intensity values for *A. rostrata* NGF-like factor ± SEM (calculated as described above from the results of two NGF-like factor and two ARP RT-PCR experiments) in untransected animals and animals 10 DPT (n=4 for each timepoint). Differences in mean relative band intensity for untransected animals and animals 10 DPT were not found to be statistically significant when compared using a two-tailed Student's *t*-test for independent samples.

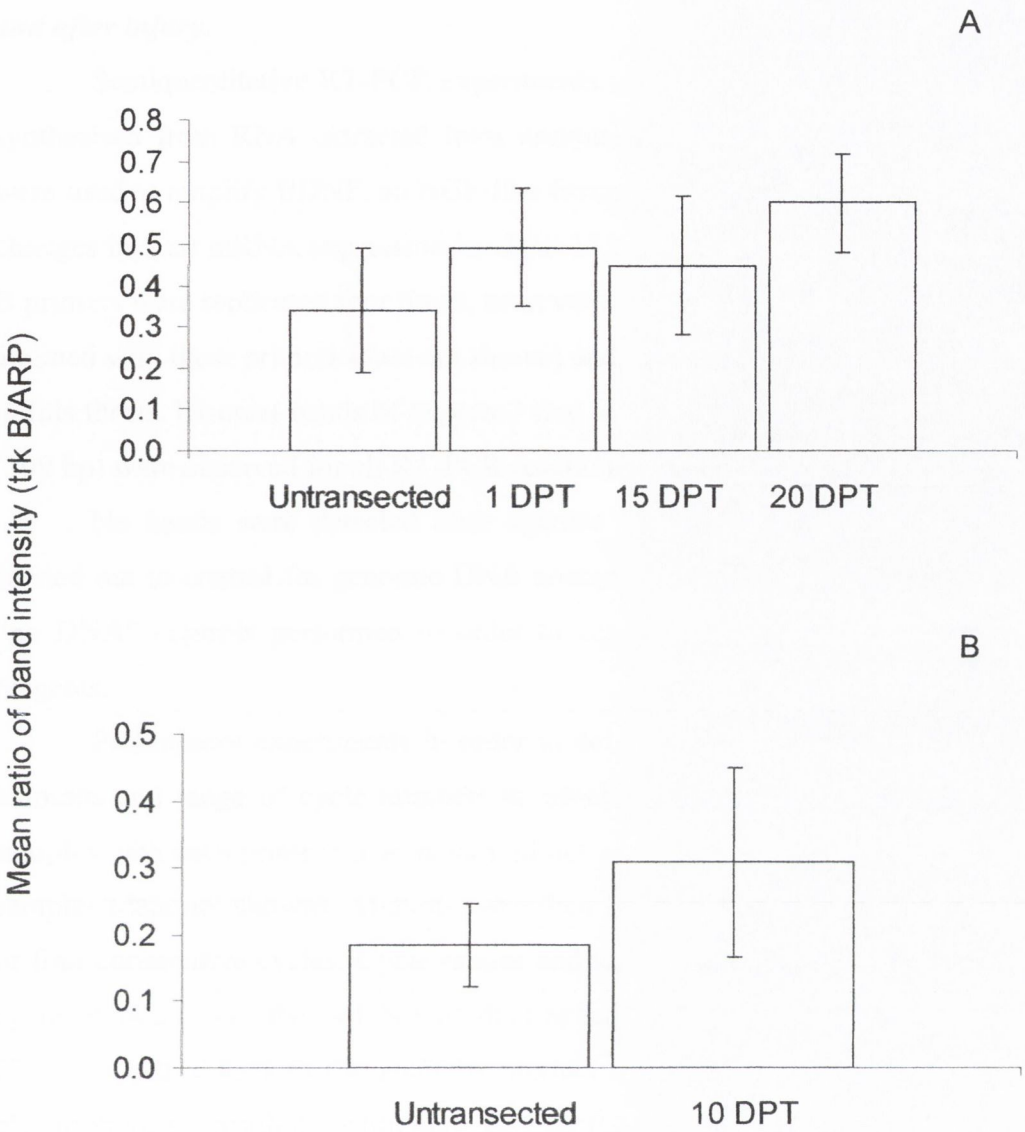


Figure 4.8. Semiquantitative RT-PCR analysis of trk B mRNA expression in the eel brain before and after spinal cord transection. **(A)** Mean relative band intensity values for *A. anguilla* trk B ± SEM in untransected animals and animals one, 15 and 20 days post transection (DPT; n = 4 for each timepoint). No statistically significant differences in mean relative band intensity values for trk B were detected at any timepoint when compared using one-way analysis of variance tests (ANOVA). Mean relative band intensities were calculated for each timepoint from the averaged values for individual samples from two independent experiments that had been normalised to the average ARP value from two replicate experiments for the same sample. **(B)** Mean relative band intensity values for *A. rostrata* trk B ± SEM (calculated as described above from the results of two trk B and two ARP RT-PCR experiments) in untransected animals and animals 10 DPT (n=4 for each timepoint). Differences in mean relative band intensity for untransected animals and animals 10 DPT were not found to be statistically significant when compared using a two-tailed Student's *t*-test for independent samples.

4.3.3 Expression levels of mRNA for BDNF, NGF and trk B in the spinal cord before and after injury.

Semiquantitative RT-PCR experiments were carried out with spinal cord sscDNA synthesised from RNA extracted from uninjured animals and animals 15 DPT. Primers were used to amplify BDNF, an NGF-like factor and trk B in order to examine post-injury changes in their mRNA expression levels at 15 DPT relative to ARP. Experiments with trk B primers were replicated four times, however consistent, reproducible results could not be attained with these primers (data not shown) and trk B results therefore will not be reported in this thesis. Discreet bands of expected size for BDNF (467 bp), NGF (277 bp) and ARP (109 bp) were observed for all RT-PCR reactions.

No bands were detected after agarose gel electrophoresis of RT-PCR reactions carried out to control for genomic DNA contamination of RNA samples and in negative, "no DNA" controls performed in order to control for DNA contamination of RT-PCR reagents.

Preliminary experiments in order to determine the approximate sscDNA template amounts and range of cycle numbers at which bands were visible for spinal cord RNA samples with each primer set were carried out as described above in section 4.3.2 for brain samples (data not shown). Aliquots were then taken from RT-PCR reactions over a range of four consecutive cycles. Cycle ranges and template amounts were as follows: 39 to 42 cycles with 2.5% of the sscDNA product as template for BDNF and NGF-like factor and 27 to 30 with 0.83% of the sscDNA product as template for ARP (see Fig. 4.9 for gel electrophoresis results). Although not perfectly exponential in all cases, a linear relationship was found between cycle number and band intensity for all samples tested indicating that the RT-PCR reaction was in the exponential phase (Freeman *et al.*, 1999; see Fig. 4.10A-C). The cycle number selected for semiquantitative RT-PCR analysis with each primer set is indicated in Fig. 4.10 (A-C) and was one which yielded a band that was clearly visible for all samples examined but at which the reaction was still within the exponential phase of amplification determined by examination of plots. A linear relationship was also seen between template amount and band intensity at 28 cycles when spinal cord sscDNA was amplified with ARP primers (see Fig. 4.9 for electrophoresis results and Fig.4.10D for plot of template amount against band intensity; The sample for 0.6 % template dilution for 15 DPT was lost during the experimental procedure therefore is not included in the graph).

Intersample variation for ARP was measured using the CV and was found to be 29.5% between band intensities for samples from untransected animals, 36.9% for samples

from animals 15 DPT and 44.3% overall (Fig. 4.11). A statistically significant change in ARP mRNA expression was not detected in animals 15 DPT ($p = 0.192$). The reproducibility of this result was confirmed by an experiment carried out by Dr S. M. Borich ($p = 0.345$; data not shown). Band intensity results for BDNF and NGF-like factor were normalised to both ARP values however ARP values were not averaged for both experiments since ARP was amplified to 30 PCR cycles by Dr S. M. Borich.

RT-PCR experiments with BDNF primers and spinal cord sscDNA were replicated twice (Fig. 4.11 shows gel electrophoresis results for one RT-PCR experiment). Statistical analysis was carried out for both experiments normalised to both sets of ARP values and revealed no significant difference in BDNF mRNA expression levels in the spinal cord 15 days after injury when compared with levels in untransected animals ($p = 0.778$; Fig. 4.12A; results are shown for one replicate only normalised to ARP values at 28 PCR cycles).

RT-PCR experiments with primers for the NGF-like factor and spinal cord sscDNA were also replicated twice (see Fig. 4.11 for electrophoresis results for one RT-PCR experiment). As for BDNF, statistical analysis of two replicate experiments normalised to both sets of ARP values revealed no statistically significant difference between expression levels in the spinal cords of untransected animals and animals 15 DPT ($p = 0.581$; Fig. 4.12B shows results for one experiment only normalised to ARP values at 28 PCR cycles).

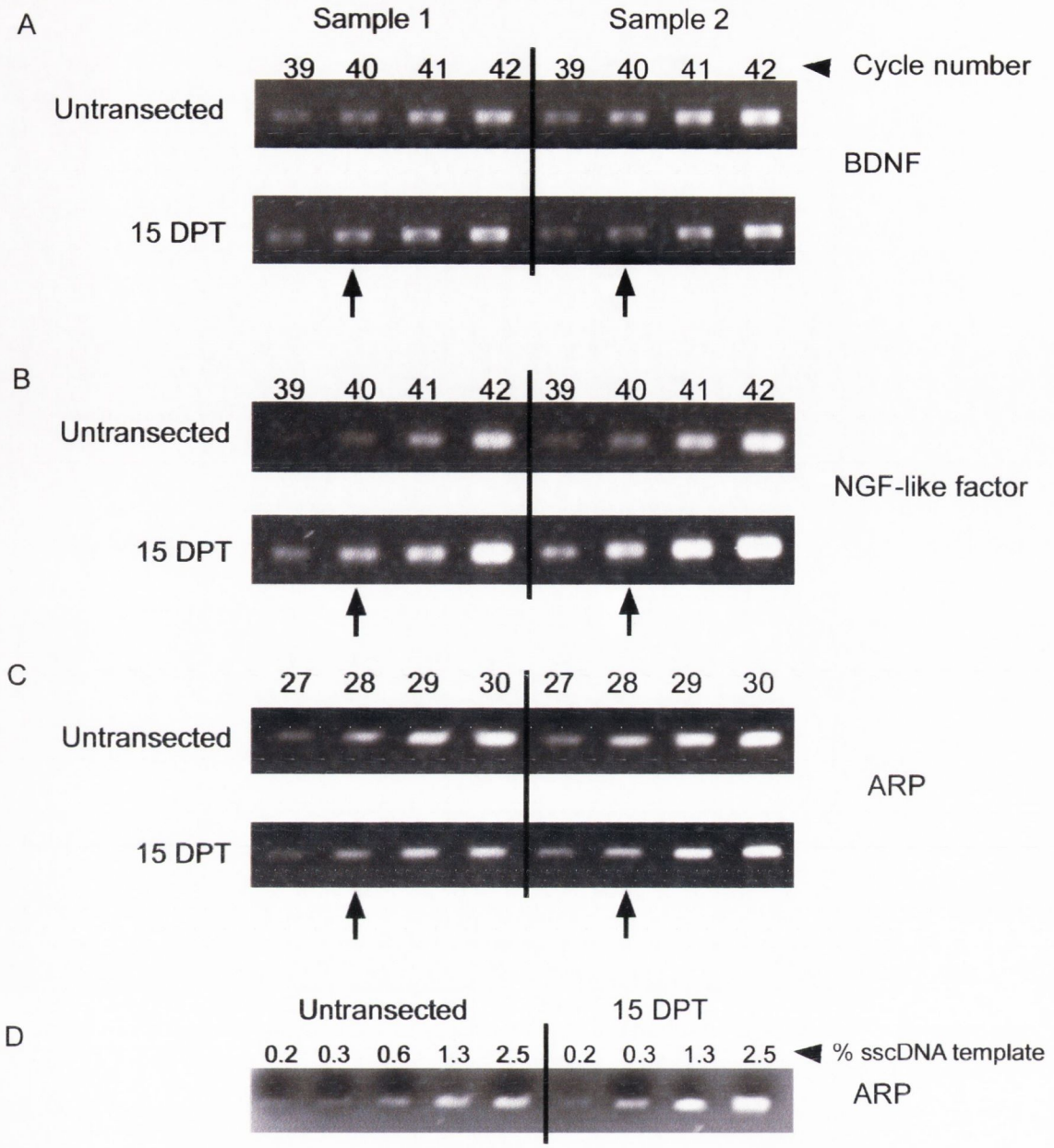


Figure 4.9. Gel electrophoresis of BDNF (A), the NGF-like factor (B) and ARP (C and D) spinal cord RT-PCR products amplified over a range of four consecutive cycles with a fixed template dilution (A-C) or from a two-fold template dilution series at 28 cycles (D). These experiments were conducted in order to determine the appropriate cycle number (A- C) and template dilution range for ARP (D) for semiquantitative analysis of mRNA expression during the exponential phase of the RT-PCR reaction. A - C: Single stranded complementary (sscDNA) from two untransected RNA samples and two 15 days post transection (DPT) RNA samples served as template for these experiments. The cycle numbers tested for each gene are given above each group of gel photographs. The arrows indicate the cycle number chosen for semiquantitative RT-PCR analysis. D: The results are shown for a two-fold dilution series of sscDNA template from one untransected RNA sample and one 15 DPT sample. The RT-PCR sample for the 0.6% template dilution for 15 DPT was lost during the experiment. A 0.83% dilution of sscDNA template was used in subsequent ARP RT-PCR experiments.

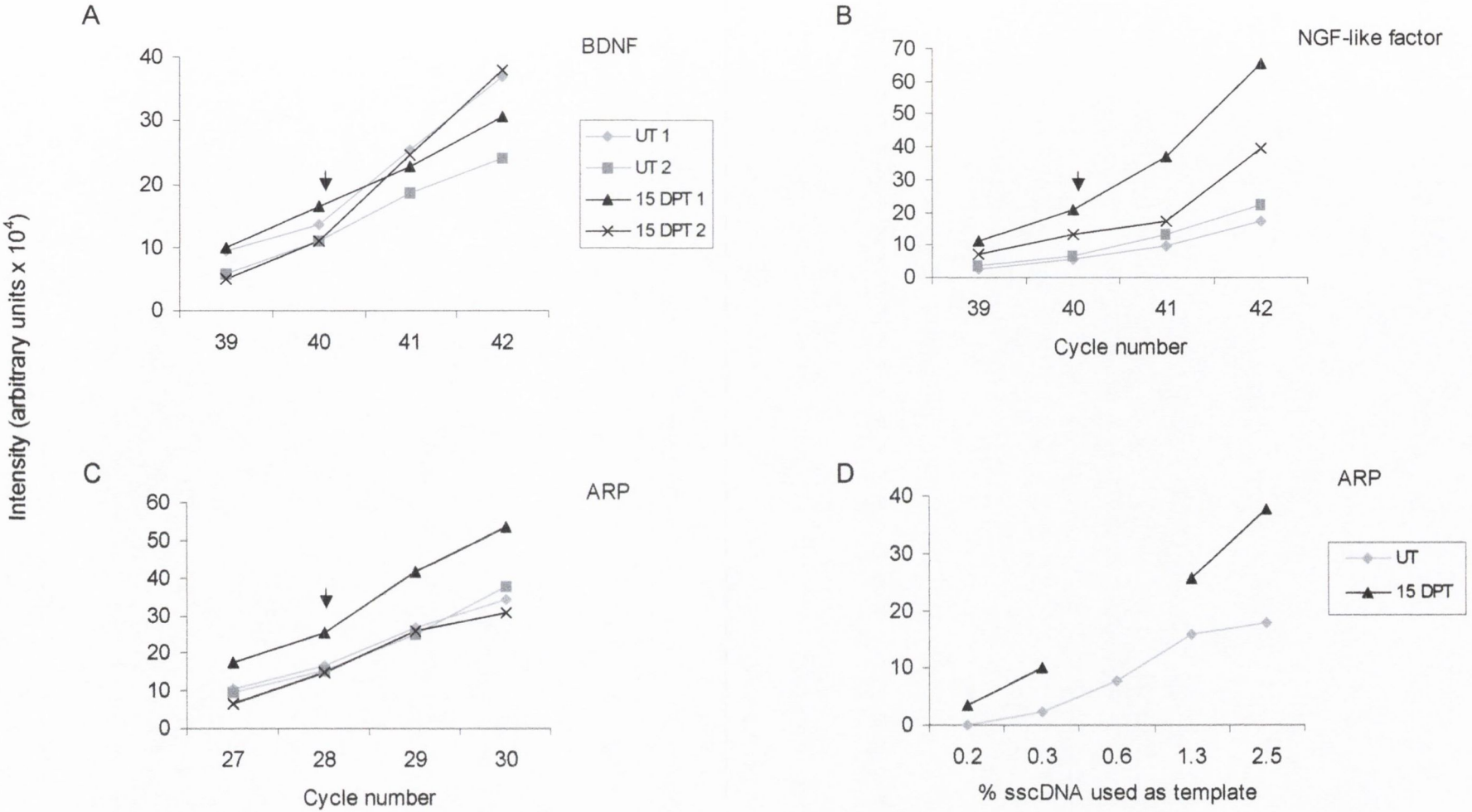


Figure 4.10. Graphs of band intensity (expressed as arbitrary units) against cycle number to verify RT-PCR quantification of mRNA levels in the eel spinal cord during the exponential phase of amplification with BDNF (A), NGF-like factor (B) and ARP (C) primers and single stranded complementary (sscDNA) template from two untransected (UT) RNA samples and two 15 days post transection (DPT) samples. Arrowheads indicate cycle number used in semiquantitative RT-PCR analysis. (D) Results for two-fold dilution series of sscDNA template at 28 PCR cycles against band intensity. Sample for 0.6 % template dilution for 15 DPT was lost during experiment.

Figure 4.11. RT-PCR analysis of BDNF, NGF-like factor (NGF) and ARP mRNA expression in spinal cord RNA samples from untransected animals and animals 15 days post spinal cord transection (DPT). BDNF, NGF and ARP: Lanes 1-3: untransected, lanes 4-6: 15 DPT. Data are shown for one RT-PCR experiment only with each primer set. Each spinal cord RNA sample was isolated from two pooled spinal cords. No bands were seen in negative, no DNA controls (data not shown).

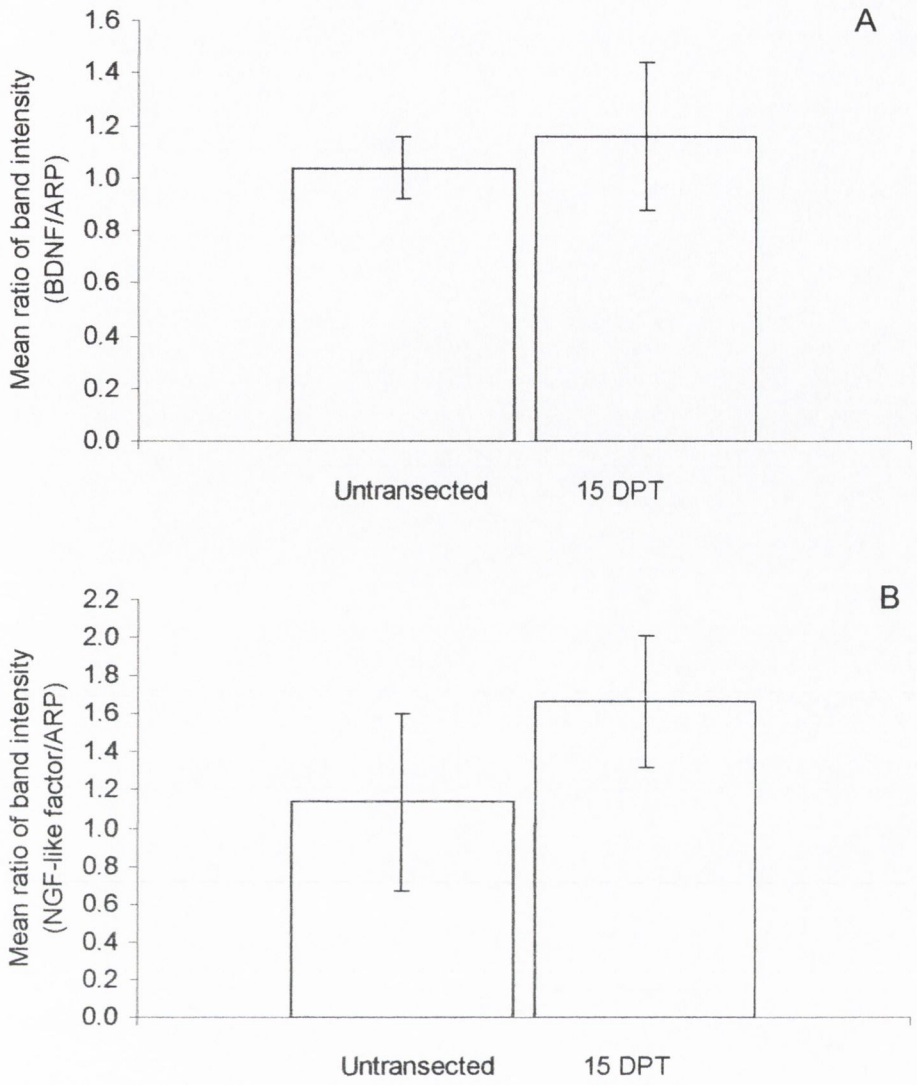


Figure 4.12. Semiquantitative RT-PCR analysis of BDNF (A) and NGF-like factor (B) mRNA expression in the eel spinal cord in untransected animals and animals 15 days post transection (DPT). RT-PCR experiments were repeated twice. Values (mean relative band intensity ± SEM) are shown for one replicate experiment only and are expressed relative to ARP values at 28 PCR cycles. Differences in mean relative band intensity for RNA samples from untransected animals (n=3) and animals 15 DPT (n=3) were not found to be statistically significant when compared using a two-tailed Student's t-test for independent samples.

4.3.4 Expression pattern of BDNF mRNA in the eel brain before and after injury.

ISH is a qualitative method and is therefore appropriate for detecting gross anatomical changes in mRNA expression patterns. In order to examine the distribution pattern of BDNF mRNA after injury, ISH was carried out on brain tissue from untransected animals and animals 10 DPT. Although the sensitivity of the procedure varied between experiments and some sections could not be included in reconstructions since they had served as negative (sense) or positive (tyrosine hydroxylase) controls, no distinct changes in the overall staining pattern for BDNF mRNA were observed between control and injured animals (see Fig. 4.13, 4.14, 4.15 and 4.16). ISH staining was restricted to cells that had a neuronal morphology and corresponding staining was not observed in negative, sense controls (data not shown). Positive, tyrosine hydroxylase controls showed staining similar to that described in chapter two (data not shown).

