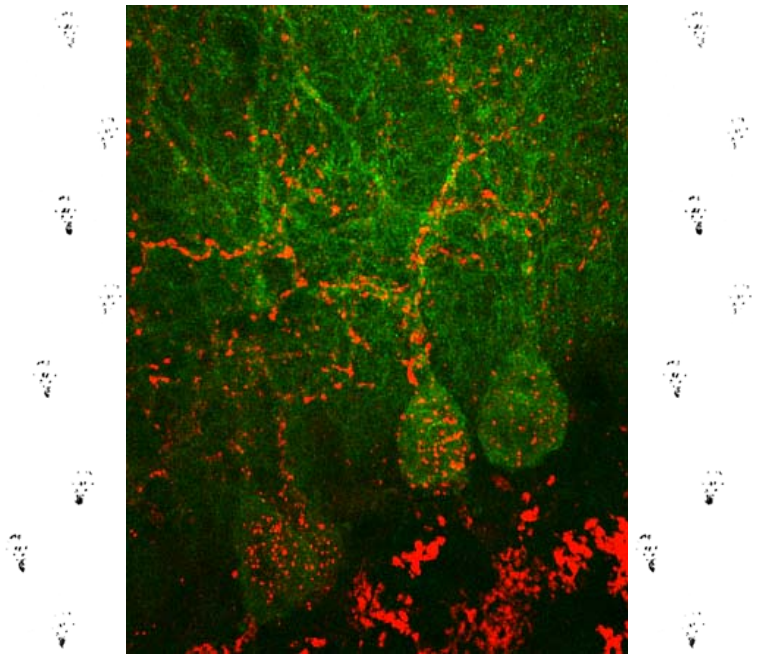
The induction and inhibition of post-lesional transcommissural climbing fibre reinnervation in the neonatal and adult rat cerebellum using Brain-derived neurotrophic factor: anatomical and functional implications



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B.App.Sci. (Biochemistry & Microbiology)
B.App.Sci. Honours I

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School of Veterinary & Biomedical Sciences
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THESIS / ANTI-THESIS / SYNTHESIS

Georg Wilhelm Friedrich Hegel, a 19th century German philosopher, was considered by his admirers to have found the key to explaining in principle, just about everything. His position was called Dialectical Idealism. "Dialectic" refers to the process of examining an idea (thesis), working out its implications, consequences and applications, and thereby finding difficulties (anti-thesis) that require the discarding of the original idea and the adoption of a modified form of it (synthesis), a new idea. We then examine the new idea (thesis) and repeat the process. The goal of the process is the final thesis, the 'Absolute'.

Georg W F Hegel (1770-1831)

PLASTICITY

The habits of an elementary particle of matter cannot change (on the principles of the atomistic philosophy), because the particle is itself an unchangeable thing; but those of a compound mass of matter can change, because they are in the last instance due to the structure of the compound, and either outward forces or inward tensions can, from one hour to another, turn that structure into something different from what it was. That is, they can do so if the body be plastic enough to maintain its integrity, and be not disrupted when its structure yields. Plasticity, then, in the wide sense of the word, means the possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once. Each relatively stable phase of equilibrium in such a structure is marked by what we may call a new set of habits. Organic matter, especially nervous tissue, seems endowed with a very extraordinary degree of plasticity of this sort; so that we may without hesitation lay down as our first proposition the following, that the phenomena of habit in living beings are due to the plasticity of the organic materials of which their bodies are composed.

William James (1842 – 1910)
Exert from ‘The Principles of Psychology’ (1980) Vol 1: Ch 4

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SIGNED STATEMENT OF SOURCES

Funding for this PhD came from a variety of sources: James Cook University (project support and a 'Doctoral Research Scheme' grant), the University of Western Australia, the University of Notre Dame and Apex Foundation for research into intellectual disabilities. Kirsty also received a travel scholarship (JCU and the School Veterinary and Biomedical Sciences) and a Woodside Neurotrauma PhD Excellence Award (UWA), which allowed attendance at two international conferences and laptop upgrades to continue thesis writing.

During this PhD Kirsty was supported by an Australian Postgraduate Award (3 years, 3 months) and received a three month extension from the School of Veterinary & Biomedical Sciences (JCU). Kirsty also received a James Cook University Doctoral Completion Scholarship.

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Laboratory and desk space was also provided by the School of Anatomy and Human Biology at the University of Western Australia (2 years).

STATEMENT ON THE CONTRIBUTION OF OTHERS

I hereby declare that this thesis is my own and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given. Furthermore, I declare that I performed all of the work in this thesis, unless stated otherwise.

Stipend support:	Australian Postgraduate Award (3 years)
Supervision:	Whilst at JCU Supervisor: Dr Sherrard (Feb 02 – Apr 04) Co-supervisor: A/Prof Bower (Feb 02 – Dec 03) Associate Supervisor: Dr Walker (Feb 02 – Mar 03) Whilst at UWA Supervisor: Dr Sherrard (Apr 04 – Nov 06) Supervisor: Dr Hodgetts (Apr 06 – Dec 06) Co-supervisor: A/Prof Fitzpatrick (Apr 04 – Dec 06)
Other collaborations:	nil
Research assistance:	nil
Any other assistance:	Dr Rachel Sherrard performed approximately 75 % of the unilateral inferior cerebellar peduncle transections. For three animals in chapter five, Dr Rachel Sherrard performed the Px30 surgery, cerebellar injections and analysed the retrograde dye transport.
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Infrastructure external to organisational unit within JCU:	Laboratory and desk space provided by School of Medicine (2 years 2 months) at James Cook University.

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Signature Date

DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Human (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University and the University of Western Australia Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A749_02) and the University of Western Australia Animal Ethics Committee (approval number 04/100/359).

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Signature Date

CONTRIBUTION OF AUTHORS ON THE CO-AUTHORED PAPER

I hereby declare that the published co-authored paper, which forms the basis of chapter five, was written mostly by Dr Rachel Sherrard. For this thesis I have removed the abstract and modified the introduction, methods and conclusion sections, however the majority of the results and discussion sections remain unchanged. The figures for this paper, which remain almost unchanged for the thesis (except figure 5.5), were created by myself in consultation with Dr Sherrard.

Signature

Date

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Bunker Bay, WA

PUBLICATIONS

Dixon K.J. and Sherrard R.M. Brain-derived neurotrophic factor induces post-lesion transcommissural growth of olivary axons that develop normal climbing fibres on mature Purkinje cells (2006) *Experimental Neurology* 202:44-56.

ABSTRACTS

Refereed

Dixon K.J. and Sherrard R.M. Brain-derived neurotrophic factor induces olivocerebellar plasticity in the mature cerebellum (2006) *Federation of European Neurosciences*

Dixon K.J. and Sherrard R.M. Brain-derived neurotrophic factor induces olivocerebellar plasticity in the mature cerebellum (2006) *Australian Neuroscience Society*

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Non-refereed

Dixon K.J. and Sherrard R.M. BDNF delays inferior olivary cell death following axotomy (2005) *Student Expo, School of Anatomy & Human Biology, UWA*

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Dixon K.J. Brain-derived neurotrophic factor induces functional olivocerebellar plasticity in the mature cerebellum (2005) *Symposium of Western Australian Neuroscience*

MEDIA RELEASE

Researcher's brainwave (15 July 2006) *Townville Bulletin*, bi-line Johnston J, p35

ABSTRACT

In the adult mammalian central nervous system, reinnervation and recovery from trauma is limited. During development however, post-lesion plasticity generates alternate paths providing models to investigate factors that promote reinnervation to appropriate targets. Following unilateral transection of the neonatal rat olivocerebellar pathway, axons from the remaining inferior olive reinnervate the denervated hemicerebellum and develop topographically organised normal climbing fibre arbors on Purkinje cells. However, the capacity to recreate this accurate target reinnervation in a mature system remains unknown. This thesis will identify whether one factor, Brain-derived neurotrophic factor (BDNF) is involved in reinnervation during the neonatal period and whether it induces similar reinnervation in the mature system. If BDNF does induce reinnervation in the mature system, this thesis will identify any return of lost function.

In rats lesioned on postnatal days 3 (P3), P15, P20 or P30 and treated with an intracerebellar injection of brain-derived neurotrophic factor (BDNF) or a BDNF blockade 24 hours following surgery, the morphology and organisation of transcommissural olivocerebellar reinnervation was examined using neuronal tracers and immunohistochemistry. The behavioural sequela of these rats was also investigated using vestibulo-spinal reflexes, simple locomotion, complex locomotion and gait synchronisation tests. Additionally, in neonatal rats (P3) with a unilateral lesion and treated with an intraolivary injection of BDNF, the survival of the axotomised inferior olivary complex and associated ipsilateral olivocerebellar pathway was examined using histochemical dyes and neuronal tracers.

In neonatal animals (P3), intracerebellar application of a BDNF blockade prevents olivocerebellar reinnervation of target Purkinje cells in the treatment area, while addition of BDNF in the mature system induces transcommissural olivocerebellar axonal growth into the denervated hemicerebellum. The distribution of BDNF-induced reinnervating climbing fibres was not confined to the injection sites, but extended throughout the denervated hemivermis and, less densely, up to 3.5 mm into the hemisphere. Transcommissural reinnervating axons were organised into parasagittal

microzones that were almost symmetrical to those in the right hemicerebellum. Reinnervating climbing fibre arbors were predominantly normal, but in the P30-lesioned group 10 % branched within the molecular layer forming a smaller secondary arbor, and in the P15-lesion group, the reinnervating arbors extended their terminals almost to the pial surface and were larger than control arbors ($p<0.02$). Behavioural testing revealed that BDNF and extensive exercise induce olivocerebellar reinnervation and that this reinnervation provides functional recovery, although this is delayed in the vehicle-treatment group. Additionally, the behavioural testing revealed that functional recovery is dependent on the age of the animal, whereby animals lesioned prior to acquiring task specific skills were developmentally disadvantaged. Lastly, during the neonatal period intraolivary BDNF transiently prevented degeneration of the axotomised inferior olivary complex, however it was unsuccessful in inducing transcommissural axonal growth of the ipsilateral olivocerebellar pathway into the denervated hemisphere.

For the first time, data from this PhD suggests that BDNF is involved in transcommissural reinnervation of denervated areas during the neonatal period and show that BDNF promotes topographically organised morphologically correct reinnervation in the mature rat cerebellum. Additionally, this reinnervation in the mature system provides functional recovery similar to sham-operated control animals. BDNF administered intraolivary however does not maintain the persistence of axotomised olivary neurons or induce transcommissural axonal growth of the ipsilateral olivocerebellar pathway. Data from this study can one day be used to contribute to a repair mechanism for traumatic brain injury, minimising long-term disabilities and on-going costs to society.

LIST OF ABBREVIATIONS USED

15B .....Pedunculotomy at postnatal day 15 and BDNF intracerebellar injection
15V .....Pedunculotomy at postnatal day 15 and vehicle intracerebellar injection
20B .....Pedunculotomy at postnatal day 20 and BDNF intracerebellar injection
20V .....Pedunculotomy at postnatal day 20 and vehicle intracerebellar injection
30B .....Pedunculotomy at postnatal day 30 and BDNF intracerebellar injection
30V .....Pedunculotomy at postnatal day 30 and vehicle intracerebellar injection
^{0}C ..... Degrees Celsius
> ..... Greater than
< .....Less than
µg ..... Microgram
µl .....Microlitre
µm ..... Micrometre
% ..... Percent
BDNF ..... Brain-derived neurotrophic factor
BSA .....Bovine serum albumin
Ca^{2+} .....Calcium
Cbr .....Cerebellar
climbing fibre .....Climbing Fibre
cm .....Centimetre
CNS .....Central nervous system
DAO .....Dorsal accessory olive
DCN ..... Deep cerebellar nuclei
DePeX ..... Di-n-butyl phthalate polystyrene xylene
E .....Embryonic day
EGL .....External germinal layer
GAP-43 .....Growth associated protein-43
GL .....Granular Layer
GTP ..... Guanosine triphosphate
IGL .....Internal granular layer
IOC .....Inferior olivary complex

IU ..... International units
Kg ..... Kilogram
M ..... Molar
MAO ..... Medial accessory olive
mg ..... Milligram
mm ..... Millimetre
MBS ..... Modified Bouins solution
ml ..... Millilitre
ML ..... Molecular layer
mRNA ..... Messenger RNA
NGF ..... Nerve growth factor
nM ..... nanoMolar
NMDA ..... N-methyl-D-aspartate
NT-3 ..... Neurotrophoin-3
NT-4/5 ..... Neurotrophin-4/5
NT-6 ..... Neurotrophin-6
NT-7 ..... Neurotrophin-7
P ..... Postnatal day
p75 ..... Pan neurotrophin receptor 75
PBS ..... Phosphate buffered saline
PC ..... Purkinje cell
pH ..... Hydrogen-ion concentration
PI-3 kinase Phosphatidylinositol-3-kinase
PO ..... Principal olive
P15 ..... Postnatal day 15
P20 ..... Postnatal day 20
P30 ..... Postnatal day 30
Px15 ..... Pedunculotomy at postnatal day 15
Px15B ..... Pedunculotomy at postnatal day 15 and BDNF intracerebellar injection
Px15V ..... Pedunculotomy at postnatal day 15 and vehicle intracerebellar injection
Px20 ..... Pedunculotomy at postnatal day 20
Px20B ..... Pedunculotomy at postnatal day 20 and BDNF intracerebellar injection

Px20V.....Pedunculotomy at postnatal day 20 and vehicle intracerebellar injection
Px30 .....Pedunculotomy at postnatal day 30
Px30B.....Pedunculotomy at postnatal day 30 and BDNF intracerebellar injection
Px30V.....Pedunculotomy at postnatal day 30 and vehicle intracerebellar injection
Rpm.....Revolutions per minute
s .....seconds
trkA .....Tropomyosin kinase receptor A
trkB.....Tropomyosin kinase receptor B
trkC.....Tropomyosin kinase receptor C
T-PBS.....TritonX-PBS
T-PBS-G.....TritonX-PBS with gelatin
UTL.....Upper time limit
VGLUT1 .....Vesicular glutamate transporter 1
VGLUT2 .....Vesicular glutamate transporter 2

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CHAPTER 1

INTRODUCTION

1.1 TRAUMATIC BRAIN INJURY: THE RESEARCH PROBLEM

Each year in Australia over 27,000 people sustain traumatic brain injuries, with the highest incidence occurring in 15 to 19 year old males (Fortune and Wen, 1999). It is estimated that 16.4 % (4,500 people per year in Australia) experience long-term disabilities (physical, cognitive and psychosocial: Fortune and Wen, 1999). While this is an obvious burden for the individual and their family and friends, it also creates a massive economic impact on society. Minimising long-term disabilities will therefore ease the individual's suffering and reduce the on-going costs to society. By its very nature however, traumatic brain injury is not easily prevented, only manageable once injury has occurred and current management is limited to extrinsic intervention such as occupational-, physio- and speech-therapies, which can help manage the deficit rather than curing the problem.

Following injury to the mature brain, neurons nearby that are not directly affected by the trauma may sprout axon terminals into their local environment (Gilson and Stensaas, 1974; Kolb et al., 2000; Rossi et al., 1991b; Zagrebelsky et al., 1996). While these axon terminals synapse on nearby neurons sometimes resembling normal terminal plexuses, this appears to be of little clinical significance as it provides limited post-lesional functional compensation (Hagg et al., 2005; Liu et al., 1999; Rossi et al., 1991b; Ruitenberg et al., 2003; Zagrebelsky et al., 1996). Conversely, the immature system appears to be better able to respond to injury (Nakamura and Bregman, 2001; Sendtner et al., 1992; Yan et al., 1992). Similar to the mature central nervous system (CNS), neurons within the immature system adjacent to the trauma also undergo changes in response to the injury (plasticity). However, these neurons may also develop completely new neuronal pathways into the area of denervation to reconnect with the denervated target cells (Angaut et al., 1985; Barth and Stanfield, 1990; Bregman and Goldberger, 1982; Cao et al., 1994; Kuang and Kalil, 1990; Naus et al., 1986; Naus et al., 1984; Sherrard et al., 1986). Importantly, these new pathways provide some return of function

from the deficits obtained from the injury (Bregman and Goldberger, 1982; Dixon et al., 2005). Therefore, if these pathways can be induced in the injured mature brain they may improve the post-lesional functional recovery i.e. minimise any long-term deficits obtained. However, there is a lack of information on what controls the development of these alternate pathways in the immature CNS.

It is known that within the CNS there is a fine balance between growth inhibition and growth promotion, which is thought to be regulated by growth inhibitory molecules located in the local extracellular environment and the intrinsic nature of neuronal populations (Spencer and Filbin, 2004). At the onset of CNS maturity, when developmental axonogenesis and synaptogenesis ceases, as well as the cessation of functionally beneficial post-lesion neuronal repair, there is an increased expression of inhibitory molecules (Rhodes et al., 2003). At first glance a simple solution to extend the post-lesional plasticity that occurs in the immature system into that of the mature system might appear to be to reduce expression of growth inhibitory molecules in the mature system. While this type of modification enables new axonal growth (as opposed to terminal sprouting), this occurs without directionality (i.e. non-specific) and does not improve functional recovery (McClellan, 1999). Thus, this method of repair has limited clinical significance.