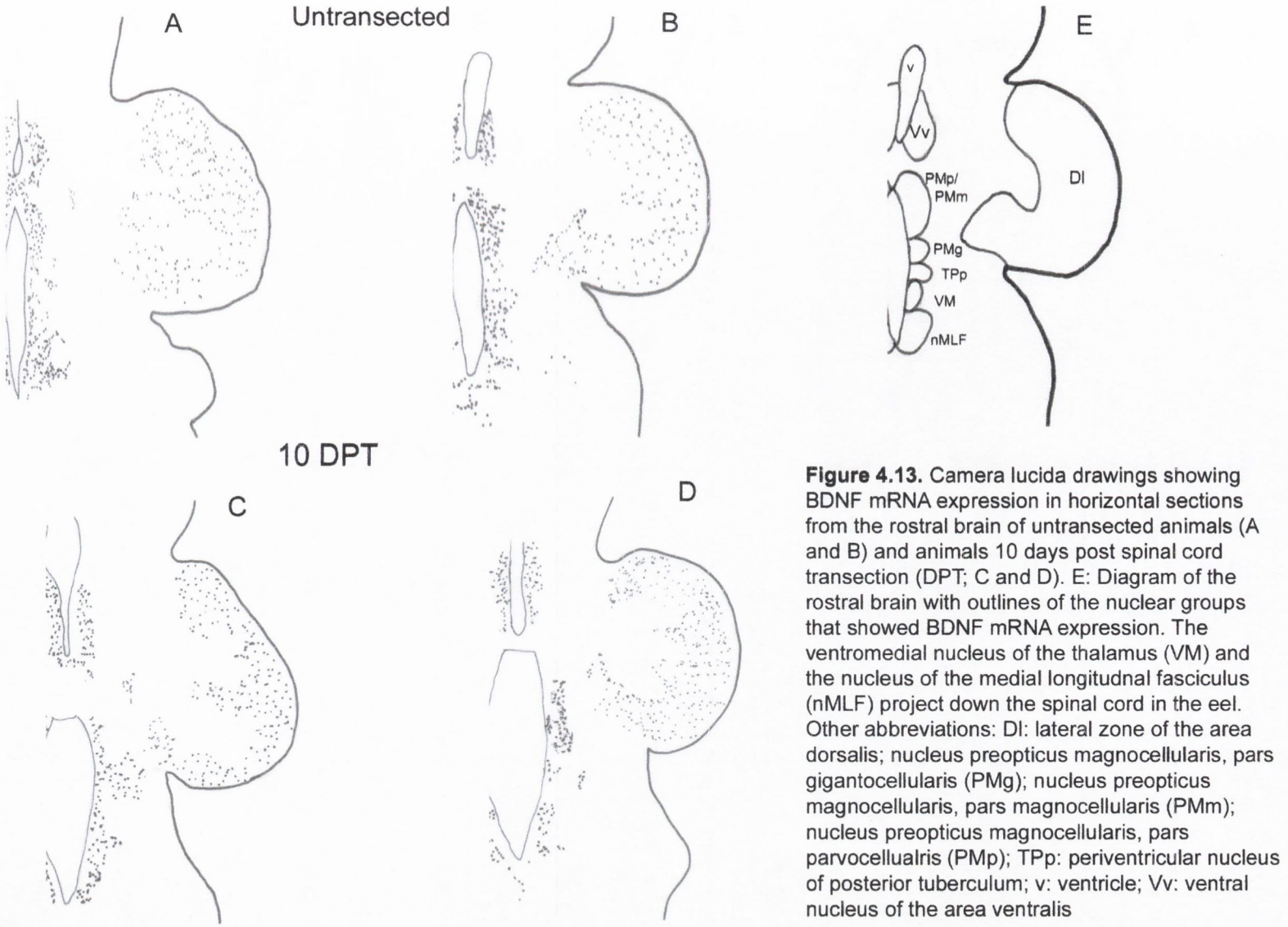


Figure 4.13. Camera lucida drawings showing BDNF mRNA expression in horizontal sections from the rostral brain of untransected animals (A and B) and animals 10 days post spinal cord transection (DPT; C and D). E: Diagram of the rostral brain with outlines of the nuclear groups that showed BDNF mRNA expression. The ventromedial nucleus of the thalamus (VM) and the nucleus of the medial longitudnal fasciculus (nMLF) project down the spinal cord in the eel. Other abbreviations: Dl: lateral zone of the area dorsalis; nucleus preopticus magnocellularis, pars gigantocellularis (PMg); nucleus preopticus magnocellularis, pars magnocellularis (PMm); nucleus preopticus magnocellularis, pars parvocellualris (PMp); TPp: periventricular nucleus of posterior tuberculum; v: ventricle; Vv: ventral nucleus of the area ventralis

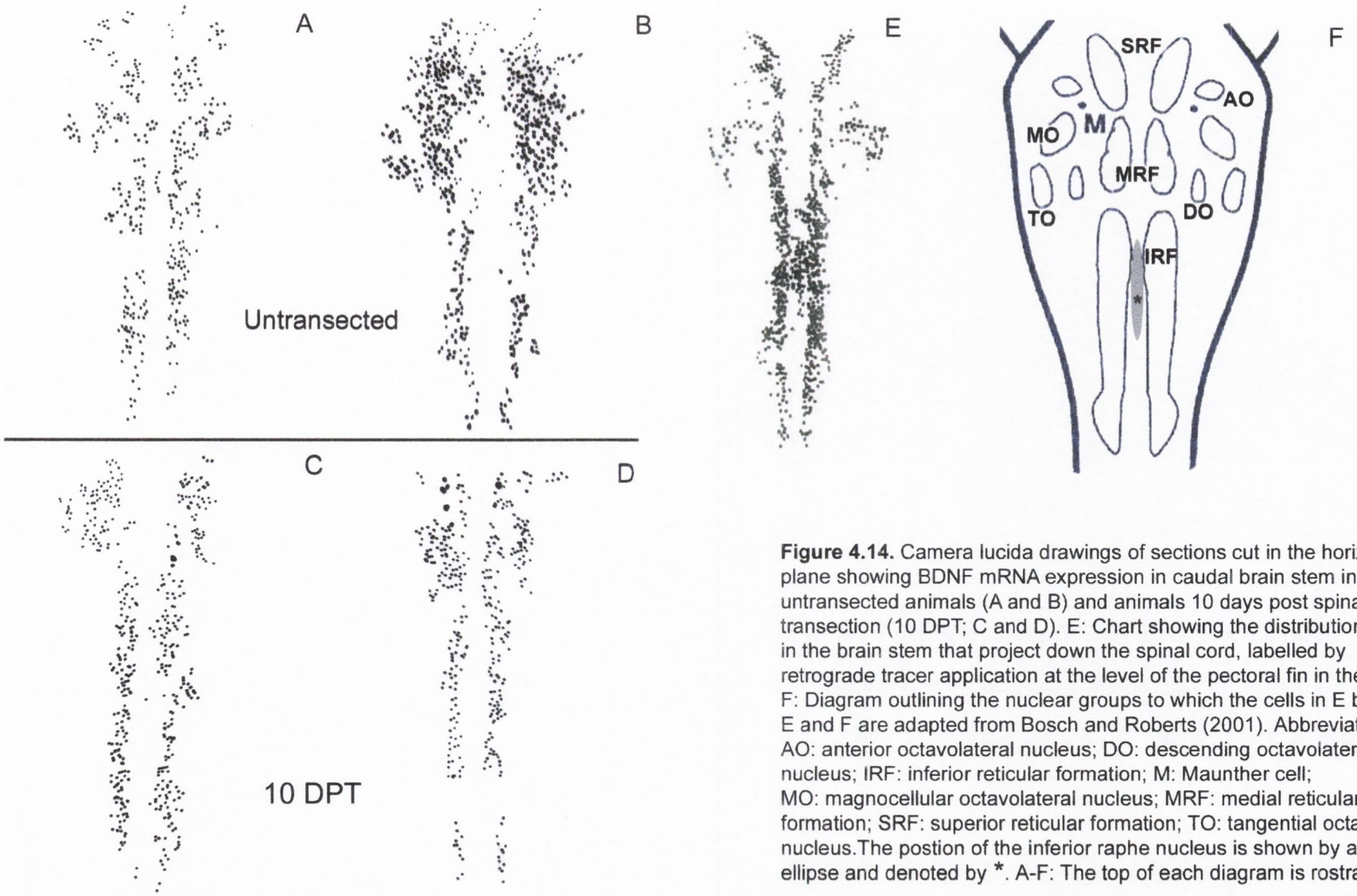


Figure 4.14. Camera lucida drawings of sections cut in the horizontal plane showing BDNF mRNA expression in caudal brain stem in untransected animals (A and B) and animals 10 days post spinal cord transection (10 DPT; C and D). E: Chart showing the distribution of cells in the brain stem that project down the spinal cord, labelled by retrograde tracer application at the level of the pectoral fin in the cord. F: Diagram outlining the nuclear groups to which the cells in E belong. E and F are adapted from Bosch and Roberts (2001). Abbreviations: AO: anterior octavolateral nucleus; DO: descending octavolateral nucleus; IRF: inferior reticular formation; M: Maunther cell; MO: magnocellular octavolateral nucleus; MRF: medial reticular formation; SRF: superior reticular formation; TO: tangential octavolateral nucleus.The postion of the inferior raphe nucleus is shown by a grey ellipse and denoted by *. A-F: The top of each diagram is rostral.

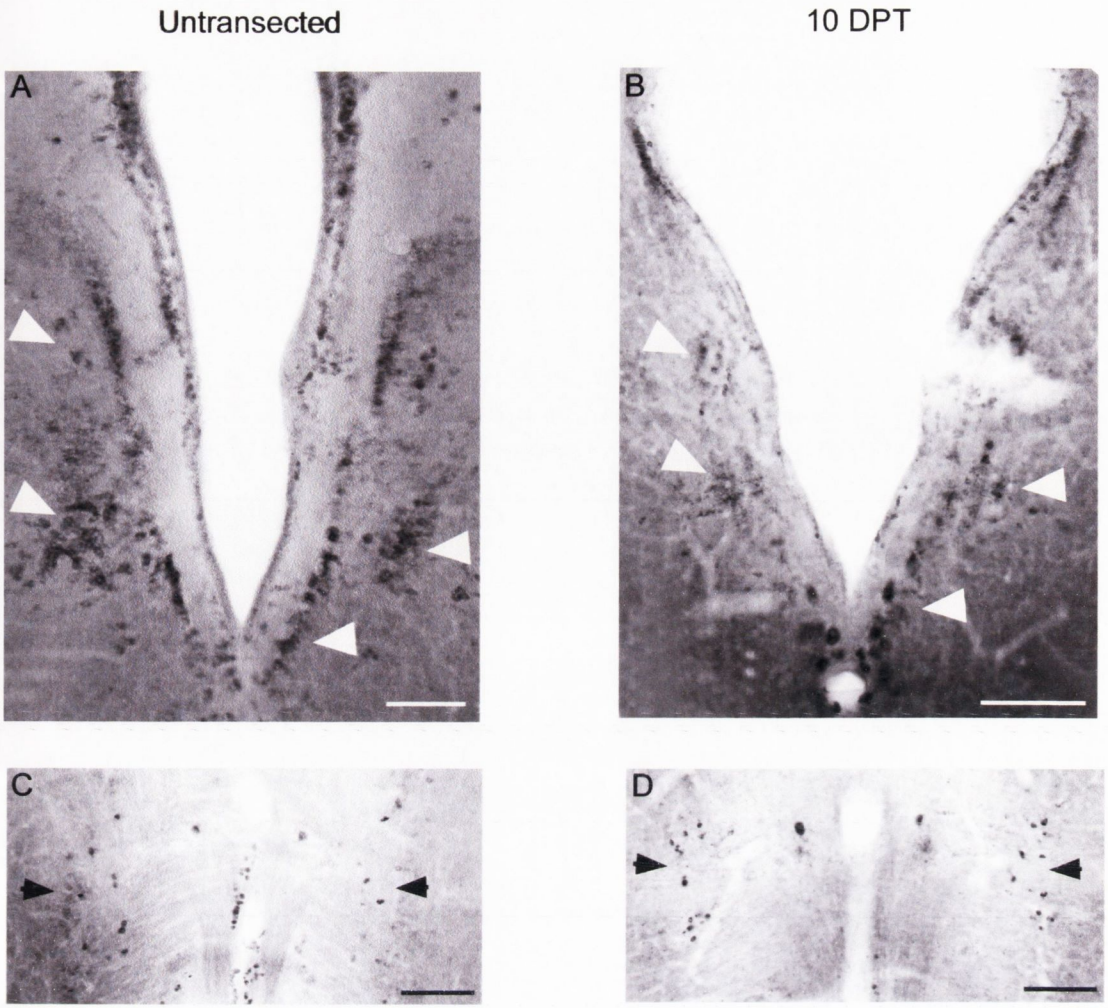


Figure 4.15. BDNF mRNA expression in the rostral brain stem (A and B) and medial reticular formation (C and D). A, B: A horizontal section of the tegmentum from an untransected animal (A) and an animal 10 days post spinal cord transection (B; 10 DPT). Similarly stained cell groups are indicated by white arrowheads. C, D: Horizontal sections showing similar staining, indicated by black arrowheads, in the medial reticular formation in the brain of an untransected animal (C) and an animal 10 DPT (D). Scale bars = 200 μm.

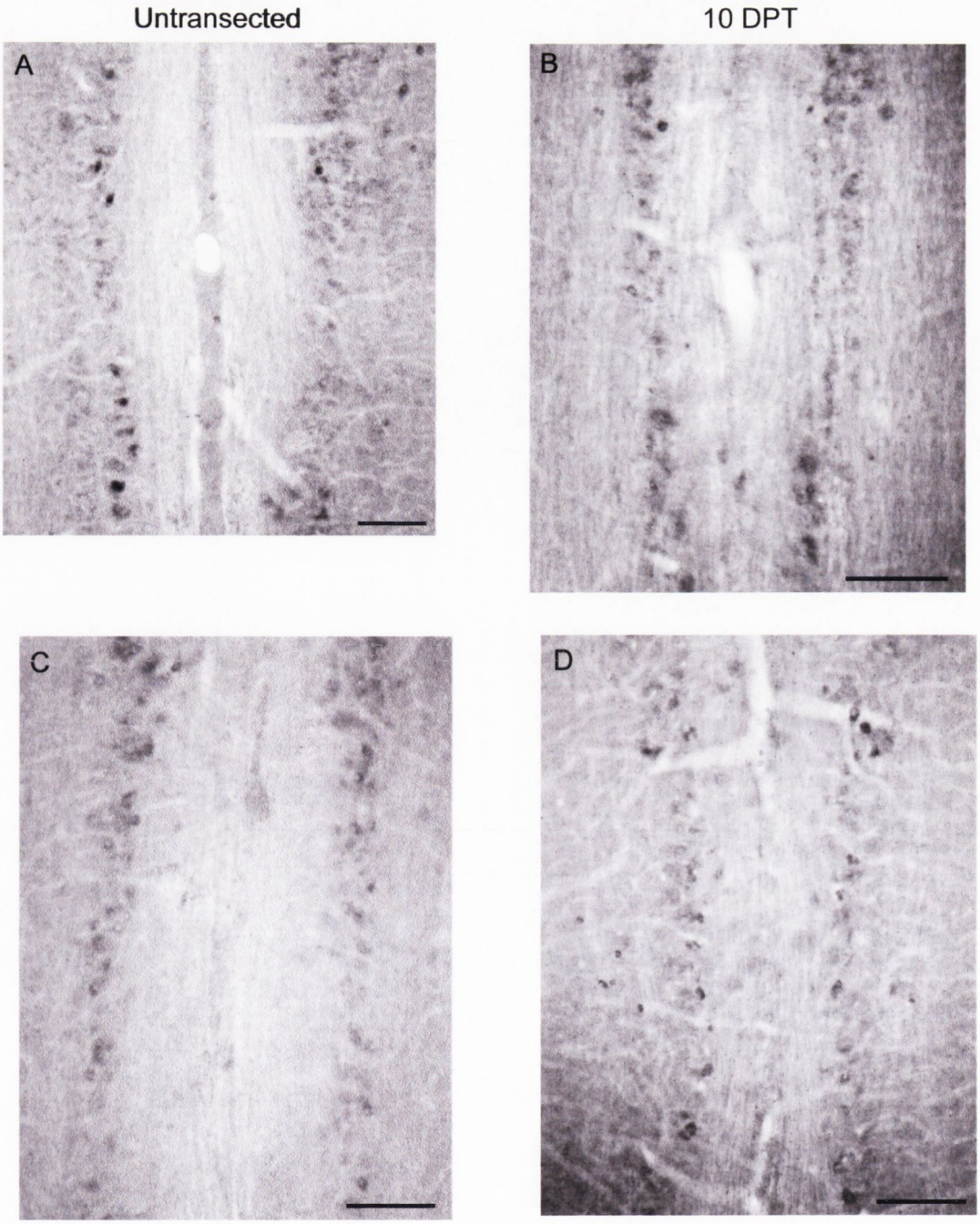


Figure 4.16. BDNF mRNA expression in horizontal sections of the rhombencephalon from an untransected animal (A and C) and an animal 10 days post spinal cord transection (DPT). A and B: Staining in the medial reticular formation (MRF). Image A has been used already in Figure 3.17 c. C and D: Staining in the inferior reticular formation (IRF).
Scale bars = 200 μm.

4.4 Discussion

A semiquantitative RT-PCR study has been set up and carried out with RNA extracted from *A. anguilla* brain and spinal cord tissue. Based on the results of this study, sources that contribute to intersample and interexperimental variability encountered when working with this species are discussed below and sample size estimations for future semiquantitative RT-PCR experiments are made.

In contrast to research conducted in mammals, the results of the semiquantitative RT-PCR and ISH experiments carried out in the present study indicate that dramatic changes in mRNA levels for BDNF, an NGF-like factor and trk B do not take place after injury in the eel CNS.

4.4.1 Sources of variability for semiquantitative RT-PCR experiments

For this study, it was necessary to use animals caught in the wild since *A. anguilla* cannot be bred in captivity. The use of outbred or wild animals in an experiment can lead to more variable intersample results than in those carried out with inbred animals since wild animals will be considerably less similar genetically than individuals derived from an inbred strain (Chen *et al.*, 1999; Festing *et al.*, 2002). In some experimental designs, this may be overcome by taking measurements from an animal before and after a treatment (Festing *et al.*, 2002). In the case of RT-PCR experiments where tissue removal requires the death of the subject, this is not possible and individual variation may be overcome by increasing animal number.

The use of high quality, intact template RNA is important for the reproducibility of RT-PCR results (Freeman *et al.*, 1999; Auer *et al.*, 2004; Bustin and Nolan, 2004; Hugett *et al.*, 2005). Conventionally, the ratio of the 28S to 18S rRNA band has been used as the primary indicator of the integrity of an RNA sample with a ratio of 1.5 to 2.0 being considered acceptable for gene expression studies (Clontech PCR-Select cDNA Subtraction Kit User Manual, 2002). Recently however, it has been noted that the relationship between rRNA profile and mRNA integrity is unclear and that a low 28S:18S rRNA band ratio of less than 1.5 does not necessarily indicate poor quality mRNA (Imbeaud *et al.*, 2005; Schroeder *et al.*, 2006).

In the present study, the integrity of the RNA samples was assessed by visualisation of the 28S and 18S rRNA bands by denaturing agarose gel electrophoresis. Sharp bands for 28S and 18S rRNA were visible for all RNA preparations used in this study and 28S:18S ratios ranged from 0.9 to 2.3 with the average being 1.4. For experiments carried out with brain tissue from *A. anguilla*, ratios for RNA samples extracted from animals one DPT were highest and ranged from 1.9 to 2.3 (see Appendix

A). The brains of these animals were dissected out and processed immediately for RNA extraction without freezing in liquid nitrogen or processing of the tissue took place one day after freezing. On the other hand, ratios for RNA preparations extracted from brain tissue that had been stored for one week to one year at -80°C ranged from 1.0 to 1.4.

It is not clear whether the quality of RNA samples, as assessed by the 28S:18S band ratios of rRNA, used in this study may have been a contributing factor to intersample and interexperimental variability seen in RT-PCR results. For RT-PCR reactions with ARP primers carried out using RNA extracted from *A. anguilla* brain tissue, a correlation between RNA sample quality and intersample and interexperimental variability was seen. The lowest intersample (CV = 6.7% for one DPT versus 25.8% for 20 DPT) and interexperimental (CV = 8.2% for one DPT versus 15.5% for 20 DPT) variability was achieved using RNA from animals one DPT. On the other hand, no correlation was seen between low intersample variation and RNA preparation quality after RT-PCR amplification with primers for BDNF, NGF-like factor and trk B. For example, in RT-PCR experiments carried out with RNA extracted from *A. anguilla* brain and BDNF primers, the average variability measured in terms of the CV for untransected animals was 12.6%; for animals one DPT: CV = 23.1%; for animals 15 DPT: CV = 54.4% and for animals 20 DPT: CV = 36.6%. In this case, the lowest intersample variation was seen for samples amplified from RNA preparations with the lowest 28S:18S ratios (1.0 – 1.3; see 28S:18S rRNA band ratios for untransected animals, Appendix A, Table A1). In addition, interexperimental variability could not be correlated with RNA preparation quality after RT-PCR amplification with primers for BDNF, NGF-like factor and trk B. In the case of the NGF-like factor, for example, interexperimental variability measured in terms of the CV ranged from 16.2 to 46.3% for untransected animals; for animals one DPT CV = 8.8 – 52.6%; for animals 15 DPT: CV = 20 – 45% and for animals 20 DPT: CV = 39.2 – 49.5%.

Obtaining high quality RNA from the eel brain and spinal cord proves difficult possibly due to small tissue size (brain and spinal cord samples weigh approximately 100 mg and 10 mg respectively) and has been optimised by Weltzien *et al.* (2005). For future experiments, the quality of RNA extracted from eel CNS tissue may be improved by use of the FastRNA Pro Green kit (Qbiogene) as recommended by Weltzien *et al.*, 2005 and by immediate processing of tissues for RNA extraction after dissection or by short term storage before processing only (1 day at -80°C). Intersample and interexperimental variability may also be reduced by more accurate determination of RNA quality than quantitation of 28S:18S rRNA band ratios by agarose gel electrophoresis such as the use of the automated system with computer software that produces user-independent assessments

of RNA quality based on the analysis of microcapillary electrophoresis traces (Auer *al.*, 2004; Imbeaud *et al.*, 2005; Schroeder *et al.*, 2006).