In addition to the increase in inhibitory molecules as the brain matures, there is also a reduction in the levels of growth permissive molecules (growth factors). These molecules are thought to increase intracellular cyclical nucleotide levels and protein kinase activation, affecting microtubule assembly and cytoskeletal protein expression (Spencer and Filbin, 2004); therefore contributing to appropriate axonogenesis and synaptogenesis. Importantly, the addition of growth factors, specifically neurotrophins, to the mature CNS following injury has been found to influence axonal growth, even in the presence of high levels of growth inhibitory molecules, when it otherwise would not occur (David and Aguayo, 1981; Lu et al., 2004; Sherrard and Bower, 2001; Sherrard and Bower, 2003). Currently however, there is a knowledge gap on whether neurotrophins influence the development of completely new alternate pathways

following trauma in the immature system, and whether the addition of neurotrophins to the mature brain can induce these pathways to reinnervate previously denervated target cells. Therefore this creates a need to assess the contribution of each neurotrophin towards CNS recovery to identify which neurotrophin might be involved in the development of these whole new post-lesion pathways and thus able to induce these pathways in the mature brain. As such, this introduction will identify which neurotrophins may be involved in post-lesional CNS recovery and discuss a suitable model to investigate the role of neurotrophins in the development of alternate neuronal pathways.

1.2 THE CENTRAL NERVOUS SYSTEM

The mammalian CNS is an extremely complex structure and encompasses the forebrain, midbrain, hindbrain and spinal cord. Development of the CNS is an extremely fine and precise process so that in the adult system there is a precise topography of neuronal connections. CNS development begins in the embryo with dorsal ectodermal cell proliferation forming the neural plate, which closes to form the neural tube, followed by the rapid division of pluripotent cells, their migration to the periphery of the neural tube, and differentiation into neural or glial cells (Zigmond et al., 1999). The final precise topographical arrangement is brought about by the intrinsic property of individual neurons and the action of guidance cues within the CNS extracellular environment. Any disruption to this developmental process may lead to abnormal CNS development ranging from mild to extreme consequences. The severity of these consequences is dependent upon the nature of the disruption, being either genetic or acquired, and the time at which the disruption occurs, be it in the embryonic, neonatal or the mature adult system.

Regarding the time at which the disruption occurs, the neonatal brain (and possibly the embryonic brain) has a special ability to re-organise its synaptic connections following injury by developing alternate pathways to limit the deficits caused by the injury (Angaut et al., 1982; Naus et al., 1984; Spear, 1995; Zagrebelsky et al., 1997). The mechanisms behind this process are unknown, however, it is believed to be the

combination of low levels of growth inhibitory factors and high levels of growth promoting factors in the immature system that allow appropriate axonogenesis and synaptogenesis (Widenfalk et al., 2001). One group of the growth promoting factors, the neurotrophins, are thought to aid the development of these alternate pathways due to their role in normal CNS development and their temporospatial expression following injury (Henderson, 1996).

1.3 THE NEUROTROPHIN FAMILY

The neurotrophins are a family of peptides that play a key role in nervous system development and maintenance. This family includes neurotrophin-3 (NT-3), BDNF, nerve growth factor (NGF), neurotrophin-4 (NT-4), neurotrophin-5 (NT-5), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7). NT-4 and NT-5 are thought to be the same protein, identified in the toad *Xenopus laevis* and mammalian species, respectively (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992). Although this has yet to be clarified, both neurotrophins are often referred to as NT-4/5. NT-6 and NT-7 have both been identified in fish (Gotz et al., 1994; Nilsson et al., 1998) but currently no mammalian equivalent is known.

1.3.1 Neurotrophin structure, biochemistry and receptor interaction

The structure and biochemistry of neurotrophins has been well documented and aids our understanding of the interactions between ligands and their cognate receptors, thus ultimately understanding their role in neuronal development, connectivity and function. Neurotrophins are synthesized as precursor proteins, which are proteolytically cleaved at dibasic residues to produce the mature protein (Selby et al., 1987). Neurotrophins are a group of dimeric polypeptides functioning *in vivo* as biologically active homodimers (Bothwell and Shooter, 1977; Gotz et al., 1994). While the peptides NT-3, BDNF and NGF are encoded by 3 distinct genes (Lewin and Barde, 1996), it is thought that NT-3 may be the neurotrophin ancestoral gene as the replacement of seven amino acid residues at strategic locations results in a neurotrophin that possesses the biological activities of NT-3, NGF and BDNF (Urfer et al., 1994). Additionally, neurotrophins are a highly conserved group of proteins among mammals, such that all neurotrophins have

the same amino acid sequence motif in the precursor protein sequence and that BDNF and NT-3 even have similar amino acid sequences in different mammalian species (Maisonpierre et al., 1992).

In regards to neurotrophin biochemistry, all neurotrophins exhibit a cluster of positively charged residues on their surface for binding to the receptors (Ibanez et al., 1992; Ryden et al., 1995), and the spatial distribution of these charges may explain some of their neurotrophin-receptor binding properties. Neurotrophins exert their effects on neurons through a two-receptor system; all neurotrophins bind to a low affinity receptor and each neurotrophin binds to at least 1 high affinity receptor.

1.3.1.1 Low affinity receptor

All neurotrophins bind to the low affinity nerve growth factor receptor, known as the pan-neurotrophin receptor (p75). This receptor is a member of the tumor necrosis factor receptor superfamily and is a 75- to 80-kilodalton single chain glycoprotein (Bibel and Barde, 2000; Hempstead, 2002). p75 has an extracellular binding domain, a single transmembrane domain and a cytoplasmic domain (Johnson et al., 1986). All neurotrophins bind to this receptor's extracellular domain with equal low affinity, but with different kinetics (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992).

Functionally, studies have shown that p75 interacts intracellularly with the inhibitory Nogo receptor, mediating inhibitory effects on axon growth (Wang et al., 2002; Wong et al., 2002). More traditionally however, ligand binding to p75 is known to activate biological functions such as promoting intracellular signalling, regulating ligand affinity and aiding ligand-receptor retrograde transport (transport from axon terminal to cell soma). In the first instance, in the absence of any secondary receptor activation, p75 influences either neuronal survival or cell death by eliciting its own intracellular signalling pathways. This might be due to availability of neurotrophins to the receptor or multiple modes of action of p75 (Casaccia-Bonnefil et al., 2002; Frade et al., 1996; Yano and Chao, 2000). In the second instance, while high affinity receptor signalling can occur in the absence of p75, in its presence the neurotrophin dose required to elicit a

neuronal response is four times less and the resulting signalling response is increased eight fold (McInnes and Sykes, 1997; Verdi et al., 1994; Yano and Chao, 2000). Therefore the presence of p75 amplifies the biological response of neurotrophins and this is thought to be due to a reduction in the number of high affinity sites available to the neurotrophins (Barker and Shooter, 1994; Weskamp and Reichardt, 1991). Lastly, in regards to ligand-receptor transport, ligand-p75 binding aids the retrograde transport of the ligand-high affinity receptor complex (Curtis et al., 1995). Therefore in summary, p75 activation plays a key role in the regulation of neurotrophin-high affinity receptor interaction.

1.3.1.2 High affinity receptors

The majority of biological effects of neurotrophins are mediated via high affinity receptors. These receptors derive their name from a proto-oncogene tropomyosin receptor kinase from which they were discovered and are now commonly referred to as trk receptors (Barbacid et al., 1991). All trk receptors possess a 66 to 68 % amino acid sequence homology (Lamballe et al., 1991), with each having an extracellular binding domain and an intracellular catalytic tyrosine kinase domain (Schneider and Schweiger, 1991). In the absence of any signal, these receptors are localised to intracellular cytoplasmic membrane vesicles (endosomes), however electrical activity, cAMP and/or the presence of calcium can stimulate fusion of these vesicles to the cell membrane followed by trk insertion into the cell surface (Du et al., 2000; Meyer-Franke et al., 1998). Neurotrophins bind to these receptors, either on the cell surface or within the intracellular endosomes to form a receptor-ligand complex that initiates receptor dimerization (Arevalo et al., 2000; Korsching, 1993; Zhang et al., 2000).

The high affinity receptors are denoted trkA (p140 trkA), trkB (p145 trkB) and trkC (p145 trkC) and in mammals are traditionally known to be activated by their cognate ligands NGF (trkA), BDNF and NT-4 (trkB) and NT-3 (trkC) (Barbacid, 1994; Klein et al., 1989; Lamballe et al., 1991). Experiments in culture have also identified that NT-3 may bind to both trkA and trkB receptors and NT-4 may also bind to trkA (Berkemeier et al., 1991; Glass et al., 1991; Squinto et al., 1991; Windisch et al., 1995). However,

any multiple binding *in vivo* is yet to be established. Despite this, multiple activation of trk receptors is important to consider as this may explain why the biological functions of each neurotrophin overlaps *in vivo*, and that blocking one neurotrophin may not prevent receptor activation.

While this 'additional binding' may be via a common functional epitope on the neurotrophins (Windisch et al., 1995), each trk receptor may have different ligand binding regions, as for example, the extracellular domain of human trkB (BDNF and NT-4 high affinity receptor) possesses two distinct ligand binding domains (Haniu et al., 1997). This identifies that different neurotrophins can activate the one receptor resulting in different intracellular cascades and thus having different biological functions.

Finally, all trk genes code for more than one receptor transcript, which results in a variety of functionally different receptor proteins. Different forms of trkB and trkC receptors have been observed that lack various amounts of the tyrosine kinase domain and while all forms mediate biological functions of the neurotrophins, it is unclear as to whether these forms interact with each other (Forooghian et al., 2001; Snider, 1994; Tsoulfas et al., 1993; Yacoubian and Lo, 2000).

1.3.2 Intracellular biological effects of neurotrophins

The binding of NGF, BDNF, NT-4 and NT-3 to their high affinity receptors results in autophosphorylation of the receptor's intracellular tyrosine kinase domain and internalisation of the ligand-receptor complex into endosomes (in this case these are activated signalling intermediates), which are retrogradely transported towards the soma of the target neuron (Ginty and Segal, 2002; Howe et al., 2001).

Formation of the ligand-trk receptor complex initiates three types of signal transduction cascades. Understanding these intracellular cascades is essential for understanding trk receptor function as two different trk receptors may activate the same intracellular signalling cascade thereby having similar effects on cell function. While the intricacies of these cascades are yet to be elucidated, one cascade regulates trk internalisation and

retrograde transport to the cell soma resulting in neuronal survival and axonogenesis, (through activation of the phosphatidylinositol–3 (PI-3) kinase signalling pathway: Huang and Reichardt, 2001; Kaplan and Cooper, 2001). A second cascade mediates axonogenesis and neuronal survival in response to injury, through activation of the mitogen-activated protein/mitogen-activated protein with extracellular-signal-regulated protein (MAP/MEK) kinase signalling pathway (Heumann, 1994; Huang and Reichardt, 2001; Kaplan and Cooper, 2001). In addition, a third cascade regulates the production of intracellular calcium and thus has an effect on cellular functions such as axonogenesis, synaptogenesis and neurotransmitter release, through activation of the phospholipase C and protein kinase C signalling pathways (Heumann, 1994; Kaplan and Cooper, 2001; Schulman and Hyman, 1999) (Figure 1.1).

Furthermore, the location of the trk receptor alters the final biological function of the neurotrophin (Whitmarsh and Davis, 2001) through activating different intracellular cascades. For example, the same growth factor induces different effects on a cell depending on whether the ligand-receptor complex is on neuronal cell body or distal axon (Watson et al., 2001). Unfortunately, very little is known about this phenomenon at the moment but it may also explain the different biological functions observed with selected neurotrophins *in vivo*, given that neurotrophins may be exogenously applied at different locations of the neuron.

1.3.3 Role of neurotrophins in CNS development

Neurotrophins have a well established role in CNS neuronal development and maintenance including cell proliferation, differentiation, axonogenesis, synaptogenesis, maturation and survival.

Evidence for neurotrophin involvement in the early stages of nervous system development comes from temporospatial patterns of neurotrophin and receptor expression and gene knockout studies. Initial embryonic expression of NT-3 coincides with the onset of neurogenesis. NT-3 is synthesized in the embryonic brain from

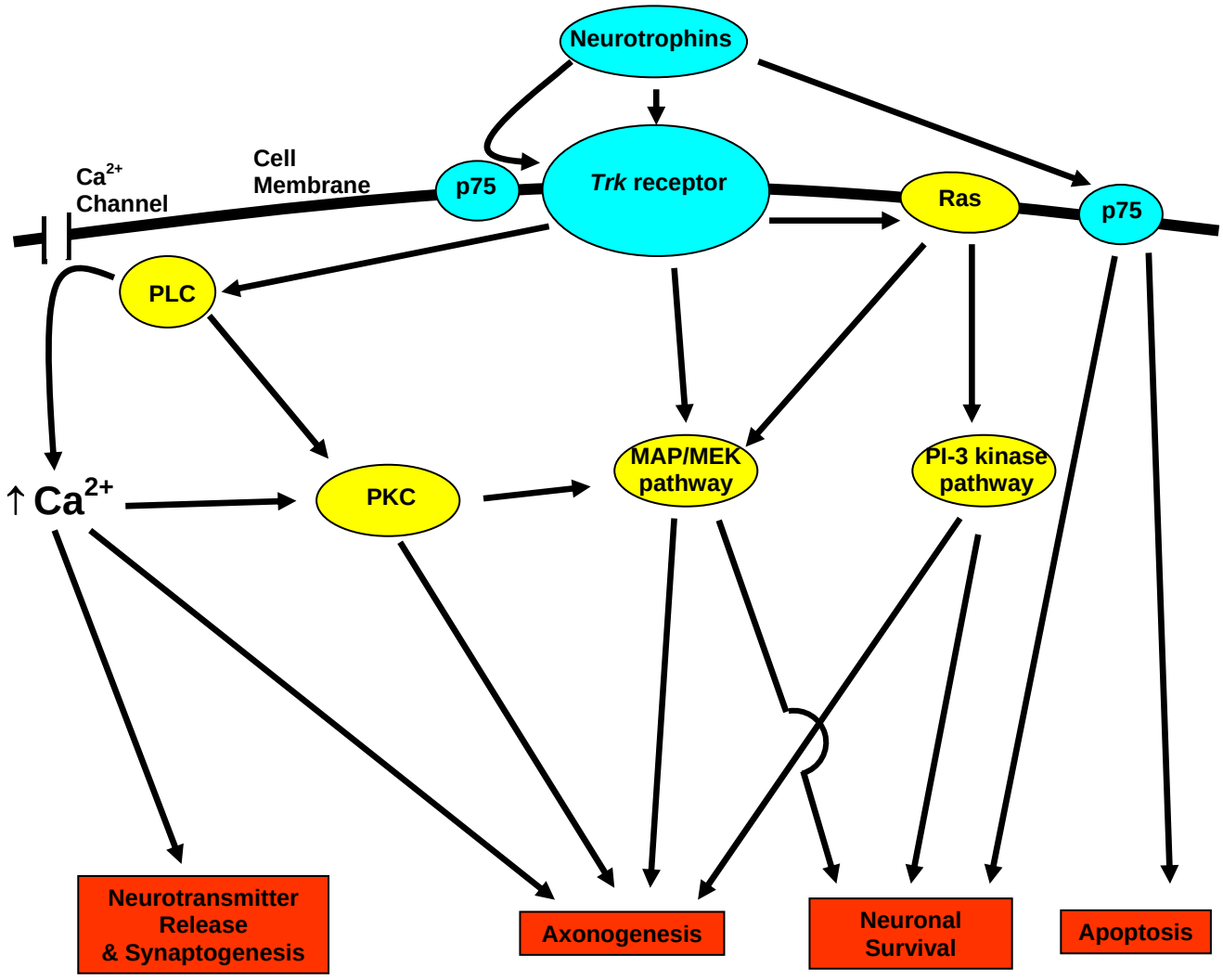

Figure 1.1 Intracellular signalling cascades activated by neurotrophins
Diagram illustrating the intracellular signal transduction cascades (yellow) activated by neurotrophins binding to the extracellular domain of their cognate receptors (Heumann, 1994; Huang and Reichardt, 2001; Schulman and Hyman, 1999; Ullrich and Schlessinger, 1990). These pathways are involved in apoptosis, neuronal survival, axonogenesis, neurotransmitter release and synaptogenesis. The p75 receptor is involved in both neuronal survival and apoptosis due to its interaction with trk receptors and inhibitory receptors (e.g. Nogo). Note the overlap between individual signalling pathways.

Key: Ca^{2+} = calcium; MAP/MEK = mitogen-activated protein/mitogen-activated protein with extracellular-signalling-regulated protein; p75 = low affinity nerve growth factor receptor; PI-3 kinase = phosphatidylinositol-3 kinase; PKC = protein kinase C; PLC = phospholipase C; Ras = membrane bound peptide involved in the intracellular signalling pathway; Trk receptor = high affinity tyrosine kinase receptor; ↑ = increase.

embryonic day 13 to 14 (E13 to E14), although the sites of synthesis are not known, NT-3 is synthesized extensively from E16 to E17 (Maisonpierre et al., 1990). NT-3 promotes neural crest progenitor cell proliferation *in vitro* and deletion of NT-3 leads to premature differentiation and subsequent cell death (Farinas et al., 1996; Kalcheim et al., 1992). Other neurotrophins involved in the early development of the CNS are BDNF and NT-4. Similarly, these neurotrophins are also associated with neuronal differentiation (Benraiss et al., 2001; Ip et al., 1993; Neveu and Arenas, 1996; Pencea et al., 2001; Sieber-Blum, 1991).

Following differentiation neurons begin to undergo neurite outgrowth to develop axons and dendrites. NT-3 (McAllister et al., 1995; Niblock et al., 2000), BDNF (Hanamura et al., 2004; McAllister et al., 1995; Schwartz et al., 1997) and NT-4 (McAllister et al., 1995; Wiklund and Ekstrom, 2000) all appear to influence axonogenesis with different neurotrophins acting on different neuronal populations. Additionally, NGF has been found to stimulate the phosphorylation of a growth-associated protein (GAP-43) (Meiri and Burdick, 1991), indicating that NGF also initiates axonogenesis (Carulli et al., 2004).

After developing neurons extend axons and dendrites, the next important stage is to make appropriate synaptic connections with neighbouring neurons. BDNF (Narisawa-Saito et al., 2002; Yamada et al., 2002) and NT-3 (Collin et al., 2001; Martinez et al., 1998) promote synaptogenesis, while NT-3 also enhances the stability of post-synaptic receptors (Blondel et al., 2000).

From early development through to adulthood, NT-3 (Castellanos et al., 2002) NT-4 (Rabacchi et al., 1999) and NGF (Frade et al., 1996; Kume et al., 2000) all prevent neuronal cell death. Likewise, BDNF is also implicated in neuronal survival although different experimental paradigms have found differing results. While administrating BDNF, up-regulating trkB, or a null mutation of trkB in juvenile or adult animals appears to delay neuronal death (Cheng et al., 2002; Gillespie et al., 2003; Pollock et al., 2003), a null mutation of BDNF does not result in a decrease of specific CNS neurons

(Ernfors et al., 1994; Segal et al., 1997). These different findings may indicate that individual neurotrophins are required for the survival of only particular neuronal populations, or that there is a change in neurotrophin-receptor responsiveness following a single neurotrophin gene knock out (causing other neurotrophins to interact with the trk receptor in the absence of the normal ligand). Additionally, studies utilising different levels of neurotrophins are likely to affect the outcome of the experiment. For example, while BDNF rescues certain neuronal populations from cell death, an excess of this peptide is known to induce NMDA excitotoxicity and thus cell death (Glazner and Mattson, 2000). This is important to consider as administering high levels of BDNF may have no effect on neuronal survival, while lower levels may possibly promote survival.

In summary, the early stages of CNS development, encompassing neurogenesis and proliferation, appear to be under the control of NT-3, while neuronal differentiation is controlled by BDNF and NGF. The later stages of CNS development, including axonal and dendritic outgrowth, synaptogenesis and neuronal survival appear to be controlled by a cocktail of all neurotrophins, but the extent to which each neurotrophin is involved is yet to be determined. In order to understand which neurotrophins may aid post-lesion CNS repair the role of each neurotrophin within the CNS will be examined following manipulation to the CNS.

1.3.4 Role of neurotrophins in CNS plasticity

As may be anticipated from their role in normal CNS development, neurotrophins are also involved in CNS plasticity. Through manipulation of these peptides useful information is gained that may be used as a powerful tool for repairing the injured CNS, or optimizing the function of remaining paths. Experimental evidence indicates that injury to the CNS alters the synthesis of neurotrophins and their receptors.

In regards to neurotrophin levels following injury, few studies have observed NT-4 or NGF expression and as such these peptides will receive little mention in this section.

Many studies have observed BDNF regulation following injury and found an upregulation of this peptide in neonatal animals following complete spinal cord transection (Nakamura and Bregman, 2001) and in the adult system following either deafferentation, axotomy or cortical contusion (Hicks et al., 1999; Kobayashi et al., 1996; Li et al., 2001; Venero et al., 2000). Unlike BDNF however, there is currently conflicting evidence in the literature as to the regulation of NT-3 following injury. Following complete spinal cord transection in neonatal animals, NT-3 expression is decreased in the spinal cord (Nakamura and Bregman, 2001), while complete transection in the adult induces NT-3 up-regulation in the spinal cord and cerebellum (Kawakami et al., 2000). This conflict may simply be that neurons respond differently at different ages, or that the post-lesion time points used in each experiment were different and therefore do not give a true description of neurotrophin expression patterns.

Equally important to neurotrophin expression following injury is the pattern of receptor expression, as increased availability of neurotrophins is only effective if the appropriate receptors are available. Following axotomy of developing neurons, or deafferentation or axotomy in the adult system, there is a transient up-regulation of p75 (Nitz et al., 2001; Turner and Perez-Polo, 1998). This is concurrent with the known role of p75 in mediating the biological effects of neurotrophins through improving neurotrophin and high affinity receptor binding (Ibanez et al., 1992; Yano and Chao, 2000). However, since p75 is also a co-receptor for the growth inhibitory Nogo receptor, whose activation causes growth cone collapse (He and Koprivica, 2004), p75 upregulation may not always aid recovery. As for the trk receptors, studies have not identified changes in trkA levels, although trkC is down-regulated in adult axotomised neurons (Lu et al., 2002). Unfortunately, as with the neurotrophins, conflict exists in the literature as to the response of trkB following injury. In the adult system trkB is upregulated in some neurons following axotomy (Lu et al., 2002; Venero et al., 2000) or cortical contusion (Hicks et al., 1999), but is downregulated following axotomy of other neuronal populations (Kobayashi et al., 1997; Spalding et al., 2005). It is possible that this different regulation (up or down) is dependent upon the type of neuron affected, since

all of these studies examined different cell populations at different post-lesion time points and may not provide an accurate view of expression patterns. Regardless of the existing conflicts, these data highlight the importance of neurotrophins and their receptors following injury, which will inevitably affect a neuronal system's capacity to repair. While much work still needs to be done, it is possible to suggest from the literature that following injury an increased expression of BDNF in the adult system up-regulates trkB, while increased expression of NT-3 down-regulates trkC: making BDNF and not other neurotrophins a candidate for beneficial post-lesional treatment.

A problem in this story exists however, in that the ability of the CNS to up-regulate neurotrophins decreases with age (Nakamura and Bregman, 2001), such that axonal repair no longer occurs in the adult brain. It follows therefore that administering neurotrophins to the adult CNS may overcome this problem and aid recovery. Indeed there is evidence to support this theory as neurotrophin administration aids axonal regeneration after complete spinal cord transection (Bregman et al., 2002). However spinal cord lesion studies utilising BDNF and NT-3 have shown only limited sprouting of neurons into the distal cord (Ruitenberg et al., 2003; Zhou et al., 2003; Zhou and Shine, 2003). While some of these studies show improved functional recovery (Coumans et al., 2001; Jin et al., 2002), the sprouting is limited and has not been correlated with the improvement in function (Ruitenberg et al., 2003).

Additionally, trauma is inevitably associated with the loss of some neurons, making regeneration of damaged axons impossible for those neurons which die (Novikova et al., 2000). To counteract this problem, administering BDNF, NT-4 or NT-3 around the cell body of neurons that have undergone axotomy or deafferentation prevents the death of certain cell populations (Gillespie et al., 2003; Sadakata et al., 2004; Tuszynski et al., 1996). This treatment however does not prevent the death of all neuronal cell populations and therefore another strategy is required to reduce the deficits obtained from the injury.

In stark contrast to the regeneration of damaged pathways, some areas of the immature CNS develop alternate neuronal pathways bypassing the area of damage and reducing the extent of functional deficits. For example alternate pathways occur in the visual cortex (Spear, 1995), the spinal cord (Barth and Stanfield, 1990; Naus et al., 1986) and in the model used for this PhD project, the cerebellum (Angaut et al., 1982; Angaut et al., 1985; Sherrard et al., 1986). These alternate pathways develop similar topography to the normal path with normal branching patterns and arborisations (Kuang and Kalil, 1990; Sugihara et al., 2003). The control of these alternate pathways is unclear, however NGF, BDNF, NT-4 and NT-3 all influence these pathways to some extent (Klau et al., 2001; Schwyzer et al., 2002; Yamada et al., 2002). Importantly, the development of these alternate pathways compensates for loss of function from the injury (Barth and Stanfield, 1990; Dixon et al., 2005; Weber and Stelzner, 1977). Therefore the question arises as to how these pathways can be induced in more mature brain. Since following injury to either the immature or adult CNS there is an up-regulation of neurotrophins and receptors in the uninjured remaining side of the brain (Hicks et al., 1999; Nitz et al., 2001), then perhaps addition of neurotrophins can manipulate these 'naturally plastic pathways' in the adult brain to improve functional recovery.

1.3.5 Neurotrophins in CNS summary

During normal CNS development NGF, NT-3, NT-4 and BDNF interact with trk receptors to induce neuronal proliferation, differentiation, axonogenesis, synaptogenesis and neuronal survival. The ability of neurotrophins to bind to more than one trk receptor and the overlapping intracellular signalling pathways probably results in each neurotrophin having more than one function. Following neuronal injury however, only those neurotrophins that influence neuronal survival, axonal growth and synaptogenesis are required to repair the injury. Since BDNF and its receptors are involved in neuronal survival, axonal growth and synaptogenesis, and upregulated following injury, this highlights the potential role of BDNF in influencing the formation of new functional pathways. Other neurotrophins and their receptors are either not involved in neuronal survival, axonogenesis or synaptogenesis during normal CNS development or are

downregulated following injury. Therefore this thesis will examine the involvement of BDNF in the development of post-lesion axonal pathways that reinnervate previously denervated regions.

1.4 THE CEREBELLUM

An ideal *in vivo* model in which to study the role of neurotrophins in plasticity is the olivocerebellar pathway in the cerebellum of rats, as this pathway can be induced to undergo post-lesional plasticity during development, but which ceases upon maturation. It is therefore necessary to understand the organisation of the cerebellum and the role of neurotrophins in cerebellar development and plasticity.

1.4.1 Cerebellar organisation

The adult rat cerebellum lies dorsal to the brainstem and can be divided into two hemispheres that are separated by a midline vermis (Larsell, 1952) (Fig 1.2A). These hemispheres can be further divided into anterior and posterior lobes, separated by the primary fissure and a ventrally placed flocculonodular lobe (Larsell, 1952; Voogd, 1967). These three lobes are further subdivided by shallow, horizontal fissures into a series of transversely orientated lobules, termed folia, which are classified in the rat by roman numeral terminology I to X (Larsell, 1952) (Fig 1.2B).

The cerebellum is composed of an outer cortical layer overlying a central white matter. This outer cortex is made up of three main layers: an inner layer of granule cell neurons called the internal granular layer, followed by a monolayer of large neurons, the Purkinje cells and then the most superficial molecular layer, which contains Purkinje cell dendritic trees and parallel fibres, the axons of granule cells, travelling in a transverse direction (Palay and Chan-Palay, 1974; Ramon y Cajal, 1911) (Fig 1.2C). Within the underlying white matter there are four pairs of deep cerebellar nuclei (DCN): medial nucleus, interpositus anterior and posterior nuclei and lateral nucleus (De Camilli et al., 1984; Palay and Chan-Palay, 1974), which form the cerebellar outflow to the rest of the CNS, along with the afferent and efferent cortical fibres (Voogd, 1995).

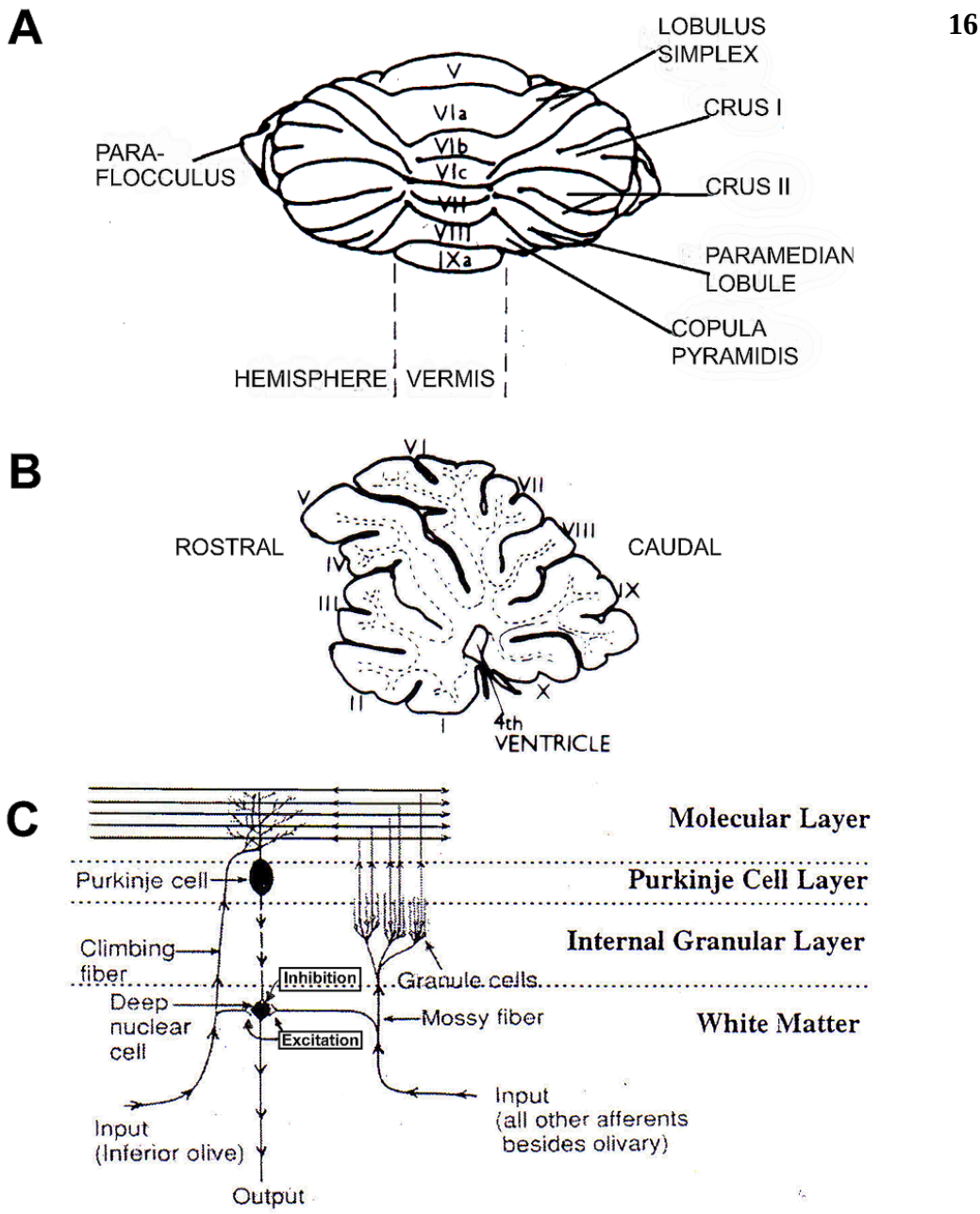

Figure 1.2 Cerebellar morphology and structure
(A) Dorsal view of a rat cerebellum displaying lobules of the vermis and left and right hemispheres. The anterior and posterior lobes of the hemispheres and vermis are divided into lobules and classified by name and roman numeral terminology from I to X (modified from Larsell, 1952). Lobules I to IV and X are not visible in this view.
(B) Midsagittal section of a rat cerebellum with numbered folia and location of the fourth ventricle (modified from Larsell, 1952). The cerebellum has an outer cortical layer overlying a central white matter (lobules I-X visible).
(C) Schematic showing the layers and circuitry of the cerebellum. Shown are the cells of the molecular layer, Purkinje cell layer and internal granular layer of the cerebellar cortex, with the deep cerebellar nuclei of the white matter (modified from Guyton, 1991).

The cerebellar cortical circuit (Fig 1.2C) is composed of (1) the Purkinje cell, which is the only outflow of the cortex terminating on the DCN, and (2) two main extra-cerebellar afferents. These afferents are olivocerebellar fibres (terminate as climbing fibres) that synapse directly onto Purkinje cells, and mossy fibres that synapse indirectly onto Purkinje cells via granule cells and their parallel fibre axons (Palay and Chan-Palay, 1974; Ramon y Cajal, 1911). Climbing fibre input activates a few Purkinje cells in a sagittal zone (Eccles et al., 1966a; Sugihara et al., 1999), while mossy fibres, by their distribution through granule cells, stimulate a broad transverse beam of Purkinje cells (Eccles et al., 1966b). Activity is modified by inhibitory interneurons located in the molecular layer, the Golgi, basket and stellate cells, which receive afferent information through parallel fibres and feedback to the granule and Purkinje cells.