The purity of RNA samples is also important for quantitative RT-PCR (Bustin and Nolan, 2004; Weltzien *et al.*, 2005). Contaminants in RNA samples such as phenol or chloroform after RNA extraction and DNase treatment may hinder the subsequent reverse transcription and PCR processes (Bustin and Nolan, 2004). Pure RNA has a ratio of 1.9 to 2.1 in 10 mM Tris-Cl, pH 7.5 (QIAGEN® OneStep RT-PCR Kit Handbook, QIAGEN). The ratio of the absorbance values at 260 and 280 nm in 10 mM Tris-Cl, pH 7.5 ranged from 1.7 to 2.1 for *A. rostrata* brain samples and 1.9 to 2.1 for *A. anguilla* brain samples. Spinal cord RNA samples had purity ratios of 1.7 to 1.8. Absorbance values for *A. rostrata* brain and *A. anguilla* spinal cord samples indicate that some contaminants may have been present in these samples and may have contributed to intersample or interexperimental variability; however, the presence of these contaminants for the most part, is controlled for by normalisation to the housekeeping gene (Bustin and Nolan, 2004).

Interexperimental variability in RT-PCR experiments is controlled for by a certain extent by use of a housekeeping gene for normalisation of band intensities. As mentioned in section 4.1 and above, the housekeeping controls for differences in RNA extraction between samples. However, other sources of interexperimental variability related to the RT-PCR reaction, such as differences from experiment to experiment in reverse transcription and PCR efficiency, may have occurred during RT-PCR process itself when using the QIAGEN® OneStep RT-PCR Kit. The housekeeping gene controls for reverse transcription efficiency when sscDNA is used as a template for RT-PCR reactions since all RT-PCR products are amplified from the same cDNA. On the other hand, a separate reverse transcription reaction takes place every time the QIAGEN® OneStep RT-PCR Kit is used with a different set of primers therefore differences in reverse transcription efficiency are not controlled for.

In order to overcome the variability due to interexperimental fluctuations in reverse transcription and PCR efficiency when using the QIAGEN® OneStep RT-PCR Kit, and fluctuations in PCR efficiency only when using sscDNA as a template for PCR reactions in future experiments, multiplex RT-PCR may be carried out in which primers with for the housekeeping gene and the gene of interest are amplified in the same RT-PCR reaction (Choquer *et al.*, 2003). Multiplex RT-PCR requires careful primer design so that primers have similar annealing temperatures but do not bind to each other (QIAGEN® OneStep RT-PCR Kit handbook). This would prove difficult in *A. anguilla* due to the relative lack of full length cDNA sequences available to facilitate primer design compared to other

species such as rodents in which multiplex RT-PCR is routinely carried out (Ruano *et al.*, 2000; Danik *et al.*, 2005; Paarmann *et al.*, 2005; Pustylnyak *et al.*, 2005; Riviere *et al.*, 2005; Sanghi *et al.*, 2005). In support of this, problems using multiplex PCR have already been reported by other authors working with *A. anguilla* (Degani *et al.*, 2003). Averaging of the results of experimental replicates as carried out by others (e.g. Briscini *et al.*, 2002; Rohatgi *et al.*, 2004; Chen *et al.*, 2005; Al-Bader and Al-Sarraf, 2005; Omura *et al.*, 2005) and in this study is a solution to overcoming interexperimental variability when multiplex RT-PCR is not appropriate.

A decrease was found in mean relative band intensity for BDNF at 15 and 20 DPT, which was statistically significant from BDNF mRNA levels in animals one DPT. Other than this result, no statistically significant changes were found in NGF, trk B at any time point or CNS region examined nor were they found in BDNF mRNA expression in the brains of animals 10 DPT and spinal cords of animals 15 DPT compared to untransected animals. However, it may be the case that any statistically significant changes in mRNA expression may not have been detected due to insufficient experimental power caused by intersample and interexperimental variability.

Estimation of sample size for future RT-PCR experiments

The power of an experiment can be defined as the probability of detecting the specified effect at the specified significance level and is usually set between 80 and 90% (Festing *et al.*, 2002; Ruxton and Colegrave, 2003). The higher the power of an experiment, the greater the likelihood that a significant difference will be detected. The greater the variability between samples the lower the power of an experiment will be. In order to amplify the power of an experiment and overcome intersample variability, sample size can be increased (Festing *et al.*, 2002; Ruxton and Colegrave, 2003). In order to estimate an advisable sample size for future RT-PCR experiments based on the results of this study, formal power analysis was carried out using nQuery Advisor (Statistical Solutions Ltd, Ireland). This software requires the user to estimate the minimal effect size to be identified by the experiment, which is essentially the minimal difference in the means being compared that would indicate a biologically significant result. It was decided for the purpose of this analysis, that a two-fold change in mean relative band intensity after RT-PCR would indicate a biologically important result since Kobayashi *et al.*, (1996) found that a two to four fold increase in BDNF mRNA expression after injury in the cell bodies of facial motoneurons of the rat led to an increase in BDNF protein which lasted for up to two weeks. In addition, for spinal cord samples, a two-fold increase in template amount led to a more than two-fold increase in band intensity for ARP (Fig, 4.10). The highest

standard deviation obtained for each data set (see Appendix B) was used to estimate a sample number than would achieve experimental power of at least 80%. For RT-PCR with *A. anguilla* brain RNA samples, an experiment with 80% possibility of detecting a two-fold change in mean relative band intensity for BDNF and NGF-like factor would require a sample size of six to seven. For trk B, nQuery Advisor estimated a required sample size of 10. For RT-PCR with *A. rostrata* brain RNA samples, an experiment carried out at the highest limits of variability with BDNF primers, would require animal numbers per treatment group of 22 to achieve experimental power of 80%. For NGF-like factor, an experiment carried out with a sample size of four would have a power of 88%. For trk B, a sample size of 50 was estimated, based on the highest standard deviation observed in these experiments, in order to achieve experimental power of 80%. For RT-PCR with spinal cord RNA samples, experiments with 80% possibility to detect a two-fold change in mean relative band intensity for BDNF would require a sample size of five. To examine NGF-like factor in spinal cord samples after injury, nQuery Advisor estimated a required sample size to 10 to attain experimental power of 80%.

It should be noted that the estimated sample sizes generated by nQuery advisor should be used only as a guideline since they were based on the highest standard deviations observed and before increasing animal numbers for experiments, every effort should be made to decrease other sources that may contribute to experimental variability (Festing *et al.*, 2002). In general, as mentioned above intersample and interexperimental variability in future RT-PCR experiments may be decreased by the use of high quality RNA with 28S:18S rRNA ratios of between 1.5 and 2.0. RNA quality may be improved by short-term storage of samples before RNA extraction and isolation of RNA as described by Weltzien *et al.* (2005). More accurate analysis of RNA quality using an automated system may also benefit the experiments (Imbeaud *et al.*, 2005; Schroeder *et al.*, 2006).

Experimental variability caused by the presence of contaminants in RNA samples may have further contributed to the variability seen in experiments carried out with *A. rostrata* brain and *A. anguilla* spinal cord tissue. All samples were phenol-chloroform extracted after DNase treatment however, for future experiments, removal of contaminants from samples with purity ratios in 10 mM Tris-HCl that are still less than 1.9 may be achieved by a further phenol-chloroform extraction.

In conclusion, for future semiquantitative RT-PCR experiments carried out with pure, high quality RNA isolated from *A. anguilla* or *A. rostrata,* a sample size of six to seven, would be recommended in order to attain experimental power of approximately 80%. Owing to the high intersample variability obtained with the trk B primers used in

this study resulting in the high estimations of required sample size for further experiments and since RT-PCR experiments with spinal cord samples were not reproducible with this primer pair, future RT-PCR experiments to investigate trk B expression may benefit from the design of a new set of trk B primers based on the published sequence for *A. anguilla* trk B (NCBI GenBank accession number DQ396402).

In future experiments, keeping the sample size of eels to a minimum without comprising scientific output is also important for ecological reasons. Freshwater eel populations are at an all time low and recent data suggest that numbers of fry in European waters have declined by up to 99% since 1980 (Stone, 2003). Reasons for this decline are unclear but may be due to overfishing, pollution and climate change (Stone, 2003).

4.4.2 BDNF, NGF-like factor and trk B receptor mRNA expression in the eel CNS before and after spinal cord transection.

The results from the literature indicate that neurotrophins such as BDNF are upregulated in the cell bodies of injured neurons in the mammalian CNS (Kokaia *et al.*, 1993; Kobayashi *et al.*, 1996; Gao *et al.*, 1997; Hirsch *et al.*, 2000; Chidlow *et al.*, 2005; Suneja *et al.*, 2005; Zhang *et al.*, 2006). A slight upregulation in BDNF mRNA expression that was not statistically significant from levels in untransected animals was seen in the eel brain one DPT ($p = 0.387$; Fig. 4.6). If the statistics reflect a real result and there is no change in BDNF mRNA expression in the eel brain one DPT, the slight trend for an increase seen may have been due to experimental variability or alternatively, it may be the case that an increase in BDNF mRNA expression one DPT is an effect experimentally induced by surgery. It has been shown for example that BDNF is upregulated due to stress (Smith *et al.*, 1995). This effect could be controlled for in future experiments by performing sham surgeries, without lesioning of the spinal cord, on eels to be used as untransected controls before tissue dissection.

On the other hand, the increase seen in mean relative band intensity for BDNF may reflect a short-term upregulation in BDNF mRNA which may lead to a longer-term, downstream increase in protein levels as suggested by the results of an experiment by Kobayashi *et al.* (1996) already mentioned above in section 4.4.1. They showed that just a two day increase in BDNF mRNA expression of two- to four-fold in the cell bodies of axotomised facial motoneurons was followed by an increase in BDNF protein production, which was sustained for approximately two weeks after injury. Preliminary western blot and immunohistochemistry experiments have been carried out on eel tissue by co-workers in our laboratory but conclusive results were not attained due to antibody specificity and sensitivity issues (Dr S. M. Borich, personal communication). Expression levels of BDNF,

NGF-like factor and Trk B protein therefore were not further investigated due to the absence of a fish-specific antibody.

The purpose of a possible initial increase in BDNF may be to promote neuronal survival and regrowth after injury by activating neurotrophin receptors coexpressed on the cell body (Kokaia *et al.*, 1993; Miranda *et al.*, 1993; Davies and Wright, 1995; McAllister *et al.*, 1999; Danzer *et al.*, 2002; Lom *et al.*, 2002) and/or by its actions as an anterogradely transported molecule (Tonra *et al.*, 1998; McAllister *et al.*, 1999; Caleo *et al.*, 2000; Chan *et al.*, 2001; Kojima *et al.*, 2002; Spalding *et al.*, 2002; Menna *et al.*, 2003; Notterpek, 2003; Omura *et al.*, 2005).

At 10 days after transection when the axons of transected neurons have begun to regrow but have not started to make functional connections (Doyle *et al.*, 2001), BDNF mRNA expression in the neuronal cell bodies in the eel brain was not found to be statistically different from that in untransected animals. At 15 and 20 DPT, axons of some injured neurons have bridged the gap created by the transection and have started to make functional connections (Doyle *et al.*, 2001). At these timepoints, a decrease in BDNF mRNA expression was then seen which was statistically significant from levels in animals one DPT (15 DPT: $p = 0.022$ and 20 DPT: $p = 0.032$). However, since the BDNF mRNA levels at 15 and 20 DPT were not found to be statistically different from levels in untransected animals (15 DPT: $p = 0.325$ and 20 DPT: $p = 0.429$), this suggests that the decrease in mRNA expression is relatively minor. The slight decrease in BDNF mRNA at 15 and 20 DPT may reflect a decline in the need for autocrine support for injured neurons as regrowing axons approach their targets and begin to receive retrogradely derived neurotrophic support.

The mRNA expression levels of the NGF-like factor and the biologically active form of the trk B receptor did not appear to change in the eel brain at any timepoint examined before or after spinal cord transection. It is possible that changes in their expression may not have been detected due to experimental variability. On the other hand, it is likely that the results of this experiment reflect the true biological situation that there is no change in NGF-like factor and trk B expression after injury since a trend like that seen for BDNF (Fig. 4.6) was not obvious when the results were compared graphically (Fig. 4.7 and 4.8). Furthermore, the values for coefficients of variation, which ranged from 14 to 60% in NGF-like factor experiments and trk B 39 to 91%, were comparable to those in other publications where statistically significant differences were detected between treatment groups using RT-PCR (Huang *et al.*, 1998; Degani *et al.*, 2003; Yuge *et al.*,

2003; Hashimoto *et al.*, 2004; Chidlow *et al.*, 2005). For example, in a study by Yuge *et al.* (2003) in *A. japonica*, the values for coefficients of variation ranged from 10 to 80%.

It should be noted that it could be the case that NGF-like factor primers are in fact amplifying NT-6/7 since they were designed based on a putative NGF-like sequence described by Dethleffsen *et al.* (2003) who state that they could not unequivocally decide whether their eel neurotrophin sequence represented an NGF-like or NT-6/7-like neurotrophin. NT-6/7 seems to be exclusive to fish and its function is as yet unknown (Heinrich and Lum, 2000).

Taken together, the minor changes in BDNF and lack of change in the NGF-like factor and trk B in the cell bodies of injured neurons observed in this study are consistent with reports on examples of CNS regeneration following optic nerve injury in other anamniotes (Caminos *et al.*, 1999; Duprey-Diaz *et al.*, 2002, 2003). Duprey-Diaz *et al.* (2002) examined BDNF and trk B receptor levels after optic nerve lesion in the adult frog (*Rana pipiens*), an animal that displays regeneration of retinal ganglion cell (RGC) axons and recovery of visual function after optic nerve lesion. They observed no overall change in BDNF mRNA levels in the retina after optic nerve injury measured by means of semiquantitative RT-PCR, although a decrease in BDNF protein expression was seen in RGCs and an increase was seen in the cells of the inner nuclear layer that project to the ganglion cell layer four to six weeks after injury. The Trk B protein expression pattern in RCG cell bodies did not change after axotomy. A similar profile of expression for NT-3 and its receptor Trk C was also seen in the retina of this species after the same injury (Duprey-Diaz *et al.*, 2003). Caminos *et al.* (1999) carried out a study to investigate changes in levels of the neurotrophins BDNF, NGF, NT-3 and neurotrophin-4/5 (NT4/5) and their receptors trk A, trk B, trk C and p75 in the tench retina, another anamniote that regains visual function due to optic nerve regeneration after injury. The only molecules whose levels increased in the cell bodies of the RGCs before and after injury were the Trk A receptor protein and its mRNA.

It could be argued that the relatively small changes in BDNF mRNA expression after spinal cord transection and indeed the lack of change detected at any timepoint in mRNA expression for NGF-like factor and trk B may be caused by the averaging effect of measuring overall levels of mRNA in the brain using RT-PCR. For example, regional changes in expression levels, whereby one brain region upregulates and another downregulates BDNF mRNA after injury could be overlooked similarly to an effect described by Chelly *et al.* (1989) and Duprey-Diaz *et al.* (2002). However, a comparison of ISH and RT-PCR results for BDNF mRNA expression levels at 10 DPT do not support

this idea. Although some individual variation was seen between animals, the overall spatial expression pattern of BDNF mRNA appeared unchanged 10 DPT in the brain stem nuclei that possess spinally projecting neurons and also in areas that house afferent neurons such as the telencephalon that project to these nuclei. In contrast, in *Rana pipens*, regional changes in BDNF mRNA expression after optic nerve transection suggested by a decline in BDNF immunoreactivity in retinal ganglion cells and an increase in BDNF immunoreactivity in amacrine cells may not have been detected by their RT-PCR experiments due to an averaging effect of RNA levels by isolation from whole retinas (Duprey-Diaz *et al.*, 2002).

At 15 DPT, regrowing axons have bridged the gap created by the spinal cord transection and synapse formation has started to take place (Doyle *et al.*, 2001) yet BDNF and NGF mRNA expression levels in the target region at this timepoint were not significantly different from those in untransected animals (Fig. 4.12). It may be the case that due to experimental variability, changes in mRNA expression for BDNF and NGF-like factor were not detected. However, similarly to the experiments carried out in this study with the RNA brain samples mentioned above, the coefficients of variability (BDNF: 20-42%; NGF: 35-72%) were not outside the range of variability in other published RT-PCR studies where statistically significant changes were detected (Huang *et al.*, 1998; Degani *et al.*, 2003; Yuge *et al.*, 2003; Hashimoto *et al.*, 2004; Chidlow *et al.*, 2005). As well as examining mRNA expression of BDNF in the cell bodies of injured neurons, Duprez-Diaz *et al.* (2002) also examined BDNF expression in the target region, the tectum, after optic nerve injury in the frog. They reported an initial decrease in BDNF mRNA expression one week after lesion but a subsequent check four weeks after retinal ganglion cell axotomy revealed a return to control levels.

BDNF has been shown to be important in synapse formation during development (McAllister, 1999, Lom *et al.*, 2002, Blum and Konnerth, 2005). BDNF and NGF are upregulated in the target region in the mammalian PNS and CNS after injury (Meyer *et al.*, 1992, Funakoshi *et al.*, 1993, Raivich and Kreutzberg, 1993, Zhang *et al.*, 2000, Ikeda *et al.*, 2001,Uchida *et al.*, 2003) and their application after injury in the target regions supports axonal regrowth during regeneration (Schnell *et al.*, 1994; Bregman *et al.*, 1997; Ye and Houle, 1997). However, in contrast to mammals, neurotrophin expression does not appear to be greatly affected in the target region or cell bodies of injured neurons after spinal cord lesion in the eel.

In summary, no dramatic changes in the expression levels of BDNF, an NGF-like factor and trk B were detected in the eel CNS post injury and during regeneration after a complete spinal cord transection.

Chapter 5
General Discussion

5. Discussion

The majority of research into spinal cord regeneration focuses on animals that cannot regenerate their CNS after injury rather than those that can (Ferretti *et al.,* 2003). However, the study of models that display CNS regeneration after injury may help to elucidate some of the key factors, such as neurotrophins, in promoting neuronal regrowth in these models and to redirect studies on non-regenerating models (Ferretti *et al.,* 2003; Mladinic *et al.,* 2005). The present study focussed on the mRNA expression of neurotrophins (BDNF, NGF and the neurotrophin receptor, trk B) in the CNS of a brain stem-spinal cord regenerating model, the European eel, after complete spinal cord transection. The eel provides a valuable model in which to investigate spinal cord regeneration owing to its relatively large body size, the well studied anguilliform nature of its swimming and its rapid morphological and functional recovery after complete spinal cord transection (Doyle *et al.,* 2001; Doyle and Roberts, 2004a, b).

5.1 Development of molecular techniques

Before this study, although the protocols for monitoring morphological and functional recovery in the eel were established in our laboratory, the molecular tools with which to monitor the intrinsic process of mRNA expression were not available and had to be developed. First, a 467 bp fragment for *A. anguilla* BDNF was cloned and is now publicly available on the NCBI GenBank database (accession number AY762996). This fragment will be invaluable for future regeneration studies in the eel and may also be important in functional genomics (Roest Crollius and Weissenbach, 2005) and phylogenetic studies in different species of the freshwater eel (Kullander *et al.*, 1997; Wirth and Bernatchez, 2001; Mank and Avise, 2003; Minegishi *et al.,* 2005) and possibly other teleosts.

Protocols for ISH to cryostat and free-floating sections were then established for *A. anguilla* and a semiquantitative RT-PCR study was also carried out with RNA isolated from this species. Aspects of the protocol that may be responsible for causing experimental variability have been identified, such as RNA template quality and purity and recommendations were made to decrease intersample and interexperimental variability such as the use of a protocol described by Weltzien *et al.,* (2005) for the isolation of high quality, pure RNA from *A. anguilla*. For future studies, in order to detect minor changes in gene expression, e.g. by a magnitude of two-fold, a sample size of six to seven per treatment was recommended for semiquantitative RT-PCR experiments with this species.

Using these techniques, two consistent findings emerged that may help us to clarify the different regenerative responses of fishes and mammals. The first is that BDNF, as well

as being expressed by many homologous cell groups in the fish and mammal, in the eel is also expressed by cell groups that are affected by spinal cord transection. The second is that neurotrophin (BDNF and NGF) and neurotrophin receptor (trk B) mRNA levels in the eel CNS are little affected by spinal cord injury.

5.2 Neurotrophins and CNS regeneration in fish

BDNF and NGF are upregulated in neuronal cell bodies (Sebert and Shooter, 1993; Kobayashi *et al.*, 1996; Gao *et al.*, 1997; Tonra *et al.*, 1998; Michael *et al.*, 1999; Hirsch *et al.*, 2000) and in the target region (Meyer *et al.*, 1992, Funakoshi *et al.*, 1993, Raivich and Kreutzberg, 1993, Zhang *et al.*, 2000, Ikeda *et al.*, 2001,Uchida *et al.*, 2003) in the mammalian PNS and CNS after injury. Their application to the cell bodies (Kobayashi *et al.*, 1997; Salie and Steeves, 2005) and the target regions (Schnell *et al.*, 1994; Bregman *et al.*, 1997; Ye and Houle, 1997) of injured neurons supports axonal regrowth during regeneration. Moreover, blockade of their activity hinders remyelination and regrowth of injured neurons (Zhang *et al.*, 2000; Boyd and Gordon, 2001). However, in contrast to mammals, as discussed in chapter four, neurotrophin expression does not appear to be greatly affected in the target region or cell bodies of injured neurons after a spinal cord lesion in the eel.

The lack of any dramatic change in neurotrophin mRNA expression after injury in the eel may be due to the fact that fish possess high levels of neurotrophins. The presence of a nerve growth promoting substance has been reported in the homogenate of the spinal axes from a wide range of uninjured fish species (Weiss, 1968). In addition, a study by Benowitz and Greene (1979) suggests that neurotrophin levels in the goldfish CNS are higher than those in the mammalian CNS; the goldfish, like the eel, displays regeneration of the CNS (Hanna *et al.*, 1998; Bernhardt *et al.*, 1989). Benowitz and Greene (1979) compared the effectiveness of homogenates from goldfish and mouse brain at promoting neurite outgrowth from pheochromocytoma (PC12) cells. They found that the goldfish brain homogenate was 35 times more effective at promoting outgrowth than the mouse homogenate, a response that was blocked by an anti-NGF antibody.

The findings of the present study also support the idea that neurotrophins levels in the fish CNS might be higher than those in the mammalian CNS, at least in some regions. As discussed in section 3.4.5, BDNF mRNA expression was observed in the reticular formation and other brain stem nuclei involved in the control of movement and that possess neurons that project down the spinal cord. In contrast, BDNF mRNA does not appear to be expressed in the equivalent nuclei in the adult mammal (Friedman *et al.*, 1991; Conner *et al.*, 1997; Galter *et al.*, 2000).