1.4.1.1 Cerebellar circuitry and function

The cerebellar cortical circuit (stated above) plays a role in the control of complex motor co-ordination. In the adult cerebellar structure, the indirect mossy fibre input to the Purkinje cells determines an animals' movement while the climbing fibre input conveys error in movement (Marr, 1969) by depressing Purkinje cell responsiveness to parallel fibre input (Granit and Phillips, 1956). Thus climbing fibre adjustments to Purkinje cell activity are made in response to an error in order to adopt new motor strategies for a particular motor task (Rondi-Reig et al., 1997). This is important for rhythmical synchronisation of previously learnt sensorimotor skills (Rondi-Reig et al., 1997).

The vermal and paravermal zones of the cerebellum regulate motor control while the lateral hemisphere and paraflocculus control spatial learning and vestibulo-ocular reflexes respectively (Billig and Balaban, 2004; Bobee et al., 2000; Joyal et al., 1996; Rushmer et al., 1976). Specifically, Purkinje cells of the vermis are activated by climbing fibres during truncal movements that involve simple equilibrium skills, while Purkinje cells of both the vermis and paravermis are activated in response to climbing fibre input, for motor skills requiring greater rhythmical synchronisation (Bobee et al., 2000; Joyal et al., 1996; Rondi-Reig et al., 1997; Rushmer et al., 1976).

1.4.1.2 Purkinje cells

The Purkinje cell is the only output neuron of the cerebellar cortex and therefore critical to transmit cerebellar cortex circuit information to other neural circuits. In the adult rat cerebellum there are 3.5 x 10^5 Purkinje cells, which on average have a soma 21 µm wide and 25 µm long, located in a monolayer (Armstrong and Schild, 1970; Palay and Chan-Palay, 1974). The Purkinje cell has a large, pale nucleus and an extensive dendritic tree that arises from the apical pole of the cell and extends directly outwards in a sagittal plane toward the surface of the folium (Palay and Chan-Palay, 1974; Ramon y Cajal, 1911). At right angles to the longitudinal axis of the folium, in the parasagittal plane, the dendritic tree is 300 to 400 µm wide, but only 15 to 20 µm wide in the coronal plane (Palay and Chan-Palay, 1974). The dendritic tree arises from 1 to 2 primary dendrites, which divide into numerous secondary and tertiary dendritic branchlets (Berry and Bradley, 1976). Located on these dendritic branches are many short dendritic spines, which are classified into 2 types (Ramon y Cajal, 1911). The first type articulates with parallel fibres and is located on the Purkinje cell tertiary spiny branchlets, while the second is found on the main trunk of the Purkinje cell primary and secondary branches and is exclusive for climbing fibre synapses (Larramendi and Victor, 1967; Ramon y Cajal, 1911; Scelfo et al., 2003).

The Purkinje cell axon arises from the basal pole of the soma and is approximately 1 µm in diameter (Palay and Chan-Palay, 1974). It descends through the internal granular layer into the white matter (Ramon y Cajal, 1911) giving off collateral branches that return upwards through the granular layer synapsing on adjacent Purkinje cells and inhibitory interneurons (Chan-Palay, 1971; De Camilli et al., 1984; O'Leary et al., 1968; Palay and Chan-Palay, 1974). The Purkinje cell axon then converges onto neurons of the DCN in a precise organization (Armstrong and Schild, 1978b; Armstrong and Schild, 1978a; Buisseret-Delmas and Angaut, 1993).

1.4.1.3 Mossy fibre-granule cell-parallel fibre system

The cerebellum has two major types of extrinsic input. The first major afferents are mossy fibres, which arise from many areas in the CNS including spinal projection

neurons, the pontine nuclei and reticular nuclei. Information to the cerebellum from these fibres is received from diverse areas such as the spinal cord, motor cortex and basal ganglia (Berry et al., 1980; Caddy et al., 1977; Sotelo and Alvarado-Mallart, 1991). Mossy fibres are thick, heavily myelinated axons, which divide numerous times as they pass through and around the DCN (Palay and Chan-Palay, 1974; Ramon y Cajal, 1911). At this point the fibres further subdivide into 20 to 30 collaterals terminating in the internal granular layer (Caddy et al., 1977; Palay and Chan-Palay, 1974; Ramon y Cajal, 1911). The terminal portion has an elongated core and expands into a flower like figure termed the mossy fibre 'rosette', with each rosette innervating several granule cells (Palay and Chan-Palay, 1974). The granule cell axon ascends into the molecular layer, bifurcates and runs transversely as parallel fibres (Palay and Chan-Palay, 1974). Parallel fibres synapse on both Purkinje cell dendritic spines and inhibitory interneurons (Chan-Palay and Palay, 1970; Mason and Gregory, 1984; Palay and Chan-Palay, 1974). The granule cell input to Purkinje cells in the rat is relatively large; for every Purkinje cell there are estimated to be approximately 897 granule cells (Lange, 1975).

1.4.1.4 Climbing fibres

Climbing fibres are the second major afferent to the cerebellum. They originate in the inferior olivary complex in the ventral medulla, cross the midline, enter the cerebellum through the contralateral inferior cerebellar peduncle and extend into the cerebellum synapsing onto Purkinje cells located in a narrow parasagittal microzone (Courville and Faraco-Cantin, 1978; Desclin, 1974; Morara et al., 2001; Sugihara et al., 1999; Sugihara et al., 2001; Sugihara and Shinoda, 2004). Olivary neurons in adult rats are 17.4 μm in diameter (Bourrat and Sotelo, 1983) and project their thick myelinated axons (2 to 3 μm) through the white matter of the cerebellum, to branch in the sagittal plane, supplying different lobules of the cortex (Ramon y Cajal, 1911; Sugihara et al., 2001). The olivocerebellar axons ascend through the internal granular layer and at the level of the Purkinje cell layer they become unmyelinated and form climbing fibre terminal arbors alongside the cell body and dendrites of Purkinje cells (Ramon y Cajal, 1911; Scheibel and Scheibel, 1954; Sugihara et al., 1999). In adults rats, single olivocerebellar axons have an average of 7 climbing fibres (Sugihara et al., 2001) and each Purkinje cell

receives information from only a single climbing fibre (Eccles et al., 1966a; Sugihara et al., 1999). This input has a powerful excitatory action, commonly termed a 'climbing fibre response' (Eccles et al., 1966a). Additionally, 91 % of olivocerebellar axons also project thin collaterals to innervate localized parasagittal strips within the DCN, which in turn receive input from Purkinje cells that are innervated by the same climbing fibre (Buisseret-Delmas, 1988a; Buisseret-Delmas, 1988b; Ruigrok and Voogd, 2000; Shinoda et al., 2000; Sugihara et al., 1996; Wiklund et al., 1990).

The olivocerebellar projection is topographically organized terminating in parasagittal zones of the cerebellar cortex, so that each strip receives terminations from a specific subnucleus of the inferior olivary complex (Buisseret-Delmas and Angaut, 1993) (Fig 1.3). Generally, neurons located laterally within the inferior olivary complex project more rostrally within the cerebellum than neurons of medial olivary origin, which project more caudally (Azizi and Woodward, 1987; Wharton and Payne, 1985). The inferior olivary complex in the rat consists of three subdivisions: the medial accessory olive (MAO), the dorsal accessory olive (DAO) and the principal olive (PO). Climbing fibres originating from the horizontal lamellae of the MAO (caudo-lateral MAO), project in a sagittal zone to the vermal anterior lobe (Azizi and Woodward, 1987; Campbell and Armstrong, 1983; Hrycyshyn et al., 1982; Sugita et al., 1989). Those climbing fibres originating from the vertical lamella of the caudal MAO, which is located medially, project to the posterior lateral vermis and the flocculus (Azizi and Woodward, 1987; Buisseret-Delmas and Angaut, 1993; Sugihara and Shinoda, 2004; Sugita et al., 1989), while the rostral lamella projects to the lateral hemispheres, paraflocculus and flocculus (Azizi and Woodward, 1987; Campbell and Armstrong, 1983). In the PO, both the dorsal and ventral lamella project to a specific sagittal strip in the lateral cerebellar hemispheres (crus I, crus II, paramedian lobule & copula pyramidis: Azizi and Woodward, 1987; Buisseret-Delmas and Angaut, 1993; Campbell and Armstrong, 1983; Furber and Watson, 1983; Sugihara and Shinoda, 2004). The dorsal fold of the DAO projects to the paravermal anterior lobe (Azizi and Woodward, 1987; Hrycyshyn et al., 1982) while the more rostral ventral fold of the DAO projects to

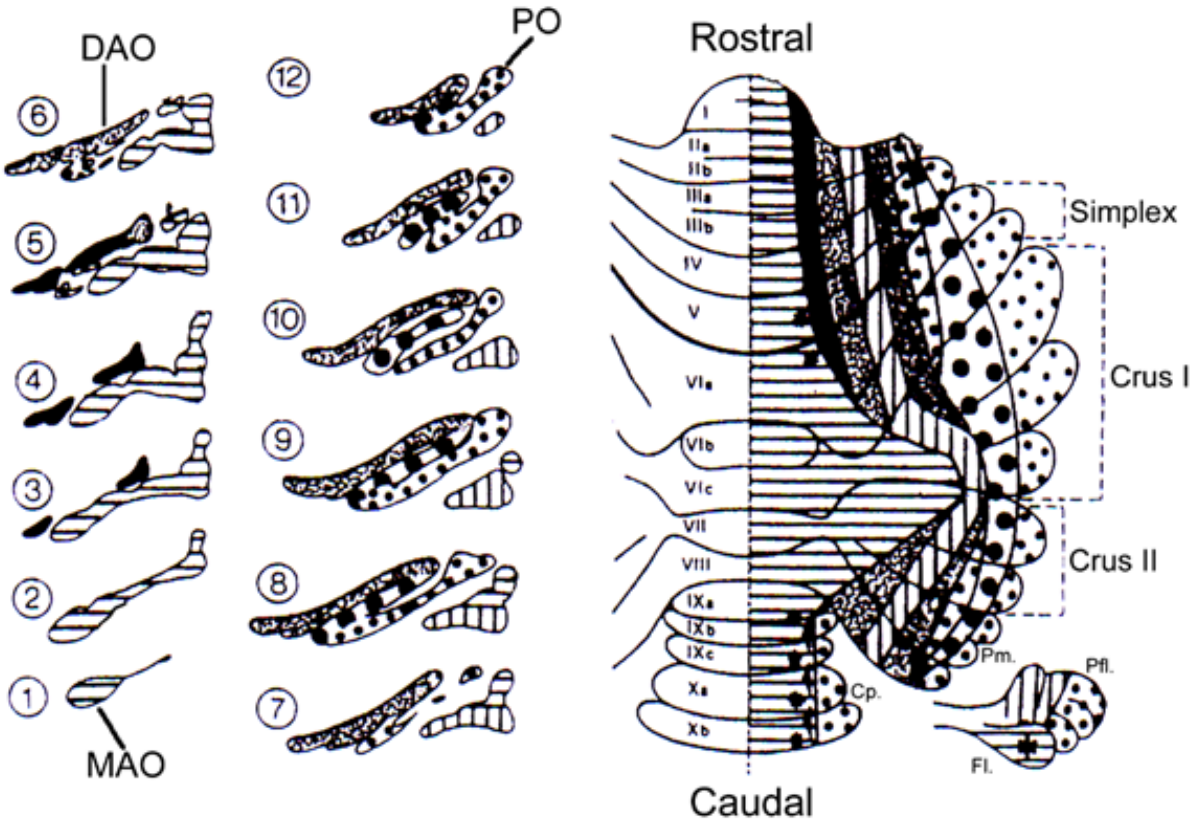

Figure 1.3 Olivocerebellar pathway topography
Diagram showing the topographical arrangement of the olivocerebellar pathway in the rat (Buisseret-Delmas and Angaut, 1993). The inferior olivary complex projects topographically organised neurons to specific regions of the cerebellum. The left hand side of the diagram shows coronal sections through the left inferior olivary complex (1-12). Section 1 is the most caudal section and section 12 is the most rostral section. The right hand side of the diagram is an unfolded cerebellar cortex, illustrating the topographical regions of the olivary innervation with respect to the subnucleui of the inferior olive. The nomenclature and lobule numbering of the flattened cerebellar cortex follows that of Larsell, 1952.

Olivary cells within sections 1 – 12 project terminal axons into the corresponding shading areas of the cerebellum on the right hand side.

Key: DAO = dorsal accessory olive; MAO = medial accessory olive; PO = principal olive.

the paravermal posterior lobe (Azizi and Woodward, 1987). This topography is important to consider as alterations to this topography map affect the animals function.

1.4.1.5 Cerebellar organisation summary

The cerebellum has two major afferents, terminating on Purkinje cells in the cortex either directly (climbing fibres) or indirectly (mossy fibres) and activate the cortical circuit. While a Purkinje cell receives activity from a single climbing fibre, organised in a precise topography, mossy fibres stimulate numerous Purkinje cells, through their granule cell-parallel fibre distribution. Activity within this cerebellar circuit is modified by the inhibitory interneurons including Golgi, basket and stellate cells, which receive afferent information though parallel fibres and terminate on both granule cells and Purkinje cell somata and dendrites. Purkinje cell axons then leave the cerebellar cortex and inhibit their target neurons in the DCN. Axons from the DCN then exit the cerebellum through both the inferior and superior cerebellar peduncles to terminate widely in other areas of the brain.

1.4.2 Cerebellar development: anatomical structure

Cerebellar development in rats is predominantly postnatal, although olivary neurons, deep cerebellar neurons, Purkinje cells and granule cell precursors are all produced embryonically in the neuroepithelium of the fourth ventricle. These neurons migrate into the developing cerebellar anlage before birth, mature and develop synaptic connections postnatally.

1.4.2.1 Granule cell development

The majority of granule cell precursors differentiate between embryonic days 11 (E11) and 21 (Altman, 1969) and migrate superficially in a rostro-dorsal direction over the surface of the cerebellar anlage, which ceases by E18 (Altman and Bayer, 1985b). From E17 the external granular layer (EGL) increases in depth so that by birth it is 6 to 8 rows thick, with 2 zones of differing morphology (Addison, 1911). The outer proliferative zone contains rounded neurons undergoing rapid division, while the inner pre-migratory zone is composed of fusiform, bipolar cells (Fig 1.4) extending their axons to form long, thin parallel fibres in a transverse plane (Addison, 1911; Altman, 1972a). These parallel

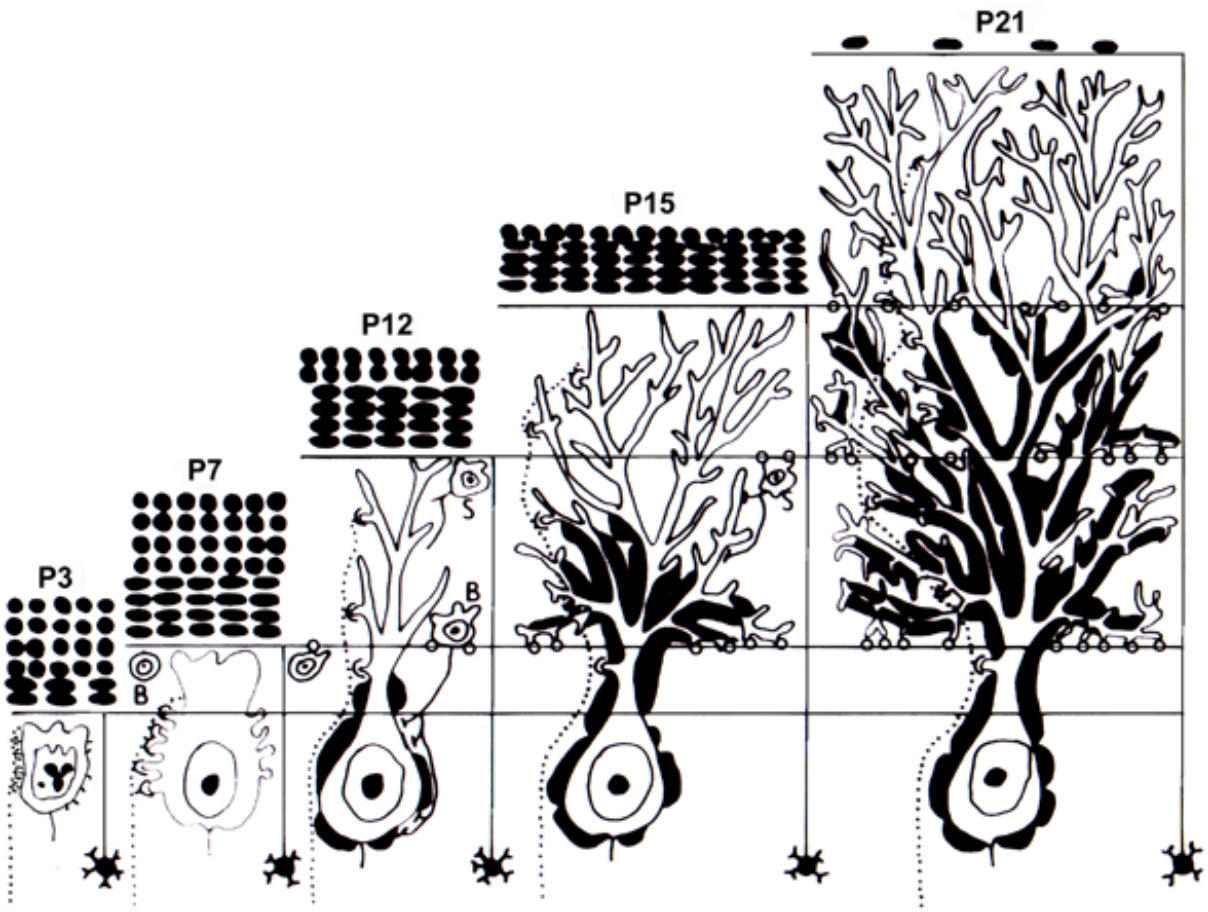

Figure 1.4 Cerebellar development
Diagram illustrating the major events in the maturation of the cerebellum from postnatal day 3 (P3) to P21 (modified from Altman, 1972b). At P3 climbing fibres have formed immature synapses on Purkinje cell transient dendrites. Cells of the external granular layer (EGL) have formed 2 zones: the outer proliferative zone and the inner pre-migratory zone. Parallel fibres synapse with Purkinje cells and granule cells are present in the internal granular layer (IGL). At P7 climbing fibres have synapsed onto Purkinje cell somatic protrusions. The EGL has become thicker and there are more parallel fibres and granule cells. From P7 to P21 the climbing fibres grow in parallel with growth of the Purkinje cell dendritic tree. The EGL diminishes and both parallel fibres and granule cells become more numerous. Single climbing fibre-Purkinje cell innervation is achieved by P15.

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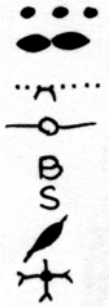


Cells of the proliferative zone
Bipolar cells of the premigratory zone
Climbing fibre and synapse
Parallel fibre and synapse
Basket cell
Stellate cell
Glial sheath
Granule cell

fibres synapse onto developing Purkinje cells and become functional from postnatal day 12 (P12: Altman, 1972a). After the parallel fibres have extended to their full length, the granule cell bodies migrate away from the EGL through the developing Purkinje cell layer to form the internal granular layer (IGL: Altman, 1972a), becoming mature and developing dendrites (Arsenio-Nunes and Sotelo, 1985). While this migration from the EGL to the IGL continues until P20 (Altman, 1972a), the first granule cell synapses with the mossy fibre afferents occur at P5 (Arsenio-Nunes and Sotelo, 1985) and become biochemically mature by P15 (Altman, 1972c). It is not until P20 however, that the IGL has reached biochemical maturity (Woodward et al., 1969).

1.4.2.2 Purkinje cell development

In addition to the embryonic neurogenesis of the EGL, the Purkinje cell plate is also formed prenatally. Purkinje cells are produced between days E12 and E14, migrate into the immature cerebellar anlage through the DCN where they leave their axons next to the neurons they will later innervate (Altman and Bayer, 1985a). In the cerebellum they settle near the surface of the cortex just below the developing EGL, forming the Purkinje cell plate (Altman, 1975).

Postnatally Purkinje cells develop an extensive dendritic tree in parallel with receiving widespread afferent synapses (Fig 1.4). From E19 to P2 a few immature non-functional synaptic connections exist between climbing fibre axon terminals and simple, pointed, transient dendrites of the most advanced but still fusiform shaped Purkinje cell somata (Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993; Morara et al., 2001). At P3 the immature Purkinje cell neurons are aligned into an irregular layer 6 to 12 cells deep, however by P5 the majority of Purkinje cells have dispersed into a monolayer (Addison, 1911; Altman, 1972b). By P7 the Purkinje cells have started to develop their primary dendrites while the transient Purkinje cell soma dendrites have disappeared (Armengol and Sotelo, 1991; Berry and Bradley, 1976; Larramendi and Victor, 1967). The Purkinje cell somata are now smoother, forming a distinct monolayer and have thick processes receiving climbing fibre synapses (Altman, 1972b; Mason et al., 1990). By P10 the perisomatic processes are reabsorbed (Berry and Bradley, 1976), growth at the

apical pole is favoured and basket cells synapse on the Purkinje cell soma (Altman, 1972b). At P12 a very large apical dendrite with a variable number of short secondary and tertiary branches has developed (Altman, 1972b). Between P12 and P15 the Purkinje cell dendritic tree grows mainly in the lateral domain because parallel fibres are synapsing on the Purkinje cell dendrites, rather than located above them (Altman, 1972a; Altman, 1972b; Berry and Bradley, 1976). By P14 however, the secondary and tertiary dendritic branches have grown to the molecular layer surface and developed spines on their distal branches as more parallel fibres are laid down above the Purkinje cell layer (Altman, 1972b). It is not until P15 that the Purkinje cell soma has an adult appearance and is covered by glial processes (Altman, 1972b). Upward dendritic growth then continues as more parallel fibres are produced and the upper synaptic domain of the Purkinje cell dendrite matures by P21 (Altman, 1972b).

1.4.2.3 Climbing fibre development

As cerebellar neurons mature, their afferents are also developing. Olivary neurons are generated in the dorsolateral and ventromedial rhombic lip (Altman and Bayer, 1987) of the neuroepithelium of the fourth ventricle between E12 and E15 (Altman and Bayer, 1978; Marchand and Poirier, 1982). Inferior olivary neurons initially migrate from the lower rhombic lip (Altman and Bayer, 1987) at E12 (Alcantara et al., 2000) ipsilaterally (Bourrat and Sotelo, 1988), towards their final adult location in the ventral medulla (Altman and Bayer, 1987). The migrating olivary cells have a long, thin process extending from the leading edge of the cell, forming the olivocerebellar axon (Bourrat and Sotelo, 1990), which crosses the midline in the ventral medulla and extends laterally. At E16 they bend dorsally, progress through the inferior cerebellar peduncle and enter the cerebellar anlage medially and terminate superficially (Chedotal and Sotelo, 1992; Wassef et al., 1992) between the Purkinje cell plate (Chedotal and Sotelo, 1992) and the spreading EGL. By E19 the axons defasciculate and surround their target Purkinje cell somata, however no synapses exist at this stage (Chedotal and Sotelo, 1992; Chedotal and Sotelo, 1993).

Immediately prior to and immediately after birth, the olivocerebellar projection is organised into broad parasagittal zones with each axon branching infrequently and terminating in bud like tips or small growth cones (Mason and Gregory, 1984; Sotelo et al., 1984). At this stage, most axon terminals surround the Purkinje cell bodies, however a few have established immature synaptic contact on Purkinje cell soma transient dendrites (Chedotal and Sotelo, 1993; Chu et al., 2000; Morara et al., 2001).

By P2 a few of the climbing fibre synapses are becoming electrically functional on the Purkinje cell temporary dendrites (Crepel, 1971; Puro and Woodward, 1977) via AMPA receptors, which are highly expressed at these synaptic sites (Zhao et al., 1998). From P3 to P4 each climbing fibre branches over several adjacent Purkinje cell perikarya so that each Purkinje cell is contacted by several climbing fibres (Crepel, 1971; Crepel et al., 1976; Mariani and Changeux, 1981a; Mason et al., 1990; Puro and Woodward, 1977) (Fig 1.4). Maximal multiple innervation of Purkinje cells by climbing fibres occurs at P5 with each Purkinje cell being contacted by an average of 3.5 climbing fibres (Mariani and Changeux, 1981b). It is during this stage that the transient dendrites of these Purkinje cells regress and the climbing fibres synapse on Purkinje cell peri-somatic protrusions (Chedotal and Sotelo, 1993; Mason et al., 1990) bearing metabotropic glutamate receptors (subtype 1 alpha: Dzubay and Otis, 2002; Lopez-Bendito et al., 2001).

From P7 to P15 climbing fibre synaptic terminals translocate to the Purkinje cell primary dendrites with the number of synaptic terminals increasing in parallel with the growth of the Purkinje cell dendritic tree (Altman, 1972b). During translocation post-synaptic receptors are a mix of ionotropic glutamate receptors (subtype 2/3), which are translocating from the Purkinje cell somata to the dendritic tree, and metabotropic glutamate receptors (subtype 1 alpha) (Dzubay and Otis, 2002; Hafidi and Hillman, 1997; Lopez-Bendito et al., 2001).

Concurrently from P7 to P15, supernumerary climbing fibre synapses disappear until single climbing fibre innervation of each Purkinje cell is established by P15 (Crepel et

al., 1976; Mariani and Changeux, 1981b). This regression of supernumerary collaterals appears to be influenced by four different mechanisms. The first mechanism is correct granule cell-Purkinje cell synaptogenesis, as the removal of granule cell precursors in the developing cerebellum (Crepel et al., 1976; Puro and Woodward, 1977; Siggins et al., 1976) or the defective development of parallel fibre-Purkinje cell synapses (Crepel et al., 1981; Hashimoto et al., 2001a; Lalouette et al., 2001), results in residual climbing fibre multi-innervation of Purkinje cells. The second mechanism influencing climbing fibre regression is the function of N-methyl-D-aspartate (NMDA) receptors located in the post-synaptic membrane of climbing fibre-Purkinje cell synapses (Hussain et al., 1991; Perkel et al., 1990; Rabacchi et al., 1992). Blockade of these receptors when climbing fibres are translocating from the Purkinje cell soma to the dendritic tree results in the persistence of multiply innervated Purkinje cells (Kakizawa et al., 2000; Rabacchi et al., 1992). Thirdly, metabotropic glutamate receptors are also implicated in collateral regression. In mutant mice lacking metabotropic receptors (subtype mGluR1), climbing fibre regression ceases early (Kano et al., 1997; Levenes et al., 1997). Also, any disruption of the intracellular cascade that mediates mGluR1 signal transduction retains multi-innervation of Purkinje cells by climbing fibres in adulthood (Chen et al., 1995; Hashimoto et al., 2001b; Kano et al., 1995; Kano et al., 1997; Kano et al., 1998; Tanaka et al., 2000). These cascades are known to include: Galphaq and/or Galpha11 GTP binding proteins, phospholipase C (β4) and protein kinase C (gamma) (Chen et al., 1995; Hashimoto et al., 2001b; Kano et al., 1995; Kano et al., 1997; Kano et al., 1998; Tanaka et al., 2000). Lastly, Bergmann glial, which usually clear synaptically released glutamate, can retract from climbing fibre-Purkinje cell synapses and lead to retained Purkinje cell multiple innervation (Ullian et al., 2004). This knowledge is important as these protein phosphorylation cascades activated in climbing fibre collateral regression may in part also be involved in the neurotrophic cascades for neurogenesis, axonogenesis, synaptogenesis and neuronal survival (Figs 1.5 & 1.6) and the addition of growth factors to manipulate plasticity may impact on normal regression processes.

Additionally, in parallel with the development of this contralateral projection an ipsilateral projection also develops, which arise from a cell population distinct from the

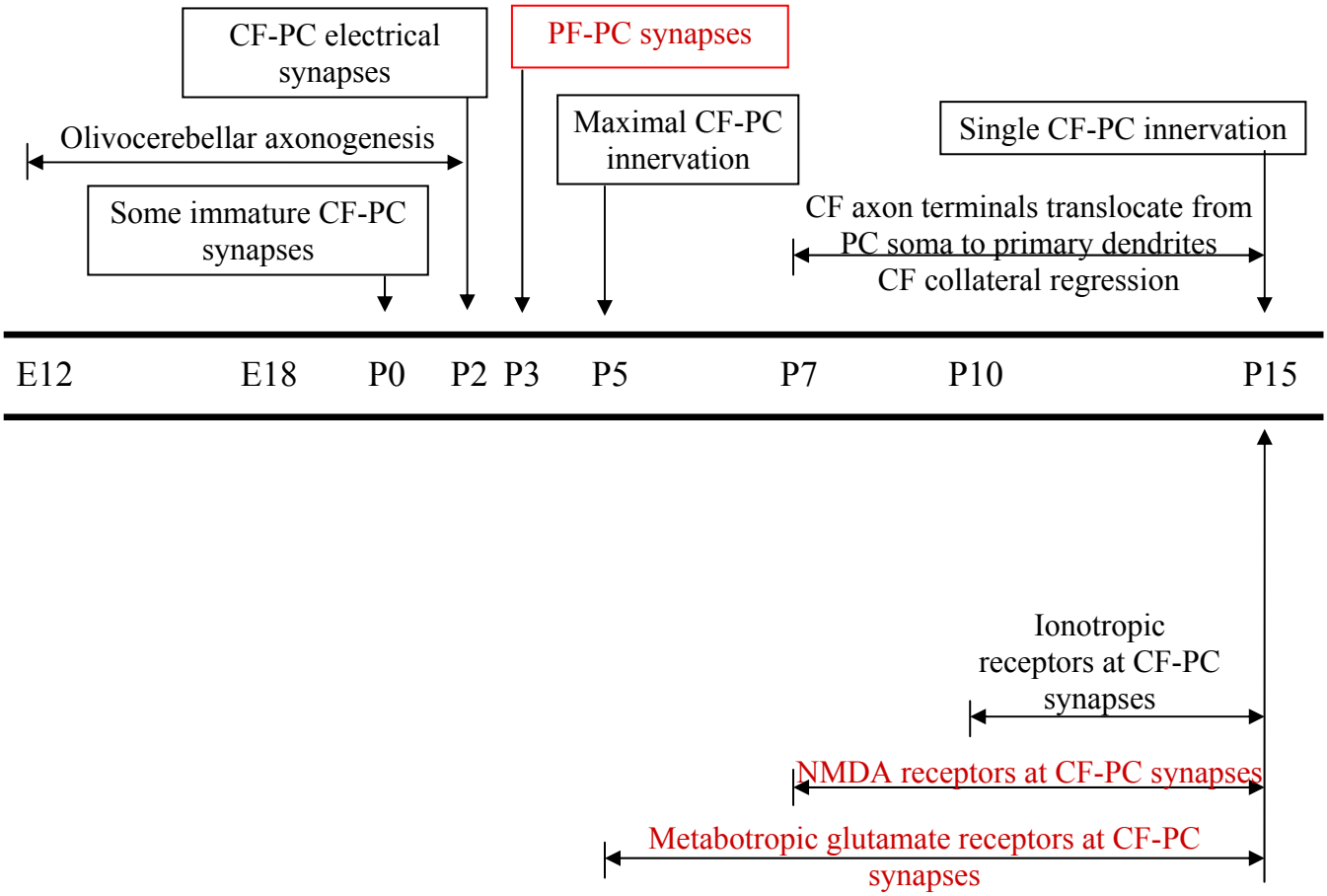


Figure 1.5 Time line of factors involved in climbing fibre development
The descriptions above the time line represent stages in climbing fibre development while the descriptions below the time line are those factors that are involved in climbing fibre development. Factors associated with climbing fibre collateral regression are in red and the remainder are in black.

Key: CF = climbing fibres; E = embryonic day; GC = granule cells; P = postnatal day; PC = Purkinje cell; NMDA = N-methyl-D-aspartate; ↑ = increase; ↓ = decrease.

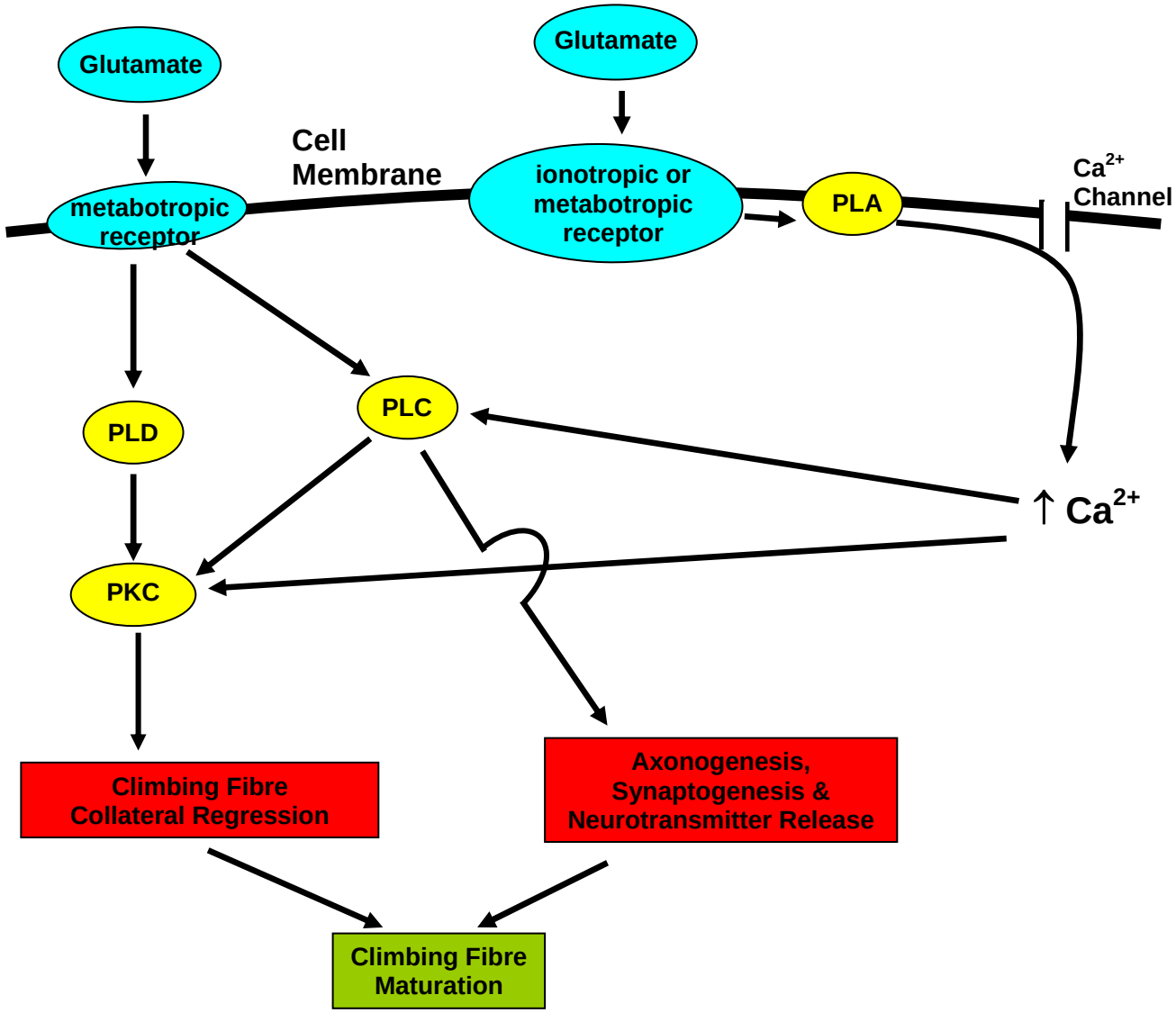


Figure 1.6 Ionotropic and metabotropic intracellular signalling pathways
Diagram showing ionotropic and metabotropic receptor intracellular signalling pathways (Chen et al., 1995; Hashimoto et al., 2001a; Kano et al., 1998; Levenes et al., 1997; Schulman and Hyman, 1999). These pathways are involved in axonogenesis, synaptogenesis, neurotransmitter release, and climbing fibre collateral regression. The neurotransmitters and their receptors are shown in blue. The proteins known to be involved in climbing fibre collateral regression are highlighted in red, while those proteins not specifically identified to be involved in climbing fibre collateral regression, yet known to be involved in neuronal signalling are in yellow.