One reason that fish may possess high levels of neurotrophins could be that fish continue to increase their body size for a relatively longer period than mammals (Holder and Clarke, 1988; Bosch and Roberts, 1994). As mentioned in chapter one, neurotrophins have important functions in development such as promotion of axonal pathfinding and elongation towards targets, and the support of synaptic plasticity (Castrén *et al.*, 1992; Cohen-Cory and Fraser, 1995; Henderson, 1996; Katz and Shatz, 1996; McAllister, 1999; Song and Poo, 1999; Fariñas *et al.*, 2002; Huang and Reichardt, 2001; Blesch *et al.*, 2002; Lom *et al.*, 2002; Gillespie, 2003; Cohen-Cory and Lom, 2004). However, expression levels of neurotrophins appear to decline as mammals reach maturity (Friedman *et al.*, 1991; Nakamura and Bregman, 2001). For instance, neurotrophin mRNA levels in the rat neonate spinal cord up to postnatal day 10 are greater than those in the adult cord but then begin to decline after this timepoint (Nakamura and Bregman, 2001). It may be the case that due of their "developmental" status, fish possess sufficient levels of neurotrophins to promote regeneration with no need for upregulation after injury.

This might be a possible explanation, therefore, for the expression of BDNF in the motor brain stem of fish and lack thereof in mammals. As eels increase in size, the cells of origin of descending projections to the spinal cord increase in size but not in number (Bosch and Roberts, 1994). BDNF may function as an autocrine or anterogradely-transported, growth promoting factor in these neurons (Kokaia *et al.*, 1993; Miranda *et al.*, 1993; Altar *et al.*, 1997; Yan *et al.*, 1997,b; Menna *et al.*, 2003). The increase in body length in eels also is accompanied by an increase in the size and number of muscle fibres (Willemse and van den Berg, 1978). The motoneurons that supply these muscle fibres respond to changes in their targets by increasing in size but not in number (Smit *et al.*, 1991). As the motoneurons become larger, the number of synapses contacting them increases (Bosch and Roberts, 1994). Anterogradely-transported BDNF may also be important therefore during the formation of new synapses between brain stem neurons and their enlarging target motoneurons in the spinal cord (Alsina *et al.*, 2001; Cohen-Cory and Lom, 2004; Hu *et al.*, 2005). After axotomy of brain stem neurons in the spinal cord, an upregulation in neurotrophin expression may not be necessary, since expression levels may already be sufficiently high in these growing neurons.

As mentioned in section 4.4.2, Caminos *et al.* (1999) investigated neurotrophin levels in the regenerating tench visual system before and after optic nerve injury. Similarly to the present study, they saw no change in neurotrophin levels after injury although they did see an increase in trk A expression (Caminos *et al.*, 1999). The tench retina continues to proliferate throughput the animal's life (Caminos *et al.*, 1999). Due to this fact, it may

be the case that neurotrophins levels are also sufficiently high in this system to promote regeneration negating the need for upregulation after neuronal insult.

In order to further investigate the idea that neurotrophin levels are higher in fish CNS than in mammalian CNS, it would be interesting to compare neurotrophin levels in the fish brain with those in an amniote such as the rat using for example enzyme-linked immunosorbent assays (ELISA). As mentioned in section 4.4.2, conclusive results for Western blot experiments and immunohistochemistry using an anti-BDNF antibody have not been attained in our laboratory due to antibody specificity and sensitivity issues (Dr S. M. Borich, personal communication). Future ELISA experiments investigating BDNF levels will depend therefore on the development of a fish-specific anti-BDNF antibody.

Furthermore, to confirm that BDNF is expressed in brain stem neurons that project down the spinal cord, axonal tracing studies combined with ISH to eel brain stem sections with a BDNF probe may be carried out. Tract tracing studies have been carried out successfully before in many fish (Becker *et al.*, 1997; Xue *et al.*, 2004) including the eel (Bosch and Roberts, 1994; Bosch and Roberts, 2001). Furthermore, Lieberoth *et al.* (2003) recently described a protocol for double labelling of brain stem neurons using a combination of retrograde axonal tracing and ISH in the adult zebrafish whereby the tracer, rhodamine dextran amine, is applied at the spinal cord level of interest. Once the tracer has been allowed to diffuse retrogradely for the desired period of time, ISH is carried out with free floating sections cut on a vibrating microtome (Lieberoth *et al.*, 2003).

Future studies will also be necessary to further elucidate the role of neurotrophins in spinal cord regeneration in fish. The various classes of spinally projecting neurons of the fish brain stem respond differently to injury (Becker *et al.*, 1998; Bosch, Maslam and Roberts, unpublished observations). For example, in the eel, neurons from some nuclear groups such as the inferior reticular formation (IRF) and the nucleus of the medial longitudinal fasciculus (nMLF) regenerate quickly after injury with axons bridging the gap created by the transection as soon as 12 days after transection. On the other hand, the neurons of the anterior and tangential (TO) octavolateral nuclei have been reported to take up to 93 days to regenerate and the Mauthner cell (M) does not appear to reconnect with its target until approximately 286 days after transection (Bosch, Maslam and Roberts, unpublished observations). In the present study, BDNF mRNA expression was seen in nuclear groups in the eel brain stem that possess spinally projecting neurons that regenerate quickly after injury such as the IRF (Fig. 3.15 e and f; Fig. 3.16 c; Fig. 3.17 c) and nMLF (Fig. 3.2K, L; Fig. 3.9 b; Table 3.3) but was also seen in the M cell (Fig. 3.2Q and Fig. 3.13 c and e) and TO (Fig. 3.2S and Fig. 3.14 a). In addition, BDNF mRNA expression

was not observed in the red nucleus (Fig. 3.2K; Table 3.3), which begins to regenerate its spinally projecting axons 35 days after injury according to Bosch, Maslam and Roberts (unpublished observations). It appears therefore that BDNF mRNA expression in the cell body of a spinally projecting neuron does not necessarily mean that it regenerates quickly after injury. Furthermore, absence of BDNF mRNA expression in a nuclear group, e.g. the red nucleus, does not prevent axonal regeneration after injury but may reflect dependence of this group on another neurotrophin such as NGF. It has been noted for example, that different neuronal populations in the mammalian CNS have different neurotrophic requirements (Giehl and Tetzlaff, 1996; Giehl *et al.*, 2001; Zhou and Shine, 2003; Salie and Steeves, 2005). For example, the application of BDNF and NT-3 but not NGF prevents atrophy of corticospinal neurons after injury (Giehl and Tetlaff, 1996). On the other hand, BDNF does not have a neurotrophic effect on the locus coeruleus (Friedman *et al.*, 1993).

Studies examining the effects of an increase in neurotrophin levels or alternatively, the prevention of neurotrophin activity in the fish CNS may help to further our understanding of the role of neurotrophins in spinal cord regeneration. For example, in order to examine the dependency of spinally projecting, axotomised brain stem neurons on autocrine, cell body derived neurotrophic support, disruption of BDNF activity might be achieved by blockade of BDNF synthesis by gene silencing at the cell body after injury although, to the best of my knowledge, an *in vivo* study in the adult fish does not appear to have been carried out before. Alternatively, endogenous BDNF may be sequestered by application of anti-BDNF antibodies (Zhang *et al.*, 2000) or BDNF scavengers in the form of Trk B-fusion proteins (Yajima *et al.*, 2005; Vaynman *et al.*, 2006) at the cell body. Disruption of BDNF activity may also be accomplished by the application of a Trk receptor antagonist in the vicinity of the cell bodies of spinally transected neurons (Rasouly *et al.*, 1995; Drake-Baumann, 2005; He *et al.*, 2005; Yajima *et al.*, 2005; Pozzo-Miller, 2006).

The lamprey, a primitive, jawless fish, possesses one neurotrophin, *Lf*-NT, which appears to be an ancestral neurotrophin to those neurotrophins found in other vertebrates (Hallböök *et al.*, 1998; Hallböök, 1999). Two receptors, *Lf*-Trk1 and *Lf*-Trk2, which are related to Trk A, B and C have also been found in the lamprey (Hallböök *et al.*, 1998). A study has already been carried out examining at the effects on neuronal regeneration of K252a application by intracellular microinjection of identifiable reticulospinal neurons in larval lamprey *in vivo* (Hall and Yao, 2000). Hall and Yao (2000) found that K252a treatment caused regenerating sprouts to be longer and narrower than in control preparations and also reduced the diameters of the axon stumps. A similar reduction in

axonal stump diameter was also seen after injection of anti-neurofilament antibodies. The authors suggested therefore that K252a may interfere with neurofilament assembly and sidearm phosphorylation leading to a reduction in neurofilament transport in axonal sprouts and stumps resulting in a decrease in stump diameter (Hall and Yao, 2000).

The authors did not attribute this effect directly to Trk receptor blockade since K252a is a broad spectrum kinase inhibitor (Hall and Yao, 2000). However, it is possible that this effect was induced by Trk receptor blockade. One of the signalling cascades initiated by Trk receptor activation is the MAP kinase pathway which has been shown to be important in axonal elongation possibly due to the ability of MAP kinases to phosphorylate cytoskeletal proteins (Segal, 2003). In addition, the results of studies by Kobayshi *et al.* (1997) and Molteni *et al.* (2004) suggest that Trk receptor activation leads to an increase in the synthesis of cytoskeletal proteins in the cell body. Moreover, Avwenagha *et al.* (2003) reported a loss of F-actin in growth cone tips in developing and regenerating retinal ganglion cell axons in culture after treatment with a TrkB-IgG scavenger protein. Thus, it is possible that the application of the Trk receptor antagonist, K252a, may have led to a decrease in neurofilament expression in the lamprey and may account for the results described by Hall and Yao (2000). It may be the case therefore, that one of the functions of neurotrophin activity in fish during neuronal regrowth is to promote the expression and phosphorylation of cytoskeletal proteins required for normal axonal elongation.

The influence of target derived neurotrophic factors at site of injury is another important area for investigation. A study monitoring functional and morphological recovery in the eel after application of anti-neurotrophin antibodies, neurotrophin scavenger proteins or Trk receptor inhibitors at the site of transection may help to provide more insight into the role of target derived neurotrophins after spinal cord injury. Application of the test substance could be carried out using a slow-release polymer, such as Elvax, as the carrier. Elvax impregnated with AP5 has previously been used in our laboratory to examine the effect of NMDA receptor blockade in the target region after injury on spinal cord regeneration in the eel (Doyle and Roberts, 2004b).

5.3 Concluding remarks

Experiments performed on simple anamniotes such as fish and amphibians form the basis of our understanding of many biological processes such as the control of movement (lamprey: Grillner and Wallén, 1985; Matsushima and Grillner, 1990; Grillner *et al.*, 1995; Deliagina *et al.*, 2000, 2002; amphibians: Dale, 1985; Dale and Roberts, 1985; Soffe and Roberts, 1989; Reith and Sillar, 1998; Delvolvé *et al.*, 1999). As stated at the beginning of

this chapter, the results of studies that elucidate the mechanisms driving neuronal regeneration in fish may be important to direct the focus of studies in mammals (Ferretti *et al.,* 2003). The findings of the current study indicate that neurotrophin mRNA expression does not change dramatically in the fish brain or spinal cord in response axonal transection of spinally projecting neurons whose cell bodies lie in the brain stem. This lack of change may be due to a persistent baseline expression of neurotrophins in these cell bodies owing to the continual growth state of fish, which in turn negates the need for neurotrophin upregulation after injury. Future experiments will be required to fully understand the role of neurotrophins in regeneration of the fish CNS.

Whilst aiding in neuronal regeneration (Kobayashi *et al.,* 1996; Zhang *et al.,* 2000; Boyd and Gordon, 2001; Molteni *et al.*, 2004; Irintchev *et al.,* 2005), it has been shown that neurotrophins alone cannot promote full morphological and functional recovery in the mammalian CNS (Schnell *et al.*, 1994; Bregman *et al.*, 1997; Kobayashi *et al.,* 1997; Ye and Houle, 1997; Zhou and Shine, 2003; Salie and Steeves, 2005). It is also likely that a combination of factors including extrinsic influences such as glial cell organisation after injury (Dervan and Roberts, 2003a, b) and the intrinsic expression of growth promoting genes, e.g. neurotrophins, contribute to regenerative regrowth in the fish CNS. Some of the tools are now in place with which to monitor the intrinsic factors associated with regeneration in the CNS of the eel. The results of studies in fish may provide insight into the conditions required by an injured neuron in the CNS to achieve successful regeneration. For successful neuronal regeneration to be accomplished in adult mammals, future studies might focus on recreating these conditions after injury in the mammalian CNS.

Appendix

Appendix A

Table A1. Summary of *A. anguilla* brain RNA preparation integrity assessed by the 28S:18S rRNA band ratio and purity of preparations estimated by calculating the ratio of the absorbance values at 260 and 280 nm in 10 mM Tris-Cl, pH 7.5.

Animal treatment	Animal number	28S:18S rRNA band ratios	Purity Abs260/280
Untransected	24	1.3	2.1
	86	1.0	2.1
	87	1.1	2.0
	88	1.0	2.1
1 DPT	82	2.0	2.0
	83	2.3	2.1
	84	1.9	2.1
	85	2.0	2.0
15 DPT	20	1.4	2.0
	21	1.2	2.0
	22	1.2	2.0
	23	1.4	1.9
20 DPT	5	1.2	2.1
	6	1.4	2.1
	7	1.2	2.0
	8	1.1	1.9

Table A2. Summary of *A. rostrata* brain RNA preparation integrity assessed by the 28S:18S rRNA band ratio and purity of preparations estimated by calculating the ratio of the absorbance values at 260 and 280 nm in 10 mM Tris-Cl, pH 7.5.

Animal treatment	Animal number	28S:18S rRNA band ratios	Purity Abs260/280
Untransected	1A	2.3	2.0
	3B	1.2	1.9
	4B	1.1	2.0
	7A	2.1	1.9
10 DPT	1B	1.6	2.0
	2B	1.2	2.1
	4A	1.3	1.7
	5A	1.7	1.9

Table A3. Summary of *A. anguilla* spinal cord RNA preparation integrity assessed by the 28S:18S rRNA band ratio and purity of preparations estimated by calculating the ratio of the absorbance values at 260 and 280 nm in 10 mM Tris-Cl, pH 7.5.

Animal treatment	Sample number	28S:18S rRNA band ratios	Purity Abs260/280
Untransected	11+14	0.9	1.8
	12+15	1.2	1.7
	13+16	1.4	1.7
15 DPT	3+4	1.16	1.7
	5+6	1.7	1.8
	8+10	1.5	1.8

Appendix B

Table B1. Summary of mean relative band intensities, standard deviations and coefficients of variation for RT-PCR experiments carried out with BDNF primers.

Template for BDNF RT-PCR experiment	Injury status §	Mean relative band intensity (mean)	Standard deviation (SD)	Coefficient of variation ((SD/mean) X 100)
A. anguilla brain RNA	Untransected	0.40	0.05	12.6%
	1 DPT	0.53	0.12	23.1%
	15 DPT	0.26	0.14	54.4%
	20 DPT	0.28	0.10	36.6%
A. rostrata brain RNA	Untransected	0.31	0.18	58.9%
	10 DPT	0.18	0.11	61.0%
A. anguilla spinal cord RNA	Untransected	1.04	0.20	19.7%
	15 DPT	1.16	0.49	42.0%

§ DPT= days post spinal cord transection

Table B2. Summary of mean relative band intensities, standard deviations and coefficients of variation for RT-PCR experiments carried out with primers for an NGF-like factor.

Template for NGF-like factor RT-PCR experiment	Injury status §	Mean relative band intensity (mean)	Standard deviation (SD)	Coefficient of variation ((SD/mean) X 100)
A. anguilla brain RNA	Untransected	0.88	0.49	55.1%
	1 DPT	0.71	0.30	41.8%
	15 DPT	0.90	0.55	60.7%
	20 DPT	0.81	0.14	34.9%
A. rostrata brain RNA	Untransected	0.46	0.06	14.1%
	10 DPT	0.47	0.16	35.2%
A. anguilla spinal cord RNA	Untransected	1.14	0.81	71.5%
	15 DPT	1.67	0.59	35.4%

§ DPT= days post spinal cord transection

Table B3. Summary of mean relative band intensities, standard deviations and coefficients of variation for RT-PCR experiments carried out with trk B primers.

Template for trk B RT-PCR experiment	Injury status §	Mean relative band intensity (mean)	Standard deviation (SD)	Coefficient of variation ((SD/mean) X 100)
A. anguilla brain RNA	Untransected	0.34	0.30	88.8%
	1 DPT	0.49	0.29	58.2%
	15 DPT	0.45	0.33	74.3%
	20 DPT	0.60	0.24	39.3%
A. rostrata brain RNA	Untransected	0.18	0.12	67.2%
	10 DPT	0.31	0.28	91.0%

§ DPT= days post spinal cord transection

References

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374(6521):450-3.

Acosta J, Carpio Y, Borroto I, Gonzalez O, Estrada MP (2005) Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J Biotechnol.119(4):324-31.

Adachi N, Kohara K, Tsumoto T (2005) Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. BMC Neurosci. 6(1):42.

Adlard PA, Perreau VM, Engesser-Cesar C, Cotman CW (2004) The timecourse of induction of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus following voluntary exercise. Neurosci Lett. 363(1):43-8.

Al-Bader MD, Al-Sarraf HA (2005) Housekeeping gene expression during fetal brain development in the rat-validation by semi-quantitative RT-PCR. Brain Res Dev Brain Res. 156(1):38-45.

Alderson RF, Alterman AL, Barde YA, Lindsay RM (1990) Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron. 5(3):297-306.

Aliaga E, Rage F, Tapia-Arancibia L, Bustos G (1999) Negative regulation of brain-derived neurotrophic factor mRNA expression by kainic acid in substantia nigra. Brain Res Mol Brain Res. 71(2):341-4.

Aliaga E, Arancibia S, Givalois L, Tapia-Arancibia L (2002) Osmotic stress increases brain-derived neurotrophic factor messenger RNA expression in the hypothalamic supraoptic nucleus with differential regulation of its transcripts. Relation to arginine-vasopressin content. Neuroscience. 112(4):841-50.

Allen GC, Earnest DJ (2005) Overlap in the distribution of TrkB immunoreactivity and retinohypothalamic tract innervation of the rat suprachiasmatic nucleus. Neurosci Lett. 376(3):200-4.

Al-Majed AA, Brushart TM, Gordon T (2000) Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. Eur J Neurosci. 12(12):4381-90.

Alsina B, Vu T, Cohen-Cory S (2001) Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. Nat Neurosci. 4(11):1093-101.

Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 389(6653):856-60.

Altar CA, DiStefano PS (1998) Neurotrophin trafficking by anterograde transport. Trends Neurosci. 21(10):433-7.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25(17):3389-402.

Angeles Luque M, Pilar Perez-Perez M, Herrero L, Torres B (2005) Involvement of the optic tectum and mesencephalic reticular formation in the generation of saccadic eye movements in goldfish. Brain Res Brain Res Rev. 49(2):388-97.

Aoyama J, Nishida M, Tsukamoto K (2001) Molecular phylogeny and evolution of the freshwater eel, genus Anguilla. Mol Phylogenet Evol. 20(3):450-9.

Aroua S, Schmitz M, Baloche S, Vidal B, Rousseau K, Dufour S (2005) Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. Neuroendocrinology. 82(3-4):221-32.

Aubert I, Ridet JL, Gage FH (1995) Regeneration in the adult mammalian CNS: guided by development. Curr Opin Neurobiol. 5(5):625-35.

Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K (2004) Chipping away at the chip bias: RNA degradation in microarray analysis. Nat Genet. 35(4):292-3.

Avwenagha O, Campbell G, Bird MM (2003) Distribution of GAP-43, beta-III tubulin and F-actin in developing and regenerating axons and their growth cones in vitro, following neurotrophin treatment. J Neurocytol. 32(9):1077-89

Bandtlow CE (2003) Regeneration in the central nervous system. Exp Gerontol. 38(1-2):79-86.

Bareyre FM, Schwab ME (2003) Inflammation, degeneration and regeneration in the injured spinal cord: insights from DNA microarrays. Trends Neurosci. 26(10):555-63.

Barnard GF, Staniunas RJ, Bao S, Mafune K, Steele GD Jr, Gollan JL, Chen LB. (1992) Increased expression of human ribosomal phosphoprotein P0 messenger RNA in hepatocellular carcinoma and colon carcinoma. Cancer Res. 52(11):3067-72.

Barron KD (2004) The axotomy response. J Neurol Sci. 220(1-2):119-21.

Bastmeyer M, Beckmann M, Schwab ME, Stuermer CA (1991) Growth of regenerating goldfish axons is inhibited by rat oligodendrocytes and CNS myelin but not but not by goldfish optic nerve tract oligodendrocytelike cells and fish CNS myelin. J Neurosci. 11(3):626-40.

Bastrop R, Strehlow B, Jurss K, Sturmbauer C (2000) A new molecular phylogenetic hypothesis for the evolution of freshwater eels. Mol Phylogenet Evol. 14(2):250-8.

Baumgart E, Schad A, Volkl A, Fahimi HD (1997) Detection of mRNAs encoding peroxisomal proteins by non-radioactive in situ hybridization with digoxigenin-labelled cRNAs. Histochem Cell Biol. 108(4-5):371-9.

Becker T, Wullimann MF, Becker CG, Bernhardt RR, Schachner M (1997) Axonal regrowth after spinal cord transection in adult zebrafish. J Comp Neurol. 377(4):577-95.

Becker T, Bernhardt RR, Reinhard E, Wullimann MF, Tongiorgi E, Schachner M (1998) Readiness of zebrafish brain neurons to regenerate a spinal axon correlates with differential expression of specific cell recognition molecules. J Neurosci. 18(15):5789-803.

Becker CG, Lieberoth BC, Morellini F, Feldner J, Becker T, Schachner M (2004) L1.1 is involved in spinal cord regeneration in adult zebrafish. J Neurosci. 24(36):7837-42.

Benani A, Vol C, Heurtaux T, Asensio C, Dauca M, Lapicque F, Netter P, Minn A (2003) Up-regulation of fatty acid metabolizing-enzymes mRNA in rat spinal cord during persistent peripheral local inflammation. Eur J Neurosci. 18(7):1904-14.

Benfey M, Bunger UR, Vidal-Sanz M, Bray GM, Aguayo AJ (1985) Axonal regeneration from GABAergic neurons in the adult rat thalamus. J Neurocytol. 14(2):279-96.