Key: Ca^{2+} = calcium; PKC = protein kinase C; PLA = phospholipase A; PLC = phospholipase C; PLD = phospholipase D; ↑ = increase.

contralateral pathway (Bower and Payne, 1987; Lopez-Roman and Armengol, 1994). While all but a few fibres of this ipsilateral projection regress before the end of cerebellar maturation (Lopez-Roman et al., 1993), granule cell eradication by x-irradiation at P5 (5500 milligrays) and neonatal unilateral climbing fibre removal maintains the persistence of this ipsilateral pathway beyond the end of the critical period (Fournier et al., 2005; Lopez-Roman and Armengol, 1994). It remains unknown however, what underlying mechanisms enable the persistence of this pathway into adulthood and possible manipulation of this ipsilateral pathway.

1.4.2.4 Cerebellar development summary

At birth climbing fibre axon tips surround Purkinje cell somata and by P2 a few have established electrical synaptic contact. By P5 climbing fibre axon terminals have become associated with metabotropic glutamate receptors on the Purkinje cell somatic protrusions. From P7 climbing fibres translocate to the Purkinje cell primary dendrites and extraneous climbing fibre synapses disappear until a one to one ratio of climbing fibre to Purkinje cells exists. The mossy fibre-granule cell-parallel fibre system becomes functional with Purkinje cells from P12 but does not reach biochemical maturity until P20. Finally, the inhibitory interneurons of the molecular and granular layers develop within the EGL, forming the mature cortical circuit by P30.

1.4.3 Cerebellar development: motor control

Rats are altricial animals such that the development of motor skills occurs postnatally. This development involves a combination of spinal reflexes, descending motor pathways and anatomical development of the cerebellum. Importantly it is the development of the cerebellar cortex and its afferents that are essential for motor learning and control (Caston et al., 1995; Le Marec and Lalonde, 1997; Marr, 1969).

The development of locomotion parallels cerebellar Purkinje cell development. At P2 there are few functional climbing fibre-Purkinje cell synapses and the animals display limited co-ordination (Altman and Sudarshan, 1975; Petrosini et al., 1990). By P7 climbing fibre terminals synapse on the immature Purkinje cell dendritic tree and the animals' postural co-ordination improves as does the development of quadruped stance.

By P8 the animal's gait is still immature as forward propulsion is regularly hindered by hindlimb slips and the animals have not yet progressed from crawling (abdomen regularly touching the ground) to walking (Altman and Sudarshan, 1975; Dixon et al., 2005; Petrosini et al., 1990; Westerga and Gramsbergen, 1990). At P15, there is the adult one-to-one climbing fibre/Purkinje cell relationship and at this time the animals' motor skills have improved to include a smooth, mature gait pattern, an increase in locomotory speed and full maturation of muscular strength, balance and dynamic postural adjustments (Dixon et al., 2005; Petrosini et al., 1990). It is only after the completion of parallel fibre-Purkinje cell synaptogenesis and the interneuronal circuit at P30 that complex motor skills are achieved (Dixon et al., 2005; Petrosini et al., 1990).

1.4.4 Cerebellar development: role of neurotrophins

The development of the cerebellum has been found to coincide with local changes in neurotrophins and their receptors, thus recent studies have focussed on the involvement of neurotrophins in the development of the cerebellum.

NGF does not appear to have a major role in cerebellar development (Table 1.1, 1.2 & 1.3). This may be due to low levels of NGF mRNA in the cerebellum (Das et al., 2001; Large et al., 1986; Lu et al., 1989; Neveu and Arenas, 1996) and the consistently low level of trkA receptor expression in the inferior olivary complex (Riva-Depaty et al., 1998) and other cerebellar afferent structures (Rabacchi et al., 1999). Therefore, this peptide will receive little mention in the following section.

1.4.4.1 Granule cell development

NT-3 is synthesized in the embryonic brain (the site of synthesis is unknown) and influences neuronal differentiation and migration of many cell types, including the differentiation and migration of granule cell precursors from the neuroepithelium of the fourth ventricle to the EGL (Lamballe et al., 1994). This is supported by the localisation of NT-3 in the EGL from E16, which probably exerts its effects through trkC receptors and p75 also located in the EGL (Carter et al., 2003; Ernfors et al., 1992; Lamballe et al., 1994; Rocamora et al., 1993; Segal et al., 1995; Yan and Johnson JR, 1988). This proposed effect of NT-3 is further supported by a lack

Table 1.1 mRNA and protein expression of neurotrophins and receptors during embryogenesis

Age	Cerebellum (location unknown)	ML	EGL	PC	IGL	IOC	DCN
Embryonic			p75 *** trkB – trkC mRNA	p75 trkB – trkC trkC mRNA	N/A	trkB mRNA **	

A summary table of the neurotrophin and neurotrophin receptor expression and protein levels within the cerebellum and inferior olivary complex during embryogenesis (Dieni and Rees, 2002; Ernfors et al., 1992).

Key: DCN = deep cerebellar nuclei; EGL = external granular layer; IGL = internal granular layer; IOC = inferior olivary complex; ML = molecular layer; P = postnatal day; PC = Purkinje cell; – = negative expression; * = low expression; ** = moderate expression; *** = high expression; **** = very high expression.

Table 1.2 mRNA and protein expression of neurotrophins and receptors during development

Age	Cerebellum (location unknown)	ML	EGL	PC	IGL	IOC	DCN
P0 – P7	NT-4 mRNA *** NT-3 ** NT-3 mRNA *** P75 mRNA *** trkB mRNA *		NT-3 mRNA **** p75 ** trkB mRNA ** trkC mRNA	trkA trkA mRNA trkC trkC mRNA p75	NT-3 mRNA trkC mRNA	p75 BDNF mRNA *** trkA mRNA trkB mRNA ** trkC mRNA **	p75 mRNA
P7 – P21	NT-3 *** NT-3 mRNA *** trkB mRNA ** trkC mRNA *	p75 –	p75 * trkB mRNA ** trkC mRNA	BDNF BDNF mRNA * NT-3 NT-3 mRNA – p75 trkA trkA mRNA trkB *** trkC trkC mRNA	NT-3 NT-3 mRNA BDNF mRNA NGF trkC trkB ** trkB mRNA trkA	p75 * BDNF mRNA ** trkA mRNA trkB mRNA trkC mRNA	p75 mRNA

A summary table of the neurotrophin and neurotrophin receptor expression and protein levels within the cerebellum and inferior olivary complex during development (Carter et al., 2003; Cohen-Cory et al., 1989; Das et al., 2001; Friedman et al., 1998; Gao et al., 1995; Katoh-Semba et al., 2000; Koh and Higgins, 1991; Lindholm et al., 1993b; Lindholm et al., 1997; Maisonpierre et al., 1990; Masana et al., 1993; Mount et al., 1994; Muller et al., 1997; Neveu and Arenas, 1996; Nitz et al., 2001; Rocamora et al., 1993; Schwartz et al., 1997; Segal et al., 1995; Timmusk et al., 1993; Tojo et al., 1995; Velier et al., 1997; Yan and Johnson JR, 1988).

Key: DCN = deep cerebellar nuclei; EGL = external granular layer; IGL = internal granular layer; IOC = inferior olivary complex; ML = molecular layer; P = postnatal day; PC = Purkinje cell; – = negative expression; * = low expression; ** = moderate expression; *** = high expression; **** = very high expression.

Table 1.3 mRNA and protein expression of neurotrophins and receptors in adulthood

Age	Cerebellum (location unknown)	ML	EGL	PC	IGL	IOC	DCN
Adult	NT-3 * trkB mRNA *** trkC mRNA **	trkA		BDNF mRNA (?) p75 – trkA * trkB mRNA –	NGF mRNA BDNF mRNA NT-3 mRNA p75 – trkA * trkB *** trkB mRNA trkC mRNA ***	trkB mRNA *	BDNF mRNA NT-3 mRNA p75 mRNA – trkB * trkB mRNA –

A summary table of the neurotrophin and neurotrophin receptor expression and protein levels within the cerebellum and inferior olivary complex during development (Barbacid, 1994; Castren et al., 1995; Dugich-Djordjevic et al., 1995; Garcia-Rocha and Avila, 1995; Hofer et al., 1990; Kaplan and Stephens, 1994; Lamballe et al., 1991; Lindholm et al., 1993a; Martinez-Murillo et al., 1997; Merlio et al., 1992; Pioro and Cuello, 1988; Riva-Depaty et al., 1998; Tsoulfas et al., 1993; Wetmore et al., 1991; Yan et al., 1997; Zhou and Rush, 1994).

Key: DCN = deep cerebellar nuclei; EGL = external granular layer; IGL = internal granular layer; IOC = inferior olivary complex; ML = molecular layer; P = postnatal day; PC = Purkinje cell; – = negative expression; * = low expression; ** = moderate expression; *** = high expression; **** = very high expression; ? = unknown.

of BDNF and its receptor trkB in the EGL at this stage (Gao et al., 1995), which in other areas of the CNS is involved in differentiation (Ip et al., 1993; Sieber-Blum, 1991). Following differentiation and migration of external granular layer precursors, granule cell proliferation occurs in the EGL and although mechanisms aiding this process are still unclear, a sole study has shown NT-3 to have no effect on this process (Gao et al., 1995). Following on from this, newly generated granule cell survival in the EGL is promoted either through BDNF induced NT-3 transcription (Leingartner et al., 1994; Shalizi et al., 2003), or directly through NT-3 (Katoh-Semba et al., 2000; Segal et al., 1992; Shalizi et al., 2003). In contrast some studies have reported that NT-3 does not aid granule cell survival (Gao et al., 1995; Kubo et al., 1995), but this may simply be a dose related issue or an age dependent association in that NT-3 only aids survival of more mature granule cells.

Throughout the literature, there is evidence that NT-3 is involved in the early stages of CNS development, while BDNF appears to have its effects later on. This paradigm appears true for the granule cell system also. Following proliferation and differentiation, presumptive granule cells undergo neurite extension and it is at this stage that the first sign of trkB mRNA in the EGL is observed (Gao et al., 1995; Masana et al., 1993; Segal et al., 1995; Zirrgiebel et al., 1995). Therefore, since trkC is already found in the EGL at this stage, it follows that NT-3, BDNF and/or NT-4 may be involved in axonal elongation (Alonso et al., 1996; Doughty et al., 1998; Gao et al., 1995; Masana et al., 1993; Schwartz et al., 1997; Segal et al., 1995). After granule cells elongate their axons, the cell bodies migrate from the EGL to the IGL and this process involves a switch in neurotrophin dependence from NT-3 to BDNF and NT-4 (Alonso et al., 1996; Coffey et al., 1997; Gao et al., 1995; Lindholm et al., 1993a; Schwartz et al., 1997; Segal et al., 1992; Segal et al., 1995). At this time however, granule cell survival appears to be influenced by BDNF and NT-4, as well as NT-3 (Bonthius et al., 2003; Coffey et al., 1997; Gao et al., 1995; Katoh-Semba et al., 2000; Minichiello and Klein, 1996; Neveu and Arenas, 1996; Nonomura et al., 1996; Schwartz et al., 1997; Zirrgiebel et al., 1995).

Once granule cells arrive in the internal granular layer, the next stage of their development involves synaptogenesis with mossy fibres. It has been proposed that the release of BDNF from granule cells acts upon trkB receptors on basilar pontine neurons directing mossy fibre growth and aiding granule cell-mossy fibre synaptogenesis (Rabacchi et al., 1999). Also at the time when granule cell- mossy fibre synaptogenesis begins in the IGL, high levels of BDNF mRNA are beginning to be expressed in the IGL (Ernfors et al., 1992; Hofer et al., 1990; Katoh-Semba et al., 2000; Lindholm et al., 1993b; Lindholm et al., 1993a; Neveu and Arenas, 1996; Rocamora et al., 1993) and this may be a possible trophic factor for Purkinje cells during development. While mossy fibre-granule cell synaptogenesis is occurring, granule cell synthesis of BDNF appears to influence granule cell-Purkinje cell synaptogenesis through p75 and trkB located on Purkinje cell tertiary branches (Morrison and Mason, 1998; Rocamora et al., 1993).

1.4.4.2 Purkinje cell development

Along with granule cells, NT-3 has also been implicated in Purkinje cell proliferation and migration embryonically, but this cannot be conclusively determined due to the unknown site of synthesis of NT-3 in the embryonic brain (Ernfors et al., 1992).

Postnatally, the extensive synthesis of NT-3 mRNA from the granule cell system and the expression of p75 and trkC on Purkinje cells (Ernfors et al., 1992; Lewin et al., 1992; Neveu and Arenas, 1996; Rocamora et al., 1993; Velier et al., 1997), suggests a role for NT-3 in Purkinje cell development. Indeed both *in vitro* and *in vivo* studies indicate that both NT-3 and granule cells are required for Purkinje cell differentiation (Baptista et al., 1994; Lindholm et al., 1993a; Mason et al., 1997; Morrison and Mason, 1998; Mount et al., 1994; Neveu and Arenas, 1996). Likewise granule cells are required for correct Purkinje cell dendritic growth by synthesising BDNF and NT-3, which activates either trkB or trkC receptors on Purkinje cell dendrites (Minichiello and Klein, 1996; Morrison and Mason, 1998; Schwartz et al., 1997; Shimada et al., 1998). Synaptogenesis on the other hand, which includes increasing the number and maturity of synaptic terminals,

appears to involve the neurotrophin triad of NT-3, BDNF and NT-4 (Morrison and Mason, 1998; Neveu and Arenas, 1996; Shimada et al., 1998).

Similarly, Purkinje cell survival also appears to depend on all three of the aforementioned peptides, NT-3, BDNF and NT-4 (Larkfors et al., 1996; Lindholm et al., 1993b; Morrison and Mason, 1998; Mount et al., 1994; Shimada et al., 1998). Since these neurotrophins are synthesised by neurons that project to the Purkinje cells, this is consistent with the role of afferent delivery of neurotrophins required for neuronal survival (Sherrard and Bower, 2002).

Less clear in Purkinje cell development is the involvement of NGF. Despite the presence of trkA receptors on Purkinje cells from postnatal day 5, *in vitro* administration of NGF appears to have no effect on Purkinje cell survival (Larkfors et al., 1996; Mount et al., 1994; Muller et al., 1997). While NGF has been localised in the Purkinje cell layer after injection into the cerebellum both *in vivo* and *in vitro* (Aloe and Vigneti, 1992), this may be due to high concentrations of NGF utilised in that study, which exceed physiological levels and thus may void any extrapolation from these results.

1.4.4.3 Climbing fibre development

Neurotrophins are also involved in the development of climbing fibre afferents to the Purkinje cells. As indicated above, inferior olivary neurons proliferate, differentiate and migrate from the neuroepithelium of the fourth ventricle to the ventral aspect of the medulla, probably under the control of NT-3 (Lamballe et al., 1994). As stated previously, this cannot be confirmed due to the unknown location of NT-3 synthesis embryonically.