Benowitz LI, Greene LA (1979) Nerve growth factor in the goldfish brain: biological assay studies using pheochromocytoma cells. Brain Res. 162(1):164-8.

Benowitz LI, Shashoua VE (1979) Immunoreactive sites for nerve growth factor (NGF) in the goldfish brain. Brain Res. 172(3):561-5.

Bernhardt R (1989) Axonal pathfinding during the regeneration of the goldfish optic pathway. J Comp Neurol. 284(1):119-34.

Bernhardt RR (1999) Cellular and molecular bases of axonal regeneration in the fish central nervous system. Exp Neurol. 157(2):223-40.

Bhattacharyya A, Watson FL, Bradlee TA, Pomeroy SL, Stiles CD, Segal RA (1997) Trk receptors function as rapid retrograde signal carriers in the adult nervous system. J Neurosci. 17(18):7007-16.

Blackmore M, Letourneau PC (2006) Changes within maturing neurons limit axonal regeneration in the developing spinal cord. J Neurobiol. 66(4):348-60.

Blesch A, Lu P, Tuszynski MH (2002) Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. Brain Res Bull. 57(6):833-8.

Blum R, Konnerth A (2005) Neurotrophin-mediated rapid signaling in the central nervous system: mechanisms and functions. Physiology 20:70-8.

Borday C, Wrobel L, Fortin G, Champagnat J, Thaeron-Antono C, Thoby-Brisson M (2004) Developmental gene control of brainstem function: views from the embryo. Prog Biophys Mol Biol. 84(2-3):89-106

Bormann P, Zumsteg VM, Roth LW, Reinhard E (1998) Target contact regulates GAP-43 and alpha-tubulin mRNA levels in regenerating retinal ganglion cells. J Neurosci Res. 52(4):405-19.

Bosch TJ, Roberts BL (1994) The size and number of neurons descending to the spinal cord in relation to body length in the European eel (*Anguilla anguilla*). Brain Behav Evol. 44(1):50-60.

Bosch TJ, Roberts BL (2001) The relationships of brain stem systems to their targets in the spinal cord of the eel, *Anguilla anguilla.* Brain Behav Evol. 57(2):106-16.

Boularand S, Biguet NF, Vidal B, Veron M, Mallet J, Vincent JD, Dufour S, Vernier P (1998) Tyrosine hydroxylase in the european eel (Anguilla anguilla): cDNA cloning, brain distribution, and phylogenetic analysis. J Neurochem. 71(2):460-70.

Boyd JG, Gordon T (2001) The neurotrophin receptors, trkB and p75, differentially regulate motor axonal regeneration. J Neurobiol. 49(4):314-25.

Boyd JG, Gordon T (2002) A dose-dependent facilitation and inhibition of peripheral nerve regeneration by brain-derived neurotrophic factor. Eur J Neurosci. 15(4):613-26.

Bozzi Y, Pizzorusso T, Cremisi F, Rossi FM, Barsacchi G, Maffei L (1995) Monocular deprivation decreases the expression of messenger RNA for brain-derived neurotrophic factor in the rat visual cortex. Neuroscience. 69(4):1133-44.

Brady R, Zaidi SI, Mayer C, Katz DM (1999) BDNF is a target-derived survival factor for arterial baroreceptor and chemoafferent primary sensory neurons. J Neurosci. 19(6):2131-42.

Braekevelt CR (1988) Retinal fine structure in the European eel Anguilla anguilla. VII. Pigment epithelium of the sexually mature silver eel stage. Anat Anz. 166(1-5):33-41.

Braford MR Jr, Northcutt RG (1974) Olfactory bulb projections in the bichir, Polypterus. J Comp Neurol. 156(2):165-78.

Braford MR Jr (1995) Comparative aspects of forebrain organization in the ray-finned fishes: touchstones or not? Brain Behav Evol. 46(4-5):259-74.

Braissant O, Wahli W (1998) A simplified In Situ Hybridization protocol using non-radioactively labelled probes to detect abundant and rare mRNAs on tissue sections. Biochemica. 1:10-16.

Bramham CR, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol. 76(2):99-125.

Bregman BS, McAtee M, Dai HN, Kuhn PL (1997) Neurotrophic factors increase axonal growth after spinal cord injury and transplantation in the adult rat. Exp. Neurol. 148:475-494

Bregman BS (1998) Regeneration in the spinal cord. Curr Opin Neurobiol. 8(6):800-7.

Breitschopf H, Suchanek G, Gould RM, Colman DR, Lassmann H (1992) In situ hybridization with digoxigenin-labeled probes: sensitive and reliable detection method applied to myelinating rat brain. Acta Neuropathol (Berl). 84(6):581-7.

Briscini L, Corradini L, Ongini E, Bertorelli R (2002) Up-regulation of ORL-1 receptors in spinal tissue of allodynic rats after sciatic nerve injury. Eur J Pharmacol. 447(1):59-65

Broglio C, Gomez A, Duran E, Ocana FM, Jimenez-Moya F, Rodriguez F, Salas C (2005) Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Res Bull. 66(4-6):277-81.

Brown C (1998) In situ hybridization with riboprobes: an overview for veterinary pathologists. Vet Pathol. 35(3):159-67.

Brown A, Ricci MJ, Weaver LC (2004) NGF message and protein distribution in the injured rat spinal cord. Exp Neurol. 188(1):115-27.

Buck CR, Seburn KL, Cope TC (2000) Neurotrophin expression by spinal motoneurons in adult and developing rats. J Comp Neurol. 416(3):309-18.

Bullock TH, Moore JK, Fields RD (1984) Evolution of myelin sheaths: both lamprey and hagfish lack myelin. Neurosci Lett. 48(2):145-8.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol.25(2):169-93.

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol. 29(1):23-39.

Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech. 15(3):155-66.

Butler AB, Hodos W (1996) Comparative Vertebrate Neuroanatomy: Evolution and Adaptation. Wiley-Liss.

Cafferty WB, Gardiner NJ, Das P, Qiu J, McMahon SB, Thompson SW (2004) Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. J Neurosci. 24(18):4432-43.

Caleo M, Menna E, Chierzi S, Cenni MC, Maffei L (2000) Brain-derived neurotrophic factor is an anterograde survival factor in the rat visual system. Curr Biol. 10(19):1155-61.

Calvo EL, Boucher C, Coulombe Z, Morisset J (1997) Pancreatic GAPDH gene expression during ontogeny and acute pancreatitis induced by caerulein. Biochem Biophys Res Commun. 235(3):636-40.

Caminos E, Becker E, Martin-Zanca D, Vecino E (1999) Neurotrophins and their receptors in the tench retina during optic nerve regeneration. J Comp Neurol. 404(3):321-31.

Caroni P (1997) Intrinsic neuronal determinants that promote axonal sprouting and elongation. Bioessays. 19(9):767-75.

Castrén E, Zafra F, Thoenen H, Lindholm D (1992) Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proc Natl Acad Sci U S A. 89(20):9444-8.

Castrén E, Thoenen H, Lindholm D (1995) Brain-derived neurotrophic factor messenger RNA is expressed in the septum, hypothalamus and in adrenergic brain stem nuclei of adult rat brain and is increased by osmotic stimulation in the paraventricular nucleus. Neuroscience. 64(1):71-80.

Ceccatelli S, Ernfors P, Villar MJ, Persson H, Hokfelt T (1991) Expanded distribution of mRNA for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the rat brain after colchicine treatment. Proc Natl Acad Sci U S A. 88(22):10352-6.

Chan JR, Cosgaya JM, Wu YJ, Shooter EM (2001) Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci U S A. 98(25):14661-8.

Chao MV, Bothwell M (2002) Neurotrophins: to cleave or not to cleave. Neuron. 33(1):9-12.

Chelly J, Concordet JP, Kaplan JC, Kahn A (1989) Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci U S A. 86(8):2617-21.

Chen DF, Jhaveri S, Schneider GE (1995) Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. Proc Natl Acad Sci U S A. 92(16):7287-91.

Chen L, Segal DM, Mash DC (1999) Semi-quantitative reverse-transcriptase polymerase chain reaction: an approach for the measurement of target gene expression in human brain. Brain Res Brain Res Protoc. 4(2):132-9.

Chen R, Li W, Lin H (2005) cDNA cloning and mRNA expression of neuropeptide Y in orange spotted grouper, *Epinephelus coioides*. Comp Biochem Physiol B Biochem Mol Biol. 142(1):79-89.

Cheng L, Sapieha P, Kittlerova P, Hauswirth WW, Di Polo A (2002) TrkB gene transfer protects retinal ganglion cells from axotomy-induced death in vivo. J Neurosci. 22(10):3977-86.

Chidlow G, Casson R, Sobrado-Calvo P, Vidal-Sanz M, Osborne NN (2005) Measurement of retinal injury in the rat after optic nerve transection: an RT-PCR study. Mol Vis. 11:387-96.

Choquer M, Boccara M, Vidal-Cros A (2003) A semi-quantitative RT-PCR method to readily compare expression levels within Botrytis cinerea multigenic families in vitro and in planta. Curr Genet. 43(4):303-9.

Chuckowree JA, Dickson TC, Vickers JC (2004) Intrinsic regenerative ability of mature CNS neurons. Neuroscientist. 10(4):280-5.

Coggeshall RE, Birse SG, Youngblood CS (1982) Recovery from spinal transection in fish. Neurosci Lett. 32(3):259-64.

Cohen J (1988) Statistcal power analysis for the behavioural sciences. Hillsdale NJ (Ed). Lawrence Erlbaum Associates.

Cohen AH, Mackler SA, Selzer ME (1988) Behavioural recovery following spinal transection: functional regeneration in the lamprey CNS. Trends Neurosci. 11(5):227-31.

Cohen-Cory S, Dreyfus CF, Black IB (1991) NGF and excitatory neurotransmitters regulate survival and morphogenesis of cultured cerebellar Purkinje cells. J Neurosci. 11(2):462-71.

Cohen-Cory S, Fraser SE (1995) Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. Nature. 378(6553):192-6.

Cohen-Cory S, Lom B (2004) Neurotrophic regulation of retinal ganglion cell synaptic connectivity: from axons and dendrites to synapses. Int J Dev Biol. 48(8-9):947-56.

Colwill RM, Raymond MP, Ferreira L, Escudero H (2005) Visual discrimination learning in zebrafish (*Danio rerio*).Behav Processes. 70(1):19-31.

Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci. 17(7):2295-313.

Connor B, Dragunow M (1998) The role of neuronal growth factors in neurodegenerative disorders of the human brain. Brain Res Brain Res Rev. 27(1):1-39.

Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, McClain J, Pan L, Helgren M, Ip NY, Boland P, et al (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature. 375(6528):235-8.

Cox KH, DeLeon DV, Angerer LM, Angerer RC (1984) Detection of mrnas in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Dev Biol. 101(2):485-502.

Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS (1997) Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nat Med. 3(1):73-6.

Cui Q, Tang LS, Hu B, So KF, Yip HK (2002) Expression of trkA, trkB, and trkC in injured and regenerating retinal ganglion cells of adult rats. Invest Ophthalmol Vis Sci. 43(6):1954-64.

Cumming R, Reaves TA Jr, Hayward JN (1982) Ultrastructural immunocytochemical characterization of isotocin, vasotocin and neurophysin neurons in the magnocellular preoptic nucleus of the goldfish. Cell Tissue Res. 223(3):685-94.

Dale N (1985) Reciprocal inhibitory interneurones in the Xenopus embryo spinal cord. J Physiol. 363:61-70.

Dale N, Roberts A (1985) Dual-component amino-acid-mediated synaptic potentials: excitatory drive for swimming in Xenopus embryos. J Physiol. 363:35-59.

Danik M, Cassoly E, Manseau F, Sotty F, Mouginot D, Williams S (2005) Frequent coexpression of the vesicular glutamate transporter 1 and 2 genes, as well as coexpression with genes for choline acetyltransferase or glutamic acid decarboxylase in neurons of rat brain. J Neurosci Res. 81(4):506-21.

Danzer SC, Crooks KR, Lo DC, McNamara JO (2002) Increased expression of brain-derived neurotrophic factor induces formation of basal dendrites and axonal branching in dentate granule cells in hippocampal explant cultures. J Neurosci. 22(22):9754-63.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science. 214(4523):931-3.

David R, Wedlich D (2001) PCR-based RNA probes: a quick and sensitive method to improve whole mount embryo in situ hybridizations. Biotechniques. 30(4):769-72, 74.

Davies AM, Wright EM (1995) Neurotrophic factors. Neurotrophin autocrine loops. Curr Biol. 5(7):723-6.

de Cremoux P, Bieche I, Tran-Perennou C, Vignaud S, Boudou E, Asselain B, Lidereau R, Magdelenat H, Becette V, Sigal-Zafrani B, Spyratos F (2004) Inter-laboratory quality control for hormone-dependent gene expression in human breast tumors using real-time reverse transcription-polymerase chain reaction. Endocr Relat Cancer. 11(3):489-95.

Degani G, Goldberg D, Tzchori I, Hurvitz A, Yom Din S, Jackson K (2003) Cloning of European eel (*Anguilla anguilla*) FSH-beta subunit, and expression of FSH-beta and LH-beta in males and females after sex determination. Comp Biochem Physiol B Biochem Mol Biol. 136(2):283-93.

De Girolamo P, Lucini C, Vega JA, Andreozzi G, Coppola L, Castaldo L (1999) Co-localization of Trk neurotrophin receptors and regulatory peptides in the endocrine cells of the teleostean stomach. Anat Rec. 256(3):219-26.

Deguchi T, Suwa H, Yoshimoto M, Kondoh H, Yamamoto N (2005) Central connection of the optic, oculomotor, trochlear and abducens nerves in medaka, Oryzias latipes. Zoolog Sci. 22(3):321-32.

Deliagina TG, Zelenin PV, Fagerstedt P, Grillner S, Orlovsky GN (2000) Activity of reticulospinal neurons during locomotion in the freely behaving lamprey. J Neurophysiol. 83(2):853-63.

Deliagina TG, Zelenin PV, Orlovsky GN (2002) Encoding and decoding of reticulospinal commands. Brain Res Brain Res Rev. 40(1-3):166-77.

Del Signore A, De Sanctis V, Di Mauro E, Negri R, Perrone-Capano C, Paggi P (2006) Gene expression pathways induced by axotomy and decentralization of rat superior cervical ganglion neurons. Eur J Neurosci. 23(1):65-74.

Delvolvé I, Branchereau P, Dubuc R, Cabelguen JM (1999) Fictive rhythmic motor patterns induced by NMDA in an in vitro brain stem-spinal cord preparation from an adult urodele. J Neurophysiol. 82(2):1074-7.

Dervan AG, Roberts BL (2003a) Reaction of spinal cord central canal cells to cord transection and their contribution to cord regeneration. J Comp Neurol. 458(3):293-306.

Dervan AG, Roberts BL (2003b) The meningeal sheath of the regenerating spinal cord of the eel, Anguilla. Anat Embryol (Berl). 207(2):157-67.

Dethleffsen K, Heinrich G, Lauth M, Knapik EW, Meyer M (2003) Insert-containing neurotrophins in teleost fish and their relationship to nerve growth factor. Mol Cell Neurosci. 24(2):380-94.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques. 37(1):112-4, 116, 118-9.

Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A (2005) The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem. 344(1):141-3.

Diekmann H, Klinger M, Oertle T, Heinz D, Pogoda HM, Schwab ME, Stuermer CA (2005) Analysis of the reticulon gene family demonstrates the absence of the neurite growth inhibitor Nogo-A in fish. Mol Biol Evol. 22(8):1635-48.

Dougherty KD, Dreyfus CF, Black IB (2000) Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiol Dis. 7(6 Pt B):574-85.

Doyle LMF, Stafford PP, Roberts BL (2001) Recovery of locomotion correlated with axonal regeneration after a complete spinal transection in the eel. Neuroscience 107 (1):169-179.

Doyle LM (2002) The impact of neuronal activity manipulations on recovery after complete spinal transaction in the eel. PhD thesis, University of Dublin.

Doyle LM, Roberts BL (2004a) Functional recovery and axonal growth following spinal cord transection is accelerated by sustained L-DOPA administration. Eur J Neurosci. 20(8):2008-14.

Doyle LM, Roberts BL (2004b) NMDA receptor blockade retards axonal growth in the transected spinal cord. Neuroreport. 15(15):2361-4.

Doyle LM, Roberts BL (2006) Exercise enhances axonal growth and functional recovery in the regenerating spinal cord. Neuroscience. 141(1):321-7.

Drew T, Prentice S, Schepens B (2004) Cortical and brainstem control of locomotion. Prog Brain Res. 143:251-61.

Dreyfus CF, Dai X, Lercher LD, Racey BR, Friedman WJ, Black IB (1999) Expression of neurotrophins in the adult spinal cord in vivo. J Neurosci Res. 56(1):1-7.

Duarte G, Segura-Noguera MM, Martin del Rio MP, Mancera JM (2001) The hypothalamo-hypophyseal system of the white seabream Diplodus sargus: immunocytochemical identification of arginine-vasotocin, isotocin, melanin-concentrating hormone and corticotropin-releasing factor. Histochem J. 33(9-10):569-78.

Duprey-Diaz MV, Soto I, Blagburn JM, Blanco RE (2002) Changes in brain-derived neurotrophic factor and trkB receptor in the adult *Rana pipiens* retina and optic tectum after optic nerve injury. J Comp Neurol. 454(4):456-69.

Duprey-Diaz MV, Blagburn JM, Blanco RE (2003) Neurotrophin-3 and TrkC in the frog visual system: changes after axotomy. Brain Res. 982(1):54-63.

Dytham C (2003) Choosing and using statistics: a biologist's guide. Blackwell Publishing.

Eberhardt KA, Irintchev A, Al-Majed AA, Simova O, Brushart TM, Gordon T, Schachner M (2006) BDNF/TrkB signaling regulates HNK-1 carbohydrate expression in regenerating motor nerves and promotes functional recovery after peripheral nerve repair. Exp Neurol. 198(2):500-10.

Edling AE, Nanavati T, Johnson JM, Tuohy VK (2004) Human and murine lymphocyte neurotrophin expression is confined to B cells. J Neurosci Res. 77(5):709-17.

Egginton S (1987) Metamorphosis of the American eel, *Anguilla rostrata* LeSeur: III. Contractile characteristics of skeletal muscle. J Exp Zool. 243(1):39-50.

Ekström P, Ostholm T, Meissl H, Bruun A, Richards JG, Mohler H (1990) Neural elements in the pineal complex of the frog, Rana esculenta, II: GABA-immunoreactive neurons and FMRFamide-immunoreactive efferent axons. Vis Neurosci. 4(5):399-412.

Ellerby DJ, Spierts IL, Altringham JD (2001) Slow muscle power output of yellow- and silver-phase European eels (*Anguilla anguilla L.*): changes in muscle performance prior to migration. J Exp Biol. 204(Pt 7):1369-79.

Fariñas I, Cano-Jaimez M, Bellmunt E, Soriano M (2002) Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res Bull. 57(6):809-16.

Fawcett JW (1992) Intrinsic neuronal determinants of regeneration. Trends Neurosci. 15(1):5-8.

Fawcett JW, Geller HM (1998) Regeneration in the CNS: optimism mounts. Trends Neurosci. 21(5):179-80.

Fawcett J (2002) Repair of spinal cord injuries: where are we, where are we going? Spinal Cord. 40(12):615-23.

Fernandez E, Pallini R, Lauretti L, Mercanti D, Serra A, Calissano P (1993) Spinal cord transection in adult rats: effects of local infusion of nerve growth factor on the corticospinal tract axons. Neurosurgery. 33(5):889-93.

Fernandez-Santos JM, Martin-Lacave I (2000) Detection of different mRnas expressed in the thyro-parathyroid complex of the rat by in situ hybridization using digoxigenin-labelled oligonucleotide probes. Histochem J. 32(4):217-22.

Ferraro GB, Alabed YZ, Fournier AE (2004) Molecular targets to promote central nervous system regeneration. Curr Neurovasc Res. 1(1):61-75.

Ferretti P, Zhang F, O'Neill P (2003) Changes in spinal cord regenerative ability through phylogenesis and development: lessons to be learnt. Dev Dyn. 226(2):245-56.

Festing MF, Overend P, Gaines Das R, Cortina Borja M, Berdoy M (2002) The design of animal experiments: reducing the use of animals in experiments through better experimental design. The Royal Society of Medicine Press Limited.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature. 381(6584):706-9.

Folgueira M, Anadon R, Yanez J (2004a) An experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). I: Olfactory bulb and ventral area. J Comp Neurol. 480(2):180-203.

Folgueira M, Anadon R, Yanez J (2004b) Experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). II: Dorsal area and preoptic region. J Comp Neurol. 480(2):204-33.

Folgueira M, Anadon R, Yanez J (2005) Experimental study of the connections of the preglomerular nuclei and corpus mamillare in the rainbow trout, Oncorhynchusmykiss. Brain Res Bull. 66(4-6):361-4.

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. Biotechniques. 26(1):112-22, 124-5.

Friedman WJ, Olson L, Persson H (1991) Cells that Express Brain-Derived Neurotrophic Factor mRNA in the Developing Postnatal Rat Brain. Eur J Neurosci. 3(7):688-697.

Friedman WJ, Ibanez CF, Hallbook F, Persson H, Cain LD, Dreyfus CF, Black IB (1993) Differential actions of neurotrophins in the locus coeruleus and basal forebrain. Exp Neurol. 119(1):72-8.

Friedman WJ, Black IB, Kaplan DR (1998) Distribution of the neurotrophins brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 in the postnatal rat brain: an immunocytochemical study. Neuroscience. 84(1):101-14.

Frisen J, Verge VM, Cullheim S, Persson H, Fried K, Middlemas DS, Hunter T, Hokfelt T, Risling M (1992) Increased levels of trkB mRNA and trkB protein-like immunoreactivity in the injured rat and cat spinal cord. Proc Natl Acad Sci U S A. 89(23):11282-6.

Frost DO, Ma YT, Hsieh T, Forbes ME, Johnson JE (2001) Developmental changes in BDNF protein levels in the hamster retina and superior colliculus. J Neurobiol. 49(3):173-87.

Fryer RH, Kaplan DR, Kromer LF (1997) Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro. Exp Neurol. 148(2):616-27.

Funakoshi H, Frisen J, Barbany G, Timmusk T, Zachrisson O, Verge VM, Persson H (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol. 123(2):455-65.

Furukawa S, Sugihara Y, Iwasaki F, Fukumitsu H, Nitta A, Nomoto H, Furukawa Y (1998) Brain-derived neurotrophic factor-like immunoreactivity in the adult rat central nervous system predominantly distributed in neurons with substantial amounts of brain-derived neurotrophic factor messenger RNA or responsiveness to brain-derived neurotrophic factor. Neuroscience. 82(3):653-70.