In line with the evidence that NT-4 and NT-3 induce growth cone motility and axonal elongation in the CNS (McCallister et al., 1999; Tucker et al., 2001; Wiklund and Ekstrom, 2000), the commencement of olivocerebellar axonogenesis coincides with the expression of NT-4 and NT-3 mRNAs in the embryonic cerebellum (Timmusk et al., 1993). This is concurrent with the embryonic expression of p75 in the olive and trkB

and trkC expression in the brainstem embryonically (the precise location is unknown) and in the olive at birth (Ernfors et al., 1992; Ringstedt et al., 1993; Riva-Depaty et al., 1998; Yan and Johnson JR, 1988). These receptors are anterogradely transported from the inferior olivary complex to the axon terminals in the cerebellum, where they form a complex with the neurotrophin and are retrogradely transported back to the olivary complex, as occurs in other regions of the CNS (Caleo et al., 2003; Spalding et al., 2002; Whitmarsh and Davis, 2001). Evidence to support this hypothesis is the accumulation of these peptides in the inferior olive from birth despite no known olivary synthesis of these peptides (Friedman et al., 1998; Rocamora et al., 1993).

Immediately prior to birth there is an interaction between climbing fibres and Purkinje cells, which influences the development of the olivocerebellar pathway (Chu et al., 2000; Morara et al., 2001). While cerebellar synthesis of BDNF is weak before P10, BDNF has been localised in Purkinje cells at birth (Das et al., 2001; Dieni and Rees, 2002; Neveu and Arenas, 1996; Rocamora et al., 1993). It has therefore been proposed that BDNF, which is strongly expressed in the inferior olive at birth (Rocamora et al., 1993), is anterogradely transported from the inferior olive to the cerebellum aiding climbing fibre-Purkinje cell interaction via trkB receptors on the Purkinje cells (Sherrard and Bower, 2002). Additionally, since in other areas of the CNS a growth associated protein (GAP-43) is known to aid axonogenesis and neurite fluidity and that the inferior olive expresses this protein (Buffo et al., 1998; Console-Bram et al., 1996; Fournier et al., 1997), cerebellar BDNF may be retrogradely transported to the inferior olive to up-regulate olivary GAP-43 aiding climbing fibre terminal fluidity, and ultimately climbing fibre-Purkinje cell synaptogenesis.

From P7, while climbing fibre maturation occurs, the synthesis of neurotrophins and receptors available in the cerebellum and inferior olivary complex are changing. In the cerebellum BDNF, NT-3 and NT-4 mRNA increases, while NGF mRNA continues to fall (Das et al., 2001; Neveu and Arenas, 1996; Rocamora et al., 1993). The location of mRNA and protein for some neurotrophins and their receptors have been identified in Table 1.1.

Climbing fibre maturation involves axonal growth in parallel with the Purkinje cell dendritic tree in addition to the regression of supernumerary collaterals. Although these processes are concurrent, they will be considered here as two separate processes. In the first, climbing fibre axonal growth in parallel with the Purkinje cell dendritic tree may still be influenced by cerebellar NT-4 (Sherrard and Bower, 2002). Additionally, climbing fibre axon terminals synapse onto ionotropic glutamate receptors at this age (Hafidi and Hillman, 1997) and this interaction may be involved in the translocation of climbing fibres from the Purkinje cell soma to the primary dendrites. It is proposed that the increase in cerebellar BDNF at this age regulates the surface expression of Purkinje cell ionotropic glutamate receptors and therefore aids climbing fibre translocation to Purkinje cell primary dendrites. This phenomenon of BDNF regulating ionotropic glutamate receptor mobility is also seen in other areas of the CNS (Narisawa-Saito et al., 2002). Additionally, evidence shows that BDNF is not only involved in synapse translocation, but also in maintaining synaptic connections (Vicario-Abejon et al., 2002); thus the large increase in cerebellar BDNF at this age may be affecting climbing fibre-Purkinje cell synapse stability.

The second process of climbing fibre maturation involves the collateral regression of supernumerary climbing fibre axon terminals, and it has been proposed that this is due to a decrease in olivary trkB expression (Riva-Depaty et al., 1998; Sherrard and Bower, 2002). The reduction of olivary trkB synthesis at this age may be restricting the action of BDNF at climbing fibre axon terminals and thus restricting signalling, creating more stable interactions with Purkinje cells (Sherrard and Bower, 2002). The net result of this restriction is observed at P15 when each Purkinje cell receives climbing fibre synapses from only one olivary axon. Additionally, it is known that granule cell-Purkinje cell synaptogenesis effects climbing fibre collateral regression, and that following granule cell removal at P5, there is a change in the levels of all trk receptors in the inferior olivary complex (Riva-Depaty et al., 1998). It is therefore possible that the granule cell-Purkinje cell interaction effects climbing fibre collateral regression by changing

neurotrophin receptor levels in the olive. Unfortunately there is little information known in this area and further investigations are required.

In regards to the receptors involved in climbing fibre development, it appears that ionotropic, metabotropic and trk receptors all interact to achieve the ultimate goal of development and maturation of the olivocerebellar pathway. Indeed as stated earlier, this may be due to the overlapping intracellular pathways between ionotropic, metabotropic and trk receptors (Fig 1.7 & 1.8). It is probable that a combination of all of these intracellular pathways are required to overlap for the correct pattern of axonogenesis, synaptogenesis, neuronal survival and collateral regression to take place.

1.4.4.4 Neurotrophins in cerebellar development summary

NT-3 in the embryonic brainstem may induce granule cell precursors, Purkinje cells and inferior olivary cells to proliferate and migrate to their appropriate location within the cerebellum or brainstem. The production of BDNF by granule cells aids granule cell-Purkinje cell synaptogenesis. Embryonic and perinatal cerebellar expression of NT-3 and NT-4 may induce olivocerebellar axonogenesis through retrograde target-derived action on the inferior olive. For climbing fibre maturation, axonal growth occurs in parallel with growth of the Purkinje cell dendritic tree possibly due to cerebellar NT-4, but more likely due to a rise in cerebellar BDNF regulating Purkinje cell-climbing fibre ionotropic relocation and stability. Climbing fibre collateral regression is probably influenced by a reduction of trkB in the inferior olivary complex, restricting GAP-43 at the axon terminals, thus leading to single climbing fibre innervation of Purkinje cells.

1.5 CEREBELLAR PLASTICITY

Following removal of olivocerebellar axons from the cerebellum there is a change in cerebellar afferent morphology and their relationship to other neurons, through reorganisation of axonal projections and thus synaptic connections.

1.5.1 Cerebellar plasticity following afferent removal

The major cell that has been studied in the plasticity of the cerebellum is the Purkinje cell and it has been observed following alterations to its afferents, the climbing fibres.

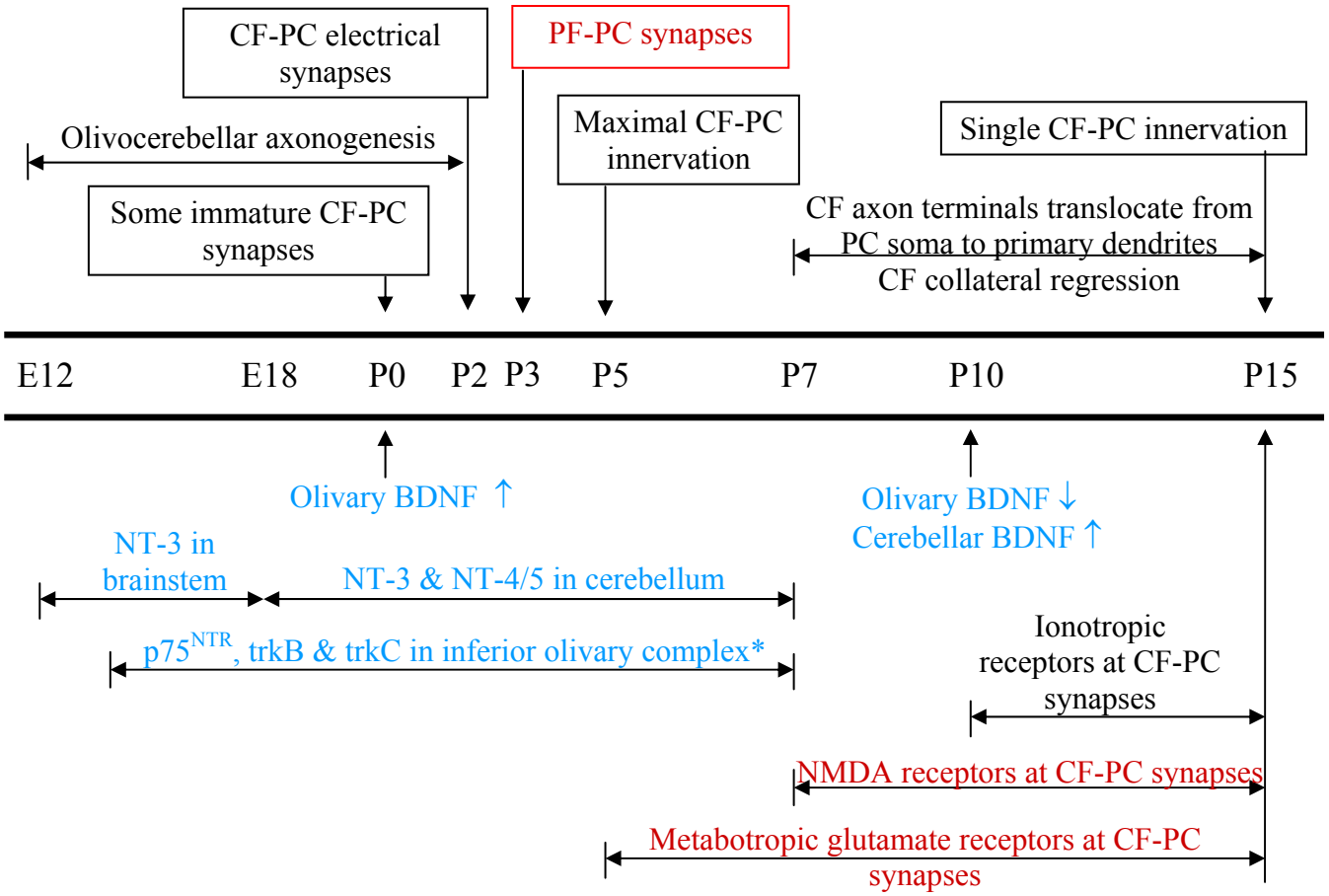


Figure 1.7 Factors involved in climbing fibre development (including neurotrophins)
The descriptions above the time line represent stages in climbing fibre development while the descriptions below the time line are those factors that are involved in climbing fibre development. Factors associated with climbing fibre collateral regression are in red, factors associated with neurotrophins are in pale blue and the remainder are in black.

* TrkB & trkC receptors are located within the brainstem from E13 to E18, although their exact location has not yet been identified. For ease of this diagram their location has been designated within the inferior olive.

Key: BDNF = brain-derived neurotrophic factor; CF = climbing fibres; E = embryonic day; GC = granule cells; NT-3 = neurotrophin-3; NT-4/5 = neurotrophin-4/5; P = postnatal day; p75 = low affinity nerve growth factor receptor; PC = Purkinje cell; trkB = high affinity tyrosine kinase receptor B; trkC = high affinity tyrosine kinase receptor C; NMDA = N-methyl-D-aspartate; ↑ = increase; ↓ = decrease.

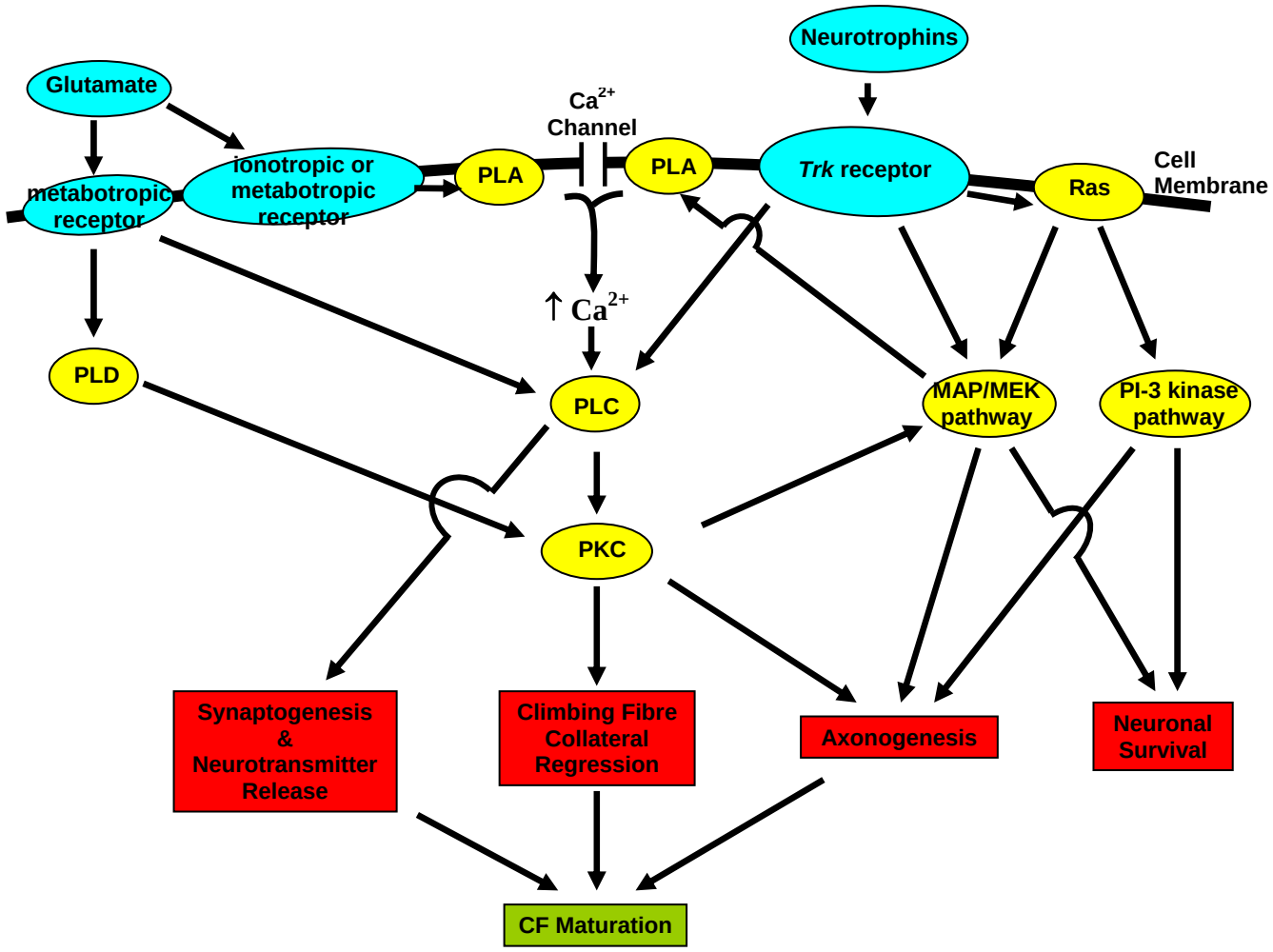


Figure 1.8 Intracellular signalling pathways of metabotropic, ionotropic and trk receptors

Diagram illustrating the intracellular pathways of metabotropic, ionotropic and trk receptors (Chen et al., 1995; Hashimoto et al., 2001a; Heumann, 1994; Huang and Reichardt, 2001; Kano et al., 1998; Levenes et al., 1997; Schulman and Hyman, 1999; Ullrich and Schlessinger, 1990). The ligands and their receptors are shown in blue and the intracellular signal transduction cascades are shown in yellow. There is a large amount of overlap between the metabotropic, ionotropic and trk receptor intracellular signalling pathways.

Key: Ca^{2+} = Calcium; MAP/MEK = mitogen-activated protein/mitogen-activated protein with extracellular-signalling-regulated protein; PI-3 kinase = phosphatidylinositol-3 kinase; PKC = protein kinase C; PLA = phospholipase A; PLC = phospholipase C; PLD = phospholipase D; Ras = membrane bound peptide involved in the intracellular signalling pathway; trk receptor = high affinity tyrosine kinase receptor; ↑ = increase.

Following permanent climbing fibre removal in both adult and neonatal animals the Purkinje cell dendritic tree becomes smaller (Bradley and Berry, 1976) and develops abnormal spines on its primary trunks. Formation of these spines is seen irrespective of whether the lesion is in organotypic culture, following inferior olivary lesions or depression of olivary electrical activity with tetrodotoxin (Baetens et al., 1982; Bravin et al., 1999; Privat and Drian, 1976; Sotelo et al., 1975; Sotelo and Arsenio-Nunes, 1976). These aberrant spines quickly become innervated by parallel fibres, which extend into the area of the Purkinje cell dendritic tree that is usually occupied by climbing fibre synapses (Privat and Drian, 1976; Sotelo et al., 1975). This is important to consider when investigating plasticity of the olivocerebellar pathway as Purkinje cell modification may restrict any subsequent climbing fibre plasticity. However, these aberrant spines retract following post-lesion climbing fibre reinnervation and thus may not restrict reinnervation.