Gahtan E, Sankrithi N, Campos JB, O'Malley DM (2002) Evidence for a widespread brain stem escape network in larval zebrafish. J Neurophysiol. 87(1):608-14.

Galter D, Unsicker K (2000) Brain-derived neurotrophic factor and trkB are essential for cAMP-mediated induction of the serotonergic neuronal phenotype. J Neurosci Res. 61(3):295-301.

Gandrillon O, Solari F, Legrand C, Jurdic P, Samarut J (1996) A rapid and convenient method to prepare DIG-labelled RNA probes for use in non-radioactive in situ hybridization. Mol Cell Probes. 10(1):51-5.

Gao H, Qiao X, Hefti F, Hollyfield JG, Knusel B (1997) Elevated mRNA expression of brain-derived neurotrophic factor in retinal ganglion cell layer after optic nerve injury. Invest Ophthalmol Vis Sci. 38(9):1840-7.

Germana A, Catania S, Cavallaro M, Gonzalez-Martinez T, Ciriaco E, Hannestad J, Vega JA (2002) Immunohistochemical localization of BDNF-, TrkB- and TrkA-like proteins in the teleost lateral line system. Anat. 200(5):477-85.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263(5153):1618-23.

Giehl KM, Tetzlaff W (1996) BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. Eur J Neurosci. 8(6):1167-75.

Giehl KM, Rohrig S, Bonatz H, Gutjahr M, Leiner B, Bartke I, Yan Q, Reichardt LF, Backus C, Welcher AA, Dethleffsen K, Mestres P, Meyer M (2001) Endogenous brain-derived neurotrophic factor and neurotrophin-3 antagonistically regulate survival of axotomized corticospinal neurons in vivo. J Neurosci. 21(10):3492-502.

Gillespie LN (2003) Regulation of axonal growth and guidance by the neurotrophin family of neurotrophic factors. Clin Exp Pharmacol Physiol. 30(10):724-33.

Ginty DD, Segal RA (2002) Retrograde neurotrophin signaling: Trk-ing along the axon. Curr Opin Neurobiol. 12(3):268-74.

Giordano S, Laessing U, Ankerhold R, Lottspeich F, Stuermer CA (1997) Molecular characterization of E587 antigen: an axonal recognition molecule expressed in the goldfish central nervous system. J Comp Neurol. 377(2):286-97.

Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods. 25(4):386-401.

Givalois L, Naert G, Tapia-Arancibia L, Arancibia S (2006) Involvement of brain-derived neurotrophic factor in the regulation of hypothalamic somatostatin in vivo. J Endocrinol. 188(3):425-33.

Gomez-Pinilla F, Ying Z, Roy RR, Molteni R, Edgerton VR (2002) Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. J Neurophysiol. 88(5):2187-95.

Gooney M, Shaw K, Kelly Á, O'Mara S M, Lynch MA (2002) Long-term potentiation and spatial learning are associated with increased phophorylation of TrkB and extracellular signal-regulated kinase (ERK) in the dentate gyrus: Evidence for a role for brain derived neurotrophic factor. Behavioural Neuroscience 116(3):455-463

Gorzelniak K, Janke J, Engeli S, Sharma AM (2001) Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. Horm Metab Res. 33(10):625-7.

Goto T, Hoshino Y (2001) Electrophysiological, histological, and behavioral studies in a cat with acute compression of the spinal cord. J Orthop Sci. 6(1):59-67.

Götz R, Raulf F, Schartl M (1992) Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. J Neurochem. 59(2):432-42.

Griesbach GS, Hovda DA, Molteni R, Wu A, Gomez-Pinilla F (2004) Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function. Neuroscience125(1):129-39.

Grillner S, McClellan A, Perret C (1981) Entrainment of the spinal pattern generators for swimming by mechano-sensitive elements in the lamprey spinal cord in vitro. Brain Res. 217(2):380-6.

Grillner S, Wallen P (1985) Central pattern generators for locomotion, with special reference to vertebrates. Annu Rev Neurosci. 8:233-61.

Grillner S, Deliagina T, Ekeberg O, el Manira A, Hill RH, Lansner A, Orlovsky GN, Wallen P (1995) Neural networks that co-ordinate locomotion and body orientation in lamprey. Trends Neurosci. 18(6):270-9.

Grosse G, Djalali S, Deng DR, Holtje M, Hinz B, Schwartzkopff K, Cygon M, Rothe T, Stroh T, Hellweg R, Ahnert-Hilger G, Hortnag H (2005) Area-specific effects of brain-derived neurotrophic factor (BDNF) genetic ablation on various neuronal subtypes of the mouse brain. Brain Res Dev Brain Res. 156(2):111-26.

Grover BG, Sharma SC (1981) Organization of extrinsic tectal connections in Goldfish (*Caraccius auratus*). J Comp Neurol. 196(3):471-88.

Grundschober C, Delaunay F, Puhlhofer A, Triqueneaux G, Laudet V, Bartfai T, Nef P (2001) Circadian regulation of diverse gene products revealed by mRNA expression profiling of synchronized fibroblasts. J Biol Chem. 276(50):46751-8.

Guertin P, Dubuc R (1997) Effects of stimulating the reticular formation during fictive locomotion in lampreys. Brain Res. 753(2):328-34.

Guiot Y, Rahier J (1995) The effects of varying key steps in the non-radioactive in situ hybridization protocol: a quantitative study. Histochem J. 27(1):60-8.

Guthrie SDM (1990) The physiology of the teleostean optic tectum. In: Douglas RH, Djamgoz MBA (Eds) The Visual System of Fish, Chapman and Hall, London, pp.279-344.

Haenisch C, Diekmann H, Klinger M, Gennarini G, Kuwada JY, Stuermer CA (2005) The neuronal growth and regeneration associated Cntn1 (F3/F11/Contactin) gene is duplicated in fish: expression during development and retinal axon regeneration. Mol Cell Neurosci. 28(2):361-74.

Hafer-Macko CE, Yu S, Ryan AS, Ivey FM, Macko RF (2005) Elevated tumor necrosis factor-alpha in skeletal muscle after stroke. Stroke. 36(9):2021-3.

Hafidi A (1999) Distribution of BDNF, NT-3 and NT-4 in the developing auditory brainstem. Int J Dev Neurosci. 17(4):285-94.

Hager G, Eckert E, Schwaiger FW (1999) Semiquantitative analysis of low levels of mRNA expression from small amounts of brain tissue by nonradioactive reverse transcriptase-polymerase chain reaction. J Neurosci Methods. 89(2):141-9.

Hall GF, Yao J (2000) Neuronal morphology, axonal integrity, and axonal regeneration in situ are regulated by cytoskeletal phosphorylation in identified lamprey central neurons. Microsc Res Tech. 1;48(1):32-46

Hallböök F, Ibanez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. Neuron. 6(5):845-58.

Hallböök F, Lundin LG, Kullander K (1998) *Lampetra fluviatilis* neurotrophin homolog, descendant of a neurotrophin ancestor, discloses the early molecular evolution of neurotrophins in the vertebrate subphylum. J Neurosci. 18(21):8700-11.

Hallböök F (1999) Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr Opin Neurobiol. 9(5):616-21.

Hammarberg H, Piehl F, Risling M, Cullheim S (2000) Differential regulation of trophic factor receptor mRNAs in spinal motoneurons after sciatic nerve transection and ventral root avulsion in the rat. J Comp Neurol. 426(4):587-601.

Hanna GF, Nawar NN, Sharma SC (1998) Regeneration of ascending spinal axons in goldfish. Brain Res. 791(1-2):235-45.

Hannestad J, Marino F, Germana A, Catania S, Abbate F, Ciriaco E, Vega JA (2000) Trk neurotrophin receptor-like proteins in the teleost *Dicentrarchus labrax*. Cell Tissue Res. 300(1):1-9.

Hartmann M, Heumann R, Lessmann V (2001) Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J. 20(21):5887-97.

Harvey PJ, Grochmal J, Tetzlaff W, Gordon T, Bennett DJ (2005) An investigation into the potential for activity-dependent regeneration of the rubrospinal tract after spinal cord injury. Eur J Neurosci. 22(12):3025-35.

Hasegawa T, Ohno K, Sano M, Omura T, Omura K, Nagano A, Sato K (2005) The differential expression patterns of messenger RNAs encoding Nogo-A and Nogo-receptor in the rat central nervous system. Brain Res Mol Brain Res. 133(1):119-30.

Hashimoto M, Heinrich G (1997) Brain-derived neurotrophic factor gene expression in the developing zebrafish. Int J Dev Neurosci. 15(8):983-97.

Hashimoto M, Ino H, Koda M, Murakami M, Yoshinaga K, Yamazaki M, Moriya H (2004) Regulation of semaphorin 3A expression in neurons of the rat spinal cord and cerebral cortex after transection injury. Acta Neuropathol (Berl). 107(3):250-6.

Hastings MH (1997) Circadian clocks. Curr Biol. 7(11):R670-2.

He J, Gong H, Luo Q (2005) BDNF acutely modulates synaptic transmission and calcium signalling in developing cortical neurons. Cell Physiol Biochem. 16(1-3):69-76.

Heerssen HM, Segal RA (2002) Location, location, location: a spatial view of neurotrophin signal transduction. Trends Neurosci. 25(3):160-5.

Heinrich G, Lum T (2000) Fish neurotrophins and Trk receptors. Int. J. Dev. Biol. 18:1-27

Henderson CE (1996) Role of neurotrophic factors in neuronal development. Curr Opin Neurobiol. 6(1):64-70.

Herrero L, Rodriguez F, Salas C, Torres B (1998) Tail and eye movements evoked by electrical microstimulation of the optic tectum in goldfish. Exp Brain Res. 120(3):291-305.

Herzog KH, Bailey K, Barde YA (1994) Expression of the BDNF gene in the developing visual system of the chick. Development. 120(6):1643-9.

Herzog KH, von Bartheld CS (1998) Contributions of the optic tectum and the retina as sources of brain-derived neurotrophic factor for retinal ganglion cells in the chick embryo. J Neurosci. 18(8):2891-906.

Heymach JV Jr, Barres BA (1997) Neurotrophins moving forward. Nature 389(6653): 789-791.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W (2002) Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J Neurosci Res. 69(2):160-8.

Higgins DG (1994) CLUSTAL V: multiple alignment of DNA and protein sequences. Methods Mol Biol. 25:307-18.

Hirsch S, Labes M, Bahr M (2000) Changes in BDNF and neurotrophin receptor expression in degenerating and regenerating rat retinal ganglion cells. Restor Neurol Neurosci. 17(2-3):125-134.

Holder N, Clarke JD (1988) Is there a correlation between continuous neurogenesis and directed axon regeneration in the vertebrate nervous system? Trends Neurosci. 11(3):94-9.

Horner PJ, Gage FH (2000) Regenerating the damaged central nervous system. Nature 407:963-70

Howe CL, Mobley WC (2005) Long-distance retrograde neurotrophic signaling. Curr Opin Neurobiol. 15(1):40-8.

Hu B, Nikolakopoulou AM, Cohen-Cory S (2005) BDNF stabilizes synapses and maintains the structural complexity of optic axons in vivo. Development. 132(19):4285-98.

Huang AM, Wang HL, Tang YP, Lee EH (1998) Expression of integrin-associated protein gene associated with memory formation in rats. J Neurosci. 18(11):4305-13.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci. 24:677-736.

Huang AM, Jen CJ, Chen HF, Yu L, Kuo YM, Chen HI (2005) Compulsive exercise acutely upregulates rat hippocampal brain-derived neurotrophic factor. J Neural Transm.

Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6(4):279-84.

Ichisaka S, Katoh-Semba R, Hata Y, Ohshima M, Kameyama K, Tsumoto T (2003) Activity-dependent change in the protein level of brain-derived neurotrophic factor but no change in other neurotrophins in the visual cortex of young and adult ferrets. Neuroscience. 117(2):361-71.

Ikeda O, Murakami M, Ino H, Yamazaki M, Nemoto T, Koda M, Nakayama C, Moriya H (2001) Acute up-regulation of brain-derived neurotrophic factor expression resulting from experimentally induced injury in the rat spinal cord.
Acta Neuropathol (Berl). 102(3):239-45.

Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C (2005) Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Res. 33(6):e56.

Inoue A, Sanes JR (1997) Lamina-specific connectivity in the brain: regulation by N-cadherin, neurotrophins, and glycoconjugates. Science. 276(5317):1428-31.

Irie S, Hachiya T, Rabizadeh S, Maruyama W, Mukai J, Li Y, Reed JC, Bredesen DE, Sato TA (1999) Functional interaction of Fas-associated phosphatase-1 (FAP-1) with p75(NTR) and their effect on NF-kappaB activation. FEBS Lett. 460(2):191-8.

Irintchev A, Simova O, Eberhardt KA, Morellini F, Schachner M (2005) Impacts of lesion severity and tyrosine kinase receptor B deficiency on functional outcome of femoral nerve injury assessed by a novel single-frame motion analysis in mice. Eur J Neurosci. 22(4):802-8.

Ito H, Murakami T, Fukuoka T, Kishida R (1986) Thalamic fiber connections in a teleost (Sebastiscus marmoratus): visual somatosensory, octavolateral, and cerebellar relay region to the telencephalon. J Comp Neurol. 250(2):215-27.

Jacobs AJ, Swain GP, Selzer ME (1996) Developmental increases in expression of neurofilament mRNA selectively in projection neurons of the lamprey CNS. J Comp Neurol. 364(3):383-401.

Jacobs AJ, Swain GP, Snedeker JA, Pijak DS, Gladstone LJ, Selzer ME (1997) Recovery of neurofilament expression selectively in regenerating reticulospinal neurons.
J Neurosci. 17(13):5206-20.

Jacobs WB, Fehlings MG (2003) The molecular basis of neural regeneration. Neurosurgery. 53(4):943-48.

Jakeman LB, Reier PJ (1991) Axonal projections between fetal spinal cord transplants and the adult rat spinal cord: a neuroanatomical tracing study of local interactions. J Comp Neurol. 307(2):311-34.

Jin L, Lloyd RV (1997) *In situ* hybridization: methods and applications. J Clin Lab Anal. 11(1):2-9.

Jin Y, Fischer I, Tessler A, Houle JD (2002) Transplants of fibroblasts genetically modified to express BDNF promote axonal regeneration from supraspinal neurons following chronic spinal cord injury. Exp Neurol. 177(1):265-75.

Johnson RA, Okragly AJ, Haak-Frendscho M, Mitchell GS (2000) Cervical dorsal rhizotomy increases brain-derived neurotrophic factor and neurotrophin-3 expression in the ventral spinal cord. J Neurosci. 20(10):RC77.

Jones KR, Farinas I, Backus C, Reichardt LF (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell. 76(6):989-99.

Jones LL, Sajed D, Tuszynski MH (2003) Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition. J Neurosci. 23(28):9276-88.

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat Neurosci. 3(4):323-9.

Kalb R (2005) The protean actions of neurotrophins and their receptors on the life and death of neurons. Trends Neurosci. 28(1):5-11.

Kanazir S, Vukosavic S, Rakic L, Ruzdijic S (1997) Optimization of in situ hybridization histochemistry for localization of GAP-43 mRNA in fetal brain during human development. Brain Res Brain Res Protoc. 1(3):280-6.

Kaneko M, Cahill GM (2005) Light-dependent development of circadian gene expression in transgenic zebrafish. PLoS Biol. 3(2):e34.

Kang H, Welcher AA, Shelton D, Schuman EM (1997) Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron. 19(3):653-64.

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol. 10(3):381-91.

Kapsimali M, Vidal B, Gonzalez A, Dufour S, Vernier P (2000) Distribution of the mRNA encoding the four dopamine D(1) receptor subtypes in the brain of the european eel (Anguilla anguilla): comparative approach to the function of D(1) receptors in vertebrates. J Comp Neurol. 419(3):320-43.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science. 274(5290):1133-8.

Katz DM (2003) Neuronal growth factors and development of respiratory control. Respir Physiol Neurobiol. 135(2-3):155-65.

Kawamoto Y, Nakamura S, Nakano S, Oka N, Akiguchi I, Kimura J (1996) Immunohistochemical localization of brain-derived neurotrophic factor in adult rat brain. Neuroscience. 74(4):1209-26.

Kelly Á, Mullany PM, Lynch MA (2000) Protein synthesis in entorhinal cortex and long-term potentiation in dentate gyrus. Hippocampus 10:431-437

Kernie SG, Liebl DJ, Parada LF (2000) BDNF regulates eating behavior and locomotor activity in mice. EMBO J. 19(6):1290-300.

Kerschensteiner M, Schwab ME, Lichtman JW, Misgeld T (2005) In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nat Med. 11(5):572-7.

Kim JY, Sutton ME, Lu DJ, Cho TA, Goumnerova LC, Goritchenko L, Kaufman JR, Lam KK, Billet AL, Tarbell NJ, Wu J, Allen JC, Stiles CD, Segal RA, Pomeroy SL (1999) Activation of neurotrophin-3 receptor TrkC induces apoptosis in medulloblastomas. Cancer Res. 59(3):711-9.

Kim HJ, Hwang JJ, Behrens MM, Snider BJ, Choi DW, Koh JY (2003) TrkB mediates BDNF-induced potentiation of neuronal necrosis in cortical culture. Neurobiol Dis. 14(1):110-9.

King VR, Bradbury EJ, McMahon SB, Priestley JV (2000) Changes in truncated trkB and p75 receptor expression in the rat spinal cord following spinal cord hemisection and spinal cord hemisection plus neurotrophin treatment. Exp Neurol. 165(2):327-41.

Klapka N, Hermanns S, Straten G, Masanneck C, Duis S, Hamers FP, Muller D, Zuschratter W, Muller HW (2005) Suppression of fibrous scarring in spinal cord injury of rat promotes long-distance regeneration of corticospinal tract axons, rescue of primary motoneurons in somatosensory cortex and significant functional recovery. Eur J Neurosci. 22(12):3047-58.

Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M (1993) Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell. 75(1):113-22.

Kobayashi NR, Bedard AM, Hincke MT, Tetzlaff W (1996) Increased expression of BDNF and trkB mRNA in rat facial motoneurons after axotomy. Eur J Neurosci. 8(5):1018-29.

Kobayashi NR, Fan DP, Giehl KM, Bedard AM, Wiegand SJ, Tetzlaff W (1997) BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talpha1-tubulin mRNA expression, and promote axonal regeneration. J. Neurosci. 17:9583-9595

Kojima M, Klein RL, Hatanaka H (2002) Pre- and post-synaptic modification by neurotrophins. Neurosci Res. 43(3):193-9.

Kokaia Z, Bengzon J, Metsis M, Kokaia M, Persson H, Lindvall O (1993) Coexpression of neurotrophins and their receptors in neurons of the central nervous system. Proc Natl Acad Sci U S A. 90(14):6711-5.

Kokaia Z, Andsberg G, Yan Q, Lindvall O (1998) Rapid alterations of BDNF protein levels in the rat brain after focal ischemia: evidence for increased synthesis and anterograde axonal transport. Exp Neurol. 154(2):289-301.

Komori T, Morikawa Y, Nanjo K, Senba E (2006) Induction of brain-derived neurotrophic factor by leptin in the ventromedial hypothalamus. Neuroscience. 139(3):1107-15.

Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci U S A. 92(19):8856-60.

Krenz NR, Weaver LC (2000) Nerve growth factor in glia and inflammatory cells of the injured rat spinal cord. J Neurochem. 74(2):730-9.

Krowczynska AM, Coutts M, Makrides S, Brawerman G (1989) The mouse homologue of the human acidic ribosomal phosphoprotein PO: a highly conserved polypeptide that is under translational control. Nucleic Acids Res. 17(15):6408.

Kruger S, Sievers J, Hansen C, Sadler M, Berry M (1986) Three morphologically distinct types of interface develop between adult host and fetal brain transplants: implications for scar formation in the adult central nervous system. J Comp Neurol. 249(1):103-16.

Kullander K, Carlson B, Hallbook F (1997) Molecular phylogeny and evolution of the neurotrophins from monotremes and marsupials. J Mol Evol. 45(3):311-21.

Kury P, Abankwa D, Kruse F, Greiner-Petter R, Muller HW (2004) Gene expression profiling reveals multiple novel intrinsic and extrinsic factors associated with axonal regeneration failure. Eur J Neurosci. 19(1):32-42.

Kwon BK, Liu J, Messerer C, Kobayashi NR, McGraw J, Oschipok L, Tetzlaff W (2002) Survival and regeneration of rubrospinal neurons 1 year after spinal cord injury. Proc Natl Acad Sci U S A. 99(5):3246-51.

Lapchak PA, Hefti F (1992) BDNF and NGF treatment in lesioned rats: effects on cholinergic function and weight gain. Neuroreport 3: 405–408.

Lawrence CB, Turnbull AV, Rothwell NJ (1999) Hypothalamic control of feeding. Curr Opin Neurobiol.9(6):778-83.

Lee RK, Eaton RC, Zottoli SJ (1993) Segmental arrangement of reticulospinal neurons in the goldfish hindbrain. J Comp Neurol. 329(4):539-56.

Lee P, Zhuo H, Helke CJ (2001a) Axotomy alters neurotrophin and neurotrophin receptor mRNAs in the vagus nerve and nodose ganglion of the rat. Brain Res Mol Brain Res. 87(1):31-41.

Lee R, Kermani P, Teng KK, Hempstead BL (2001b) Regulation of cell survival by secreted proneurotrophins. Science. 294(5548):1945-8.

Lees MB, Brostoff SW (1984) Proteins of myelin, In: Morell P (Ed) Myelin, Plenum Press, New York.

Lefebvre PP, Malgrange B, Staecker H, Moghadass M, Van de Water TR, Moonen G (1994) Neurotrophins affect survival and neuritogenesis by adult injured auditory neurons in vitro. Neuroreport. 5(8):865-8.

Lessmann V, Heumann R (1998) Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. Neuroscience. 86(2):399-413.

Lessmann V, Gottmann K, Malcangio M (2003) Neurotrophin secretion: current facts and future prospects. Prog Neurobiol. 69(5):341-74.

Levi AD, Dancausse H, Li X, Duncan S, Horkey L, Oliviera M (2002) Peripheral nerve grafts promoting central nervous system regeneration after spinal cord injury in the primate. J Neurosurg. 96(2 Suppl):197-205.

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237(4819):1154-62.

Lewin GR, Barde YA (1996) Physiology of the neurotrophins. Annu Rev Neurosci. 19:289-317

Li YY, Inoue K, Takei Y (2003) Steroidogenic acute regulatory protein in eels: cDNA cloning and effects of ACTH and seawater transfer on its mRNA expression. Zoolog Sci. 20(2):211-9.

Liang FQ, Sohrabji F, Miranda R, Earnest B, Earnest D (1998a) Expression of brain-derived neurotrophic factor and its cognate receptor, TrkB, in the rat suprachiasmatic nucleus. Exp Neurol. 151(2):184-93.

Liang FQ, Walline R, Earnest DJ (1998b) Circadian rhythm of brain-derived neurotrophic factor in the rat suprachiasmatic nucleus. Neurosci Lett. 242(2):89-92.

Liang FQ, Allen G, Earnest D (2000) Role of brain-derived neurotrophic factor in the circadian regulation of the suprachiasmatic pacemaker by light. J Neurosci. 20(8):2978-87.

Lieberoth BC, Becker CG, Becker T (2003) Double labeling of neurons by retrograde axonal tracing and non-radioactive in situ hybridization in the CNS of adult zebrafish. Methods Cell Sci. 25(1-2):65-70.

Liebl DJ, Huang W, Young W, Parada LF (2001) Regulation of Trk receptors following contusion of the rat spinal cord. Exp Neurol. 167(1):15-26.

Lin YS, Poh YP, Tzeng CS (2001) A phylogeny of freshwater eels inferred from mitochondrial genes. Mol Phylogenet Evol. 20(2):252-61.

Liu X, Ernfors P, Wu H, Jaenisch R (1995) Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. Nature. 375(6528):238-41.

Liu Y, Kim D, Himes BT, Chow SY, Schallert T, Murray M, Tessler A, Fischer I (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. J Neurosci. 19(11):4370-87.

Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR (2006a) Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. Brain Res. 1067(1):1-12.

Liu NA, Liu Q, Wawrowsky K, Yang Z, Lin S, Melmed S (2006b) Prolactin receptor signaling mediates the osmotic response of embryonic zebrafish lactotrophs. Mol Endocrinol. 20(4):871-80.

Lom B, Cohen-Cory S (1999) Brain-derived neurotrophic factor differentially regulates retinal ganglion cell dendritic and axonal arborization in vivo. J Neurosci. 19(22):9928-38.

Lom B, Cogen J, Sanchez AL, Vu T, Cohen-Cory S (2002) Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci. 22(17):7639-49.

Lu J, Ashwell KW, Waite P (2000) Advances in secondary spinal cord injury: role of apoptosis. Spine. 25(14):1859-66.

Lu P, Blesch A, Tuszynski MH (2001) Neurotrophism without neurotropism: BDNF promotes survival but not growth of lesioned corticospinal neurons. J Comp Neurol. 436(4):456-70.

Lu B, Pang PT, Woo NH (2005) The yin and yang of neurotrophin action. Nat Rev Neurosci. 6(8):603-14.

Lucini C, Maruccio L, De Girolamo P, Vega JA, Castaldo L (2001) TrkA and TrkB neurotrophin receptor immunoreactivity in the teleost (Scorpaena porcus) endocrine pancreas. Anat Rec. 263(2):113-7.

Lum T, Huynh G, Heinrich G (2001) Brain-derived neurotrophic factor and TrkB tyrosine kinase receptor gene expression in zebrafish embryo and larva. Int J Dev Neurosci. 19(6):569-87.

Mackler SA, Selzer ME (1985) Regeneration of functional synapses between individual recognizable neurons in the lamprey spinal cord. Science. 229(4715):774-6.

Mackler SA, Yin HS, Selzer ME (1986) Determinants of directional specificity in the regeneration of lamprey spinal axons. J Neurosci. 6(6):1814-21.

Mackler SA, Selzer ME (1987) Specificity of synaptic regeneration in the spinal cord of the larval sea lamprey. J Physiol. 388:183-98.

Majdan M, Miller FD (1999) Neuronal life and death decisions functional antagonism between the Trk and p75 neurotrophin receptors. Int J Dev Neurosci. 17(3):153-61.

Malkovska I, Kernie SG, Parada LF (2006) Differential expression of the four untranslated BDNF exons in the adult mouse brain. J Neurosci Res. 83(2):211-21.

Mank JE, Avise JC (2003) Microsatellite variation and differentiation in North Atlantic eels. J Hered. 94(4):310-4.

Mansur NR, Meyer-Siegler K, Wurzer JC, Sirover MA (1993) Cell cycle regulation of the glyceraldehyde-3-phosphate dehydrogenase/uracil DNA glycosylase gene in normal human cells. Nucleic Acids Res. 21(4):993-8.

Marin O, Smeets WJ, Gonzalez A (1997) Basal ganglia organization in amphibians: catecholaminergic innervation of the striatum and the nucleus accumbens. J Comp Neurol 378: 50-69.

Markus A, Patel TD, Snider WD (2002) Neurotrophic factors and axonal growth. Curr Opin Neurobiol. 12(5):523-31.

Martin SC, Marazzi G, Sandell JH, Heinrich G (1995) Five Trk receptors in the zebrafish. Dev Biol. 169(2):745-58.

Mason MR, Lieberman AR, Anderson PN (2003) Corticospinal neurons up-regulate a range of growth-associated genes following intracortical, but not spinal, axotomy. Eur J Neurosci. 18(4):789-802.

Matsukawa T, Arai K, Koriyama Y, Liu Z, Kato S (2004) Axonal regeneration of fish optic nerve after injury. Biol Pharm Bull. 27(4):445-51.

Matsushima T, Grillner S (1990) Intersegmental co-ordination of undulatory movements--a "trailing oscillator" hypothesis. Neuroreport. 1(2):97-100.

Matsuyama K, Mori F, Nakajima K, Drew T, Aoki M, Mori S (2004) Locomotor role of the corticoreticular-reticulospinal-spinal interneuronal system. Prog Brain Res. 143:239-49.

McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. Neuron. 15(4):791-803.

McAllister AK, Katz LC, Lo DC (1999) Neurotrophins and synaptic plasticity. Annu Rev Neurosci. 22:295-318.

McKerracher L, Essagian C, Aguayo AJ (1993) Temporal changes in beta-tubulin and neurofilament mRNA levels after transection of adult rat retinal ganglion cell axons in the optic nerve. J Neurosci. 13(6):2617-26.

McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J Neurosci. 11(11):3398-411.

Medhurst AD, Harrison DC, Read SJ, Campbell CA, Robbins MJ, Pangalos MN (2000) The use of TaqMan RT-PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. J Neurosci Methods. 98(1):9-20.

Medina M, Reperant J, Dufour S, Ward R, Le Belle N, Miceli D (1994) The distribution of GABA-immunoreactive neurons in the brain of the silver eel (Anguilla anguilla L.). Anat Embryol (Berl). 189(1):25-39.

Meek J (1983) Functional anatomy of the tectum mesencephali of the goldfish. An explorative analysis of the functional implications of the laminar structural organization of the tectum. Brain Res. 287(3):247-97.

Meek J (1990) Tectal morphology: connections, neurones and synapses. In: Douglas RH, Djamgoz MBA (Eds) The Visual System of Fish, Chapman and Hall, London, pp.239-278.

Menei P, Montero-Menei C, Whittemore SR, Bunge RP, Bunge MB (1998) Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. Eur J Neurosci. 10(2):607-21.

Menna E, Cenni MC, Naska S, Maffei L (2003) The anterogradely transported BDNF promotes retinal axon remodeling during eye specific segregation within the LGN. Mol Cell Neurosci. 24(4):972-83.

Meredith GE, Roberts BL (1986) The relationship of saccular efferent neurons to the superior olive in the eel, Anguilla anguilla. Neurosci Lett. 68(1):69-72

Meredith GE, Roberts BL (1987) Distribution and morphological characteristics of efferent neurons innervating end organs in the ear and lateral line of the European eel. J Comp Neurol. 265(4):494-506.

Meredith GE, Roberts BL, Maslam S (1987) Distribution of afferent fibers in the brainstem from end organs in the ear and lateral line in the European eel. J Comp Neurol. 265(4):507-20.

Meredith GE (1988) Comparative view of the central organization of afferent and efferent circuitry for the inner ear. Acta Biol Hung. 39(2-3):229-49.

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol. 119(1):45-54.

Michael GJ, Averill S, Shortland PJ, Yan Q, Priestley JV (1999) Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. European Journal of Neuroscience 11:3539-3551

Middlemas DS, Lindberg RA, Hunter T (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol. 11(1):143-53.

Miller FD, Kaplan DR (2001) On Trk for retrograde signaling. Neuron. 32(5):767-70.

Minegishi Y, Aoyama J, Inoue JG, Miya M, Nishida M, Tsukamoto K (2005) Molecular phylogeny and evolution of the freshwater eels genus *Anguilla* based on the whole mitochondrial genome sequences. Mol Phylogenet Evol. 34(1):134-46.

Miranda RC, Sohrabji F, Toran-Allerand CD (1993) Neuronal colocalization of mRNAs for neurotrophins and their receptors in the developing central nervous system suggests a potential for autocrine interactions. Proc Natl Acad Sci U S A. 90(14):6439-43.

Miura T, Ando N, Miura C, Yamauchi K (2002) Comparative studies between in vivo and in vitro spermatogenesis of Japanese eel (Anguilla japonica). Zoolog Sci. 19(3):321-9.

Mladinic M, Wintzer M, Del Bel E, Casseler C, Lazarevic D, Crovella S, Gustincich S, Cattaneo A, Nicholls J (2005) Differential expression of genes at stages when regeneration can and cannot occur after injury to immature mammalian spinal cord. Cell Mol Neurobiol. 25(2):407-26.

Mo X, Yokoyama A, Oshitari T, Negishi H, Dezawa M, Mizota A, Adachi-Usami E (2002) Rescue of axotomized retinal ganglion cells by BDNF gene electroporation in adult rats. Invest Ophthalmol Vis Sci. 43(7):2401-5.

Molist P, Maslam S, Velzing E, Roberts BL (1993) The organisation of cholinergic neurons in the mesencephalon of the eel, *Anguilla anguilla,* as determined by choline acetyltransferase immunohistochemistry and acetylcholinesterase enzyme histochemistry. Cell Tissue Res. 271:555-566.

Molteni R, Zheng JQ, Ying Z, Gomez-Pinilla F, Twiss JL (2004) Voluntary exercise increases axonal regeneration from sensory neurons. Proc Natl Acad Sci U S A. 101(22):8473-8.

Montero M, Vidal B, King JA, Tramu G, Vandesande F, Dufour S, Kah O (1994) Immunocytochemical localization of mammalian GnRH (gonadotropin-releasing hormone) and chicken GnRH-II in the brain of the European silver eel (Anguilla anguilla L.). J Chem Neuroanat. 7(4):227-41.

Morita Y, Murakami T, Ito H (1983) Cytoarchitecture and topographic projections of the gustatory centers in a teleost, Carassius carassius. J Comp Neurol. 218(4):378-94.

Morrison ME, Mason CA (1998) Granule neuron regulation of Purkinje cell development: striking a balance between neurotrophin and glutamate signaling. J Neurosci. 18(10):3563-73.

Mufson EJ, Kroin JS, Sendera TJ, Sobreviela T (1999) Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative diseases. Prog Neurobiol. 57(4):451-84.

Mukuda T, Ando M (2003) Brain Atlas of the Japanese Eel: Comparison to Other Fishes. Mem. Fac. Integrated Arts and Sci., Hiroshima Univ. Ser. IV, 29 1-25.

Muller T, Varadi B, Horn P, Bercsenyi M (2003) Effects of various hormones on the sexual maturity of European eel (*Anguilla anguilla* L.) females from farm and lakes. Acta Biol Hung. 54(3-4):313-22.

Muragaki Y, Chou TT, Kaplan DR, Trojanowski JQ, Lee VM (1997) Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express TrkA receptors. J Neurosci. 17(2):530-42.

Murer MG, Boissiere F, Yan Q, Hunot S, Villares J, Faucheux B, Agid Y, Hirsch E, Raisman-Vozari R (1999) An immunohistochemical study of the distribution of brain-derived neurotrophic factor in the adult human brain, with particular reference to Alzheimer's disease. Neuroscience. 88(4):1015-32.

Murer MG, Yan Q, Raisman-Vozari R (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. Prog Neurobiol. 63(1):71-124.

Murphy K, Borich SM, Boyce, A, Doyle L, Roberts BL (2000) Immuno-histochemical detection of the number and distribution of GAP-43 positive neurons in the brain of the spinal-transected European eel, *Anguilla anguilla*. Experimental Neurology 163 (1), 292.

Murphy P, Kolsto A (2000) Expression of the bZIP transcription factor TCF11 and its potential dimerization partners during development. Mech Dev. 97(1-2):141-8.

Naert G, Ixart G, Tapia-Arancibia L, Givalois L (2006) Continuous i.c.v. infusion of brain-derived neurotrophic factor modifies hypothalamic-pituitary-adrenal axis activity, locomotor activity and body temperature rhythms in adult male rats. Neuroscience. 139(2):779-89.

Nakamura M, Bregman BS (2001) Differences in neurotrophic factor gene expression profiles between neonate and adult rat spinal cord after injury. Experimental Neurology 169(2):407-15.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman CW (1996) Physical activity increases mRNA for brain-derived neurotrophic factor and nerve growth factor in rat brain. Brain Res. 726(1-2):49-56.

Neumann S, Skinner K, Basbaum AI (2005) Sustaining intrinsic growth capacity of adult neurons promotes spinal cord regeneration. Proc Natl Acad Sci U S A. 102(46):16848-52.

Neumann S, Woolf CJ (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron. 23(1):83-91.

Nicholls J, Saunders N (1996) Regeneration of immature mammalian spinal cord after injury. Trends Neurosci. 19(6):229-34.

Nieuwenhuys R, Pouwels E (1983) The brain of actinopterygian fishes. In: Northcutt RG and Davis RE (Eds), Fish Neurobiology, Ann Arbor: University of Michigan Press, pp. 25-88.

Nieuwenhuys R, Meek J (1990) The telencephalon of sarcopterygian fishes. In: Jones E, Peters A (Eds). Cerebral Cortex, Plenum Press New York, pp.75-104.

Noga BR, Kettler J, Jordan LM (1988) Locomotion produced in mesencephalic cats by injections of putative transmitter substances and antagonists into the medial reticular formation and the pontomedullary locomotor strip. J Neurosci. 8(6):2074-86.

Nona, SN (1998) Repair in goldfish central nervous system. Restorative neurology and neurscience 12:1-11.

Northcutt RG, Braford MR Jr (1980) New observations on the organization and evolution of the telencephalon of actinopterygian fishes. In: Ebbesson SOE (Ed). Comparative neurology of the telencephalon. Plenum, New York, pp.41-98.

Northcutt RG (2006) Connections of the lateral and medial divisions of the goldfish telencephalic pallium. J Comp Neurol. 494(6):903-43.

Northmore DP (1991) Visual responses of nucleus isthmi in a teleost fish (Lepomis macrochirus). Vision Res. 31(3):525-35.

Notterpek L (2003) Neurotrophins in myelination: a new role for a puzzling receptor. Trends Neurosci. 26(5):232-4.

Novikova LN, Novikov LN, Kellerth JO (2002) Differential effects of neurotrophins on neuronal survival and axonal regeneration after spinal cord injury in adult rats. J Comp Neurol. 452(3):255-63.

Nykjaer A, Willnow TE, Petersen CM (2005) p75NTR--live or let die. Curr Opin Neurobiol. 15(1):49-57.

Obata K, Yamanaka H, Kobayashi K, Dai Y, Mizushima T, Katsura H, Fukuoka T, Tokunaga A, Noguchi K (2006) The effect of site and type of nerve injury on the expression of brain-derived neurotrophic factor in the dorsal root ganglion and on neuropathic pain behavior. Neuroscience. 137(3):961-70.

O'Brien E (2004) A degenerate PCR-based approach to cloning Trk genes of the European eel, *Anguilla anguilla.* Moderatorship thesis, University of Dublin.

Ohara O, Gahara Y, Teraoka H, Kitamura T (1992) A rat brain-derived neurotrophic factor-encoding gene generates multiple transcripts through alternative use of 5' exons and polyadenylation sites. Gene. 121(2):383-6.

Okubo K, Suetake H, Aida K (2002) Three mRNA species for mammalian-type gonadotropin-releasing hormone in the brain of the eel *Anguilla japonica.*
Mol Cell Endocrinol. 192(1-2):17-25.

Oliff HS, Berchtold NC, Isackson P, Cotman CW (1998) Exercise-induced regulation of brain-derived neurotrophic factor (BDNF) transcripts in the rat hippocampus. Brain Res Mol Brain Res. 61(1-2):147-53.

Olivereau M, Olivereau J, Vandesande F (1990) Localization of growth hormone-releasing factor-like immunoreactivity in the hypothalamo-hypophysial system of some teleost species. Cell Tissue Res. 259(1):73-80.

Olivereau M, Moons L, Olivereau J, Vandesande F (1988) Coexistence of corticotropin-releasing factor-like immunoreactivity and vasotocin in perikarya of the preoptic nucleus in the eel. Gen Comp Endocrinol. 70(1):41-8.

Omura T, Sano M, Omura K, Hasegawa T, Doi M, Sawada T, Nagano A (2005) Different expressions of BDNF, NT3, and NT4 in muscle and nerve after various types of peripheral nerve injuries. J Peripher Nerv Syst. 10(3):293-300.

Paarmann I, Frermann D, Keller BU, Villmann C, Breitinger HG, Hollmann M (2005) Kinetics and subunit composition of NMDA receptors in respiratory-related neurons. J Neurochem. 93(4):812-24.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron. 16(6):1137-45.

Peiris TS, Machaalani R, Waters KA (2004) Brain-derived neurotrophic factor mRNA and protein in the piglet brainstem and effects of Intermittent Hypercapnic Hypoxia. Brain Res. 1029(1):11-23.

Pelleymounter MA, Cullen MJ, Wellman CL (1995) Characteristics of BDNF-induced weight loss. Exp Neurol. 131(2):229-38.

Pierce AL, Dickey JT, Larsen DA, Fukada H, Swanson P, Dickhoff WW (2004) A quantitative real-time RT-PCR assay for salmon IGF-I mRNA, and its application in the study of GH regulation of IGF-I gene expression in primary culture of salmon hepatocytes. Gen Comp Endocrinol. 135(3):401-11.

Plenz G, Weissen B, Steffen I (2002) Detection of mRNAs on cryosections of the cardiovascular system using DIG-labelled RNA probes. In: Eisel D, Grunewald-Janho S, Kruchen B, Roche Applied Science DIG Application Manual For Non-Radioactive *In Situ* Hybridization. Roche Diagnostics GmbH, pp.189-196.

Prasada Rao PD, Jadhao AG, Sharma SC (1993) Topographic organization of descending projection neurons to the spinal cord of the goldfish, Carassius auratus. Brain Res. 620(2):211-20.

Prasada Rao PD, Jadhao AG, Sharma SC (1987) Descending projection neurons to the spinal cord of the goldfish, Carassius auratus. J Comp Neurol. 265(1):96-108.

Prechtl JC, von der Emde G, Wolfart J, Karamursel S, Akoev GN, Andrianov YN, Bullock TH (1998) Sensory processing in the pallium of a mormyrid fish. J Neurosci. 18(18):7381-93.

Puissant C, Bayat-Sarmadi M, Devinoy E, Houdebine LM (1994) Variation of transferrin mRNA concentration in the rabbit mammary gland during the pregnancy-lactation-weaning cycle and in cultured mammary cells. A comparison with the other major milk protein mRNAs. Eur J Endocrinol. 130(5):522-9.

Pustylnyak VO, Gulyaeva LF, Lyakhovich VV (2005) CAR expression and inducibility of CYP2B genes in liver of rats treated with PB-like inducers. Toxicology. 216(2-3):147-53.

Radak Z, Toldy A, Szabo Z, Siamilis S, Nyakas C, Silye G, Jakus J, Goto S (2006) The effects of training and detraining on memory, neurotrophins and oxidative stress markers in rat brain. Neurochem Int. 49(4):387-92.

Rage F, Givalois L, Marmigere F, Tapia-Arancibia L, Arancibia S (2002) Immobilization stress rapidly modulates BDNF mRNA expression in the hypothalamus of adult male rats. Neuroscience. 112(2):309-18.

Rage F, Riteau B, Alonso G, Tapia-Arancibia L (1999) Brain-derived neurotrophic factor and neurotrophin-3 enhance somatostatin gene expression through a likely direct effect on hypothalamic somatostatin neurons. Endocrinology. 140:909-916

Raivich G, Kreutzberg GW (1993) Peripheral nerve regeneration: role of growth factors and their receptors. Int J Dev Neurosci. 11(3):311-24.

Rasouly D, Lazarovici P, Stephens RM, Kaplan DR (1995) Signal transduction of neurotrophin receptors: basic concepts and available pharmacological tools. Promega notes magazine 50:24-26

Rattiner LM, Davis M, French CT, Ressler KJ (2004) Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J Neurosci. 24(20):4796-806.

Rattray M, Michael GJ (1998) Oligonucleotide probes for *in situ* hybridization. In: Wilkinson DG (Ed), *In situ* hybridization: a practical approach. Oxford University Press, Oxford, pp.23-66.

Reiner A, Medina L, Veenman CL (1998) Structural and functional evolution of the basal ganglia in vertebrates. Brain Res Brain Res Rev. 28(3):235-85.

Reiner A, Northcutt RG (1992) An immunohistochemical study of the telencephalon of the senegal bichir (Polypterus senegalus). J Comp Neurol. 319(3):359-86.

Reiner A, Northcutt RG (1987) An immunohistochemical study of the telencephalon of the African lungfish, Protopterus annectens. J Comp Neurol. 256(3):463-81.

Reith CA, Sillar KT (1998) A role for slow NMDA receptor-mediated, intrinsic neuronal oscillations in the control of fast fictive swimming in Xenopus laevis larvae. Eur J Neurosci. 10(4):1329-40.

Rich BE, Steitz JA (1987) Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. Mol Cell Biol. 7(11):4065-74.

Richardson PM, McGuinness UM, Aguayo AJ (1980) Axons from CNS neurons regenerate into PNS grafts. Nature 284(5753):264-5.

Rink E, Wullimann MF (2004) Connections of the ventral telencephalon (subpallium) in the zebrafish (Danio rerio). Brain Res. 1011(2):206-20.

Rink E, Wullimann MF (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain Res. 889(1-2):316-30.

Rink E, Wullimann MF (1998) Some forebrain connections of the gustatory system in the goldfish Carassius auratus visualized by separate DiI application to the hypothalamic inferior lobe and the torus lateralis. J Comp Neurol. 394(2):152-70.

Riviere G, Michaud A, Breton C, VanCamp G, Laborie C, Enache M, Lesage J, Deloof S, Corvol P, Vieau D (2005) Angiotensin-converting enzyme 2 (ACE2) and ACE activities display tissue-specific sensitivity to undernutrition-programmed hypertension in the adult rat. Hypertension. 46(5):1169-74.

Roberts BL, Meredith GE, Maslam S (1989) Immunocytochemical analysis of the dopamine system in the brain and spinal cord of the European eel, Anguilla anguilla. Anat Embryol (Berl). 180(4):401-12.

Roberts, B.L. and Meredith, G.E. The efferent innervation of the ear: variations on an enigma. In: Webster DB, Fay RA, Popper AN (Eds) The evolutionary biology of hearing Springer, New York, pp.185 -210.

Roberts BL, Mos W (1992) Neural circuits for speed change in swimming fish. In Neurobiology of motor programme selection edited by Kien, J, McCrohan, C R, Winlow, W. Pergamon Press, chapter 8, pp 123-146

Rocamora N, Welker E, Pascual M, Soriano E (1996) Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation. J Neurosci. 16(14):4411-9.

Rodriguez F, Lopez JC, Vargas JP, Gomez Y, Broglio C, Salas C (2002) Conservation of spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. J Neurosci. 22(7):2894-903.

Roest Crollius H, Weissenbach J (2005) Fish genomics and biology. Genome Res. 15(12):1675-82.

Rohatgi T, Henrich-Noack P, Sedehizade F, Goertler M, Wallesch CW, Reymann KG, Reiser G (2004) Transient focal ischemia in rat brain differentially regulates mRNA expression of protease-activated receptors 1 to 4.
J Neurosci Res. 75(2):273-9.

Rossi A, Palombi F (1976) The saccus vasculosus of *Anguilla anguilla* (L.) from larva to adult. I. Ultrastructural modifications of the coronet cells. Cell Tissue Res. 167(1):11-21.

Rossi FM, Bozzi Y, Pizzorusso T, Maffei L (1999) Monocular deprivation decreases brain-derived neurotrophic factor immunoreactivity in the rat visual cortex. Neuroscience. 90(2):363-8.

Roubert C, Sagne C, Kapsimali M, Vernier P, Bourrat F, Giros B (2001) A Na(+)/Cl(-)-dependent transporter for catecholamines, identified as a norepinephrine transporter, is expressed in the brain of the teleost fish medaka (Oryzias latipes). Mol Pharmacol. 60(3):462-73.

Roux PP, Bhakar AL, Kennedy TE, Barker PA (2001) The p75 neurotrophin receptor activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway. J Biol Chem. 276(25):23097-104.

Ruano D, Letang V, Biton B, Avenet P, Benavides J, Scatton B, Vitorica J (2000) Subunit composition of rat ventral spinal cord GABA(A) receptors, assessed by single cell RT-multiplex PCR. Neuroreport. 11(14):3169-73.

Rudge JS, Silver J (1990) Inhibition of neurite outgrowth on astroglial scars in vitro. J Neurosci. 10(11):3594-603.

Salie R, Steeves JD (2005) IGF-1 and BDNF promote chick bulbospinal neurite outgrowth in vitro. Int J Dev Neurosci. 23(7):587-98.

Sanghi S, Kumar R, Smith M, Baker KM, Dostal DE (2005) Activation of protein kinase A by atrial natriuretic peptide in neonatal rat cardiac fibroblasts: role in regulation of the local renin-angiotensin system. Regul Pept. 132(1-3):1-8.

Sawai H, Clarke DB, Kittlerova P, Bray GM, Aguayo AJ (1996) Brain-derived neurotrophic factor and neurotrophin-4/5 stimulate growth of axonal branches from regenerating retinal ganglion cells. J Neurosci. 16(12):3887-94.

Sayer FT, Oudega M, Hagg T (2002) Neurotrophins reduce degeneration of injured ascending sensory and corticospinal motor axons in adult rat spinal cord. Exp Neurol. 175(1):282-96.

Schellart, NAM (1990) The visual pathways and central non-tectal processing. In: Douglas RH, Djamgoz MBA (Eds) The Visual System of Fish, Chapman and Hall, London, pp.345-372

Schinder AF, Poo M (2000) The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci. 23(12):639-45.

Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature 367(6459):170-3.

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol. 31;7:3.

Schuster S, Wohl S, Griebsch M, Klostermeier I (2006) Animal cognition: how archer fish learn to down rapidly moving targets. Curr Biol. 16(4):378-83.

Schwab ME (1990) Myelin-associated inhibitors of neurite growth. Exp Neurol. 109(1):2-5.

Schwab ME (2004) Nogo and axon regeneration. Curr Opin Neurobiol. 14(1):118-24.

Sebert ME, Shooter EM (1993) Expression of mRNA for neurotrophic factors and their receptors in the rat dorsal root ganglion and sciatic nerve following nerve injury. J Neurosci Res. 36(4):357-67.

Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci. 26:299-330.

Sekhon LH, Fehlings MG (2001) Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine. 26(24 Suppl):S2-12.

Selzer ME (2003) Promotion of axonal regeneration in the injured CNS. Lancet Neurol. 2(3):157-66.

Seroogy KB, Lundgren KH, Tran TM, Guthrie KM, Isackson PJ, Gall CM (1994) Dopaminergic neurons in rat ventral midbrain express brain-derived neurotrophic factor and neurotrophin-3 mRNAs. J Comp Neurol. 342(3):321-34.

Sharma SC, Jadhao AG, Rao PD (1993) Regeneration of supraspinal projection neurons in the adult goldfish. Brain Res. 620(2):221-8.

Sharpe J (2004) Optical projection tomography. Annu Rev Biomed Eng. 6:209-28.

Shashoua VE (1977) Brain protein metabolism and the acquisition of new patterns of behavior. Proc Natl Acad Sci U S A. 74(4):1743-7.

Shen H, Chung JM, Chung K (1999) Expression of neurotrophin mRNAs in the dorsal root ganglion after spinal nerve injury. Brain Res Mol Brain Res. 64(2):186-92.

Shen S, Wiemelt AP, McMorris FA, Barres BA (1999) Retinal ganglion cells lose trophic responsiveness after axotomy. Neuron. 23(2):285-95.

Sherwood NT, Lo DC (1999) Long-term enhancement of central synaptic transmission by chronic brain-derived neurotrophic factor treatment. J Neurosci. 19(16):7025-36.

Shifman MI, Selzer ME (2000) In situ hybridization in wholemounted lamprey spinal cord: localization of netrin mRNA expression. J Neurosci Methods. 104(1):19-25.

Shimizu T, Nakagaki M, Nishi Y, Kobayashi Y, Hachimori A, Uchiumi T (2002) Interaction among silkworm ribosomal proteins P1, P2 and P0 required for functional protein binding to the GTPase-associated domain of 28S rRNA. Nucleic Acids Res. 30(12):2620-7.

Simpson DA, Feeney S, Boyle C, Stitt AW (2000) Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. Mol Vis. 6:178-83.

Skene JH (1989) Axonal growth-associated proteins. Annu Rev Neurosci. 12:127-56.

Skup M, Dwornik A, Macias M, Sulejczak D, Wiater M, Czarkowska-Bauch J (2002) Long-term locomotor training up-regulates TrkB(FL) receptor-like proteins, brain-derived neurotrophic factor, and neurotrophin 4 with different topographies of expression in oligodendroglia and neurons in the spinal cord. Exp Neurol. 176(2):289-307.

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature. 368(6468):246-9.

Smit WA, Roberts BL, Velzing EH (1991) Changes in size and number of spinal motoneurons in relation to growth of the musculature in the eel, Anguilla. Brain Res Dev Brain Res. 58(1):73-80.

Snider WD, Zhou FQ, Zhong J, Markus A (2002) Signaling the pathway to regeneration. Neuron 35(1):13-6.

Sobreviela T, Pagcatipunan M, Kroin JS, Mufson EJ (1996) Retrograde transport of brain-derived neurotrophic factor (BDNF) following infusion in neo- and limbic cortex in rat: relationship to BDNF mRNA expressing neurons. J Comp Neurol. 375(3):417-44.

Soffe SR, Roberts A (1989) The Influence of Magnesium Ions on the NMDA Mediated Responses of Ventral Rhythmic Neurons in the Spinal Cord of Xenopus Embryos. Eur J Neurosci. 1(5):507-515.

Song HJ, Poo MM (1999) Signal transduction underlying growth cone guidance by diffusible factors. Curr Opin Neurobiol. 9(3):355-63.

Spalding KL, Rush RA, Harvey AR (2004) Target-derived and locally derived neurotrophins support retinal ganglion cell survival in the neonatal rat retina. J Neurobiol. 60(3):319-27.

Spalding KL, Tan MM, Hendry IA, Harvey AR (2002) Anterograde transport and trophic actions of BDNF and NT-4/5 in the developing rat visual system. Mol Cell Neurosci. 19(4):485-500.

Steven C, Lehnen N, Kight K, Ijiri S, Klenke U, Harris WA, Zohar Y (2003) Molecular characterization of the GnRH system in zebrafish (Danio rerio): cloning of chicken GnRH-II,

adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. Gen Comp Endocrinol. 133(1):27-37.

Stone R (2003) Ecology: Freshwater eels are slip-sliding away. Science. 9;304(5668):209-11.

Sundin L, Turesson J, Burleson M (2003) Identification of central mechanisms vital for breathing in the channel catfish, Ictalurus punctatus. Respir Physiol Neurobiol. 138(1):77-86.

Suneja SK, Yan L, Potashner SJ (2005) Regulation of NT-3 and BDNF levels in guinea pig auditory brain stem nuclei after unilateral cochlear ablation. J Neurosci Res. 80(3):381-90.

Swain GP, Jacobs AJ, Frei E, Selzer ME (1994) A method for in situ hybridization in wholemounted lamprey brain: neurofilament expression in larvae and adults. Exp Neurol. 126(2):256-69.

Tachibana T, Noguchi K, Ruda MA (2002) Analysis of gene expression following spinal cord injury in rat using complementary DNA microarray. Neurosci Lett. 327(2):133-7.

Takei Y, Inoue K, Ando K, Ihara T, Katafuchi T, Kashiwagi M, Hirose S (2001) Enhanced expression and release of C-type natriuretic peptide in freshwater eels. Am J Physiol Regul Integr Comp Physiol. 280(6):R1727-35.

Tamai TK, Vardhanabhuti V, Arthur S, Foulkes NS, Whitmore D (2003) Flies and fish: birds of a feather. J Neuroendocrinol. 15(4):344-9.

Tapia-Arancibia L, Rage F, Givalois L, Arancibia S (2004) Physiology of BDNF: focus on hypothalamic function. Front Neuroendocrinol. 25(2):77-107.

Tetzlaff W, Alexander SW, Miller FD, Bisby MA (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci. 11(8):2528-44.

Theiss C, Gunturkun O (2001) Distribution of BDNF, NT-3, trkB and trkC in the developing retino-tectal system of the pigeon (*Columba livia*). Anat Embryol (Berl). 204(1):27-37.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol. 75(2-3):291-5.

Thoby-Brisson M, Cauli B, Champagnat J, Fortin G, Katz DM (2003) Expression of functional tyrosine kinase B receptors by rhythmically active respiratory neurons in the pre-Botzinger complex of neonatal mice. J Neurosci. 23(20):7685-9.

Tierney TS, P Doubell T, Xia G, Moore DR (2001) Development of brain-derived neurotrophic factor and neurotrophin-3 immunoreactivity in the lower auditory brainstem of the postnatal gerbil. Eur J Neurosci. 14(5):785-93.

Tonra JR, Curtis R, Wong V, Cliffer KD, Park JS, Timmes A, Nguyen T, Lindsay RM, Acheson A, DiStefano PS (1998) Axotomy upregulates the anterograde transport and

expression of brain-derived neurotrophic factor by sensory neurons. J Neurosci. 18(11):4374-83.

Torborg CL, Feller MB (2005) Spontaneous patterned retinal activity and the refinement of retinal projections. Prog Neurobiol. 76(4):213-35. Epub 2005 Nov 8.

Tsukamoto K (2006) Oceanic biology: spawning of eels near a seamount. Nature. 23;439(7079):929.

Turner JE, Schwab ME, Thoenen H (1982) Nerve growth factor stimulates neurite outgrowth from goldfish retinal explants: the influence of a prior lesion. Brain Res. 256(1):59-66.

Uchida K, Baba H, Maezawa Y, Furukawa S, Omiya M, Kokubo Y, Kubota C, Nakajima H (2003) Increased expression of neurotrophins and their receptors in the mechanically compressed spinal cord of the spinal hyperostotic mouse (twy/twy). Acta Neuropathol (Berl). 106(1):29-36.

Valter K, Bisti S, Gargini C, Di Loreto S, Maccarone R, Cervetto L, Stone J (2005) Time course of neurotrophic factor upregulation and retinal protection against light-induced damage after optic nerve section. Invest Ophthalmol Vis Sci. 46(5):1748-54.

Van Beek J, Chan P, Bernaudin M, Petit E, MacKenzie ET, Fontaine M (2000) Glial responses, clusterin, and complement in permanent focal cerebral ischemia in the mouse. Glia. 31(1):39-50.

Vanholme B, De Meutter J, Tytgat T, Gheysen GD, Vanhoutte I, Gheysen GD (2002) An improved method for whole-mount in situ hybridization of Heterodera schachtii juveniles. Parasitol Res. 88(8):731-3.
van Raamsdonk W, Smit-Onel MJ, Maslam S, Velzing E, de Heus R (1998) Changes in the synaptology of spinal motoneurons in zebrafish following spinal cord transection. Acta Histochem. 100(2):133-48.

Vaynman SS, Ying Z, Yin D, Gomez-Pinilla F (2006) Exercise differentially regulates synaptic proteins associated to the function of BDNF. Brain Res. 27;1070(1):124-30.

Vaynman S, Gomez-Pinilla F (2005) License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabil Neural Repair. 19(4):283-95.

Venero JL, Vizuete ML, Revuelta M, Vargas C, Cano J, Machado A (2000) Upregulation of BDNF mRNA and trkB mRNA in the nigrostriatal system and in the lesion site following unilateral transection of the medial forebrain bundle. Exp Neurol. 161(1):38-48.

Venkatesh B, Brenner S (1995) Structure and organization of the isotocin and vasotocin genes from teleosts. Adv Exp Med Biol. 395:629-38.

Vicario-Abejon C, Owens D, McKay R, Segal M (2002) Role of neurotrophins in central synapse formation and stabilization. Nat Rev Neurosci. 3(12):965-74.

von Bartheld CS, Williams R, Lefcort F, Clary DO, Reichardt LF, Bothwell M (1996) Retrograde transport of neurotrophins from the eye to the brain in chick embryos: roles of the p75NTR and trkB receptors. J Neurosci. 16(9):2995-3008.

Wang L, Calle M, Roubos EW (2005) Brain-Derived Neurotrophic Factor in the Hypothalamo-Hypophyseal System of *Xenopus laevis*. Ann N Y Acad Sci. 1040:512-4.

Watkins TA, Barres BA (2002) Integrins as developmental switches. Nat Cell Biol. 4(11):E253-5.

Weis JS (1968) Analysis of the development of the nervous system of the zebrafish, Brachydanio rerio. II. The effect of nerve growth factor and its antiserum on the nervous system of the zebrafish. J Embryol Exp Morphol. 19(2):121-35.

Weis JS (1968) The occurrence of nerve growth factor in teleost fishes. Experientia. 24(7):736-7.

Weltzien FA, Pasqualini C, Vernier P, Dufour S (2005) A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. Gen Comp Endocrinol. 142(1-2):134-42.

Wetmore C, Olson L (1995) Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. J Comp Neurol. 353(1):143-59.

Wetmore C, Ernfors P, Persson H, Olson L (1990) Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. Exp Neurol. 109(2):141-52.

Whelan PJ (1996) Control of locomotion in the decerebrate cat. Prog Neurobiol. 49(5):481-515.

Widenfalk J, Lundstromer K, Jubran M, Brene S, Olson L (2001) Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. J Neurosci 21(10):3457-75.

Widenfalk J, Olson L, Thoren P (1999) Deprived of habitual running, rats downregulate BDNF and TrkB messages in the brain. Neurosci Res. 34(3):125-32.

Wilkinson DG (1995) RNA detection using non-radioactive *in situ* hybridization. Curr Opin Biotechnol. 6(1):20-3.

Wilkinson DG (1998) The theory and practice of *in situ* hybridization. In: Wilkinson DG (Ed), *In situ* hybridization: a practical approach. Oxford University Press, Oxford, pp.1-21.

Willemse JJ, van den Berg PG (1978) Growth of striated muscle fibres in the M. lateralis of the European eel *Anguilla anguilla* (L.) (Pisces, Teleostei). J Anat.125(Pt 3):447-60.

Williams FE, White D, Messer WS (2002) A simple spatial alternation task for assessing memory function in zebrafish. Behav Processes. 58(3):125-132.

Winer J, Jung CK, Shackel I, Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem. 270(1):41-9.

Wintzer M, Mladinic M, Lazarevic D, Casseler C, Cattaneo A, Nicholls J (2004) Strategies for identifying genes that play a role in spinal cord regeneration. J Anat. 204(1):3-11.

Wirth T, Bernatchez L (2001) Genetic evidence against panmixia in the European eel. Nature. 409(6823):1037-40.

Wood M, Cohen M (1979) Synaptic regeneration in identified neurons of the lamprey spinal cord. Science. 206:344-7.

Wood MR, Cohen MJ (1981) Synaptic regeneration and glial reactions in the transected spinal cord of the lamprey. J Neurocytol. 10(1):57-79.

Wu S, Storey KB (2005) Up-regulation of acidic ribosomal phosphoprotein P0 in response to freezing or anoxia in the freeze tolerant wood frog, Rana sylvatica. Cryobiology. 50(1):71-82.

Wu X, Yoo S, Wrathall JR (2005) Real-time quantitative PCR analysis of temporal-spatial alterations in gene expression after spinal cord contusion. J Neurochem. 93(4):943-52.

Wullimann MF, Rupp B, Reichert H (1996) Neuroanatomy of the Zebrafish Brain A Topological Atlas. Springer Verlag.

Wullimann MF, Rink E (2002) The teleostean forebrain: a comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. Brain Res Bull. 57(3-4):363-70.

Xu XM, Guenard V, Kleitman N, Aebischer P, Bunge MB (1995) A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. Exp Neurol. 134(2):261-72.

Xu Q, Wilkinson DG (1998) *In situ* hybridization of mRNA with hapten labelled probes. In: Wilkinson DG (Ed), *In situ* hybridization: a practical approach. Oxford University Press, Oxford, pp.87-106.

Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF (2000a) The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J Neurosci. 20(18):6888-97.

Xu B, Zang K, Ruff NL, Zhang YA, McConnell SK, Stryker MP, Reichardt LF (2000b) Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron. 26(1):233-45.

Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH, Reichardt LF (2003) Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci. 6(7):736-42.

Xue HG, Yamamoto N, Yoshimoto M, Yang CY, Ito H (2001) Fiber connections of the nucleus isthmi in the carp (Cyprinus carpio) and tilapia (Oreochromis niloticus). Brain Behav Evol. 58(4):185-204.

Xue HG, Yang CY, Ito H (2004) The anterograde and retrograde axonal transport of biotinylated dextran amine and biocytin in the nervous system of teleosts. Brain Res Brain Res Protoc. 13(2):106-14.

Yadav VK, Muraly P, Medhamurthy R (2004) Identification of novel genes regulated by LH in the primate corpus luteum: insight into their regulation during the late luteal phase. Mol Hum Reprod. 10(9):629-39.

Yajima Y, Narita M, Usui A, Kaneko C, Miyatake M, Narita M, Yamaguchi T, Tamaki H, Wachi H, Seyama Y, Suzuki T (2005) Direct evidence for the involvement of brain-derived neurotrophic factor in the development of a neuropathic pain-like state in mice. J Neurochem. 93(3):584-94.

Yamaguchi T (2004) The central pattern generator for forelimb locomotion in the cat. Prog Brain Res. 143:115-22.

Yan Q, Radeke MJ, Matheson CR, Talvenheimo J, Welcher AA, Feinstein SC (1997a) Immunocytochemical localization of TrkB in the central nervous system of the adult rat. J Comp Neurol. 378(1):135-57.

Yan Q, Rosenfeld RD, Matheson CR, Hawkins N, Lopez OT, Bennett L, Welcher AA (1997b) Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience. 78(2):431-48.

Yang Y, Xie Y, Chai H, Fan M, Liu S, Liu H, Bruce I, Wu W. (2006) Microarray analysis of gene expression patterns in adult spinal motoneurons after different types of axonal injuries. Brain Res. 1075(1):1-12.

Ye JH, Houle JD (1997) Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Exp Neurol. 143(1):70-81.

Yeomans JS, Frankland PW (1995) The acoustic startle reflex: neurons and connections. Brain Res Brain Res Rev. 21(3):301-14.

Yin HS, Mackler SA, Selzer ME (1984) Directional specificity in the regeneration of lamprey spinal axons. Science. 224(4651):894-6.

Ying Z, Roy RR, Edgerton VR, Gomez-Pinilla F (2005) Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. Exp Neurol. 193(2):411-9.

Yip HK, Grafstein B (1982) Effect of nerve growth factor on regeneration of goldfish optic axons. Brain Res. 238(2):329-39.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV (1998) Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci. 18(9):3273-81.

Yuge S, Inoue K, Hyodo S, Takei Y (2003) A novel guanylin family (guanylin, uroguanylin, and renoguanylin) in eels: possible osmoregulatory hormones in intestine and kidney.J Biol Chem. 278(25):22726-33.

Zhang L, Schmidt RE, Yan Q, Snider WD (1994) NGF and NT-3 have differing effects on the growth of dorsal root axons in developing mammalian spinal cord. J Neurosci. 14(9):5187-201.

Zhang JY, Luo XG, Xian CJ, Liu ZH, Zhou XF (2000) Endogenous BDNF is required for myelination and regeneration of injured sciatic nerve in rodents. Eur J Neurosci. 12(12):4171-80.

Zhang FX, Lai CH, Li JL, Shum DK, Chan YS (2006) Tyrosine kinase receptor immunoreactivity in trigeminal mesencephalic and motor neurons following transection of masseteric nerve of the rat. Neuroscience. 139(3):921-30.

Zhou L, Shine HD (2003) Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury. J Neurosci Res. 74(2):221-6.

Zupanc MM, Wellbrock UM, Zupanc GK (2006) Proteome analysis identifies novel protein candidates involved in regeneration of the cerebellum of teleost fish. Proteomics. 6(2):677-96.

Zweifel LS, Kuruvilla R, Ginty DD (2005) Functions and mechanisms of retrograde neurotrophin signalling. Nat Rev Neurosci. 6(8):615-25.