Furthermore, in adult animals the complete loss of climbing fibres from the cerebellum also leads to mossy fibre elongation and sprouting into the molecular layer synapsing on interneurons, but not Purkinje cells (Murase, 1995). This mossy fibre plasticity increases Purkinje cell inhibition by increasing interneuron activation, which may replicate the action of climbing fibres in depressing Purkinje cell responsiveness to parallel fibre input (Granit and Phillips, 1956). Comparatively, only partial destruction of the inferior olivary complex in adult animals and thus only partial removal of climbing fibres from the cerebellar hemisphere, leads to localized climbing fibre terminal sprouting (Rossi et al., 1989; Rossi et al., 1991b; Rossi et al., 1991a; Zagrebelsky et al., 1996). These climbing fibres sprout collaterals within the molecular layer, forming new climbing fibre arbors on adjacent Purkinje cells in a topographically organized manner (Rossi et al., 1991b; Zagrebelsky et al., 1996). Unfortunately however, this form of climbing fibre plasticity does not confer behavioural function, as these animals have motor-skill deficiencies and poor sensorimotor abilities at tasks requiring moderate skills (Rondi-Reig et al., 1997).

In contrast, Purkinje cell afferent plasticity is much greater during development. In neonatal rats surgical transection of one olivocerebellar pathway (pedunculotomy), results in the ipsilateral inferior olivary neurons producing an alternate pathway that reinnervates the denervated hemicerebellum (Angaut et al., 1982; Angaut et al., 1985) (Fig 1.9). Unfortunately, the reinnervating climbing fibres do not completely replace the lesioned paths, as reinnervated Purkinje cells are more numerous in the vermis and paravermis than in the lateral hemispheric regions (Angaut et al., 1982; Angaut et al., 1985; Sherrard et al., 1986; Sugihara et al., 2003). Importantly in the lesioned animals the climbing fibre-Purkinje cell synapses display normal climbing fibre synaptic function and the cerebellar cortex and its circuitry appears normal (Angaut et al., 1982; Sherrard et al., 1986; Sugihara et al., 2003). This re-innervation has been attributed to transcommissural sprouting of climbing fibres where the climbing fibres from the inferior olive leave the intact hemicerebellum and cross the midline to innervate previously denervated Purkinje cells (Angaut et al., 1982; Sherrard et al., 1986; Zagrebelsky et al., 1997). Contrary to these findings, it has been suggested that these alternate transcommissural climbing fibres are a consequence of the persistence of a transient, transcommissural projection retained by suppression of axonal competition (Angaut et al., 1985). However, the time course of reinnervation indicates that they are sustained by newly formed transcommissural axons and not by the retention of pre-existing fibres (Lohof et al., 2005; Zagrebelsky et al., 1997). Accordingly, lesioned animals with reinnervation of the medial hemispheric region perform complex locomotory tasks as well as control animals, but with a side preference ipsilateral to the lesion (Dixon et al., 2005). These animals also have a reduction of sensorimotor skills when performing difficult tasks, as compared to control animals (Dixon et al., 2005). This reduced ability is probably due to the limited amount of reinnervation in the paravermis and lateral hemisphere (Sugihara et al., 2003), as innervation of Purkinje cells in these areas is necessary to perform motor tasks requiring rhythmical synchronisations (Bobee et al., 2000; Joyal et al., 1996; Rondi-Reig et al., 1997; Rushmer et al., 1976). Despite these minor deficits, climbing fibre reinnervation improves the animals' functional recovery, in comparison to those animals without reinnervation; therefore it would be ideal to induce this reinnervation in mature animals

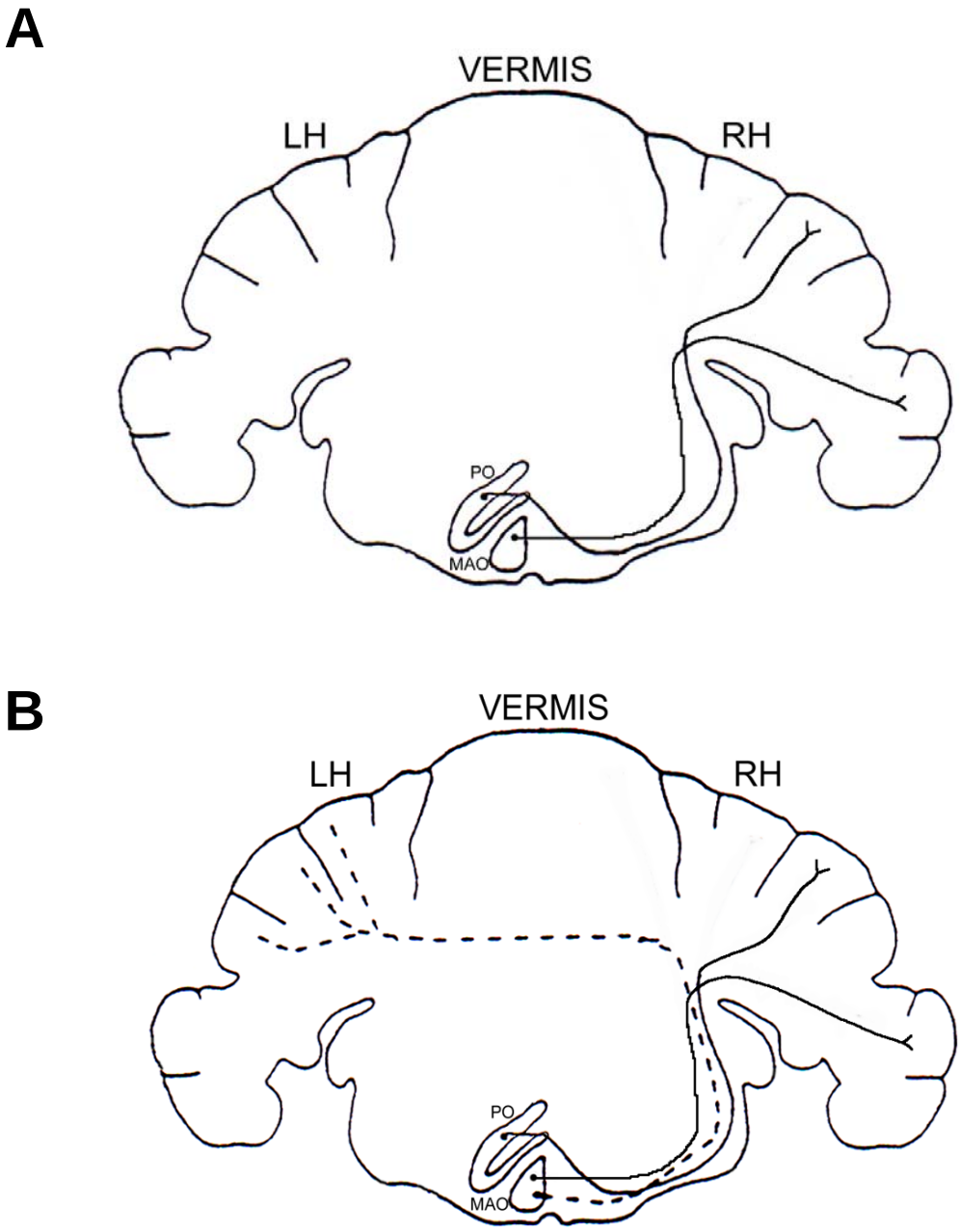


Figure 1.9 Contralateral and transcommissural olivocerebellar pathways
Schematic diagrams of the cerebellum and brainstem (modified from Sherrard et al., 1986) illustrating the normal contralateral (solid lines in A and B) and reinnervating transcommissural (dotted line in B) olivocerebellar pathways. Only the left inferior olivary complex has been drawn and, for clarity, it is out of proportion to the brainstem.

Key: LH = left hemisphere; MAO = medial accessory olive; PO = principal olive; RH = right hemisphere.

to reduce post-lesion functional deficits. Unfortunately, however, it is currently unknown what controls the development of these alternate pathways in the neonatal system.

It is known that the ability to generate these pathways decreases with increasing age of the animal. Transection of the olivocerebellar projection within 48 hours after birth generates a new path with an exact mirror image of the normal olivocerebellar pathway, thus reproducing the normal climbing fibre topography (Angaut et al., 1985; Zagrebelsky et al., 1997). However, if the pedunculotomy is performed at postnatal day 3 (P3), the topography of reinnervation differs from control animals, where fibres of the new pathway are only a good mirror image of the normal pathway in control animals (Sugihara et al., 2003), while if performed at P7 the symmetrical distribution is poor (Sherrard et al., 1986). If the pedunculotomy is performed on or after P10, then reinnervation no longer occurs (Sherrard et al., 1986).

The above pattern of reinnervation is perhaps best explained by the developmental stage of the cerebellar system. At birth the climbing fibres have reached the Purkinje cell plate (Chedotal and Sotelo, 1992), but have established only a few temporary climbing fibre-Purkinje cell synapses (Chedotal and Sotelo, 1993; Chu et al., 2000; Morara et al., 2001). This age is only just beyond normal olivocerebellar axonogenesis and therefore the same climbing fibre guidance cues located within the cerebellum must still be present producing an almost identical topographic map to the normal olivocerebellar pathway. From P3 onwards more climbing fibre-Purkinje cell synapses have now formed at the base of the Purkinje cell soma (Altman, 1972b; Crepel, 1971; Woodward et al., 1971) and as such a decline in the expression of climbing fibre axonal growth cues in the cerebellum would be expected. While there is a reduction in olivary p75 expression during this stage (Nitz et al., 2001), which coincides with the progressive reduction of similarity between the normal climbing fibre topography and the topography of the reinnervating fibres, there is no reduction in neurotrophin expression

in the postnatal cerebellum (Table 1.1). However, changes in neurotrophin expression have been observed in the cerebellum following injury.

1.6 ROLE OF NEUROTROPHINS IN OLIVOCEREBELLAR PLASTICITY

Given that neurotrophins appear to be involved in olivocerebellar development and that olivary neurotrophin and receptor expression decreases with maturation of the pathway, it is possible that these neurotrophins also influence olivocerebellar plasticity following injury. Indeed, evidence for this comes from alterations in neurotrophin and receptor expression during post-lesion plasticity of the olivocerebellar pathway.

One of these changes to the olivocerebellar pathway can be made indirectly by unilateral labyrinthectomy (deafferentation of the inner ear). This procedure removes labyrinthine input both to the inferior olive and the cerebellum (Balaban and Romero, 1998) and in adult rats leads to the acute appearance of behavioural symptoms including vertigo and ataxia (Li et al., 2001; Maingay et al., 2000). These symptoms are closely followed by a process known as vestibular compensation, which is a re-organisation of neuronal circuits in the brainstem and cerebellum, thought to involve olivocerebellar plasticity (Li et al., 2001). Following unilateral labyrinthectomy the expression of BDNF mRNA increases in sub-regions of the inferior olivary complex that undergo plasticity (Li et al., 2001). This increase in BDNF synthesis is consistent with the normal neonatal olivary BDNF expression (Rocamora et al., 1993), which may influence olivocerebellar axonogenesis and synaptogenesis. As stated earlier, it is proposed that inferior olivary BDNF is anterogradely transported to the cerebellum and released from the olivocerebellar presynaptic terminals acting in an autocrine fashion enabling neurite fluidity through the actions of GAP-43 (Sherrard and Bower, 2002). Therefore, this up-regulation of BDNF mRNA expression in the inferior olivary complex is likely to reorganise the input of climbing fibres to Purkinje cells in the projections that were affected by the unilateral labyrinthectomy. Once this synaptic reorganisation has occurred, it is expected that olivary BDNF mRNA would decline as climbing fibre plasticity is no longer required. Indeed, this is the case, as within 72 hours of the labyrinthectomy olivary BDNF mRNA levels revert to normal (Li et al., 2001), once

functional improvement has occurred. Importantly, this timeframe of plasticity is comparable to the timeframe taken to generate an alternate olivocerebellar pathway following unilateral pedunculotomy.

Similarly, following unilateral transection of the olivocerebellar pathway in neonatal rats there is an up-regulation of p75 in the uninjured inferior olivary complex undergoing modification (i.e. plasticising inferior olivary complex) (Nitz et al., 2001). This is concurrent with other studies of the CNS, where p75 is upregulated following injury (Rende et al., 1993; Sebert and Shooter, 1993; Turner and Perez-Polo, 1998). Effectively, increased p75 in the plasticising olivary complex increases its availability for anterograde transport to the axon terminals in the cerebellum mediating the effects of neurotrophins on the plasticising olivocerebellar axon terminals.

The up-regulation of BDNF mRNA and p75 in the olivary complex following labyrinthectomy and pedunculotomy implicates BDNF and p75 in olivocerebellar plasticity and reinnervation. Indeed the administration of BDNF to the left hemicerebellum 24 hours after unilateral pedunculotomy at P11 generates an alternate pathway that is topographically organised like that which occurs during neonatal plasticity (Sherrard and Bower, 2001). However, it is currently unknown whether BDNF can induce this pathway in a mature system. Therefore, there is a knowledge gap on whether BDNF is involved in neonatal climbing fibre reinnervation and whether this peptide can induce climbing fibre reinnervation in the mature cerebellum. Additionally, if BDNF can induce climbing fibre reinnervation onto previously denervated Purkinje cells in the mature cerebellum, it is still uncertain whether any functional recovery can be achieved by this type of repair mechanism. Therefore, the aims of this PhD will attempt to fill these knowledge gaps.

1.7 THESIS AIMS

The goal of this PhD is to determine whether BDNF plays a role in climbing fibre reinnervation of denervated Purkinje cells in the cerebellum of Wistar rats, and if so,

does this improve the animals' functional recovery? This goal will be fulfilled by collecting and analysing data relating to 4 aims outlined below.

While alternate pathways can be induced in the mature nervous system by neurotrophins, this creates an imprecise neuronal circuit, which has limited post-lesion return of function. At the end of development however, supernumerary ipsilateral pathways regress once the normal pathway has been established. While growth factors can prevent naturally occurring cell death it remains unknown whether growth factors can also prevent the death of these ipsilateral olivocerebellar pathways and manipulate their growth to create normal neuronal circuitry post-lesion, albeit through an abnormal route. Therefore, the first aim of this PhD is to identify whether BDNF injected into the right inferior olivary complex, 24 hours following left unilateral olivocerebellar pathway transection at P3, can prevent the degeneration of olivary cells within the right inferior olive (and thus prevent degeneration of the right ipsilateral pathway). Furthermore, if BDNF can indeed prevent inferior olivary cell death, then can BDNF also induce reinnervation of the left denervated hemisphere by climbing fibre terminals belonging to the right ipsilateral pathway?

Secondly, it has been hypothesised that neurotrophins, specifically BDNF, play a role in the development of the alternate olivocerebellar pathway during the neonatal period. Therefore, the second aim is to determine whether BDNF induces climbing fibre reinnervation of denervated Purkinje cells following left unilateral olivocerebellar transection at P3 by neutralising BDNF in the cerebellum.

Recently it has been demonstrated that BDNF induces the post-lesion development of an alternate olivocerebellar pathway, just beyond the close of the critical period at P10. However the morphology of this projection within the cerebellar cortex and the role of BDNF remain unknown, i.e. whether the alternate circuitry can be reproduced when target neurons have developed complex adult morphology. Therefore, the third aim is to determine whether BDNF injection into the left hemicerebellum induces topographically and morphologically correct climbing fibre reinnervation of mature Purkinje cells.

Lastly, if BDNF can induce post-lesion climbing fibre reinnervation in the mature cerebellum, then the fourth aim is to determine whether this reinnervation improves the animals' post-lesion deficits. Since the cerebellum is involved in motor coordination and gait synchronisation, then the animals' functional recovery will be assessed by observing the animals on a series of behavioural tasks specific to motor coordination and gait synchronisation.

The data provided by each of these aims will fill the knowledge gap on whether BDNF induces functional climbing fibre reinnervation of previously denervated Purkinje cells.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 ANIMALS

Experiments were performed on Albino rats *Rattus rattus norvegicus* (Wistar Strain) under licence from James Cook University Animal Experimentation Ethics Committee (AEEC A749) and The University of Western Australia Ethics Committee (AEC 04/100/359), which are in accordance with the "*Code of practice for the care and use of animals for scientific purposes"* of the National Health and Medical Research Council of Australia. Animals were housed in a 12 hr light/dark cycle with food and water *ad libitum*. The day of birth was designated as P0 and litters were weaned at P28.

2.2 ANAESTHETIC

Rats weighing less than 100 g were anaesthetised with diethyl ether (20 ml within a container approximately 1000 ml: BDH, Poole UK) and older animals weighing more than 100 g were anaesthetised with an intraperitoneal injection of ketamine (50 mg/kg, Parnell, NSW Australia), xylazine (10 mg/kg, Ilium, NSW Australia) and acepromazine (0.75 mg/kg, Delvet, NSW Australia). This is because younger animals are easily susceptible to an overdose of ketamine/xylazine/acepromazine, while older animals require more time for surgical procedures (i.e. the surgical procedures in older animals take longer).

There was an exception to the above rule however. Some procedures required anaesthetic 2 days in a row, and it was found that young adult rats are susceptible to a diethyl ether overdose if given on 2 sequential days (i.e. smaller therapeutic window on the second day). Therefore, P30 rodents less than 100 g were anaesthetised with diethyl ether on the first day (P30) and a reduced dose of ketamine (35 mg/kg), xylazine (7 mg/kg) and acepromazine (0.05 mg/kg) on the second day (P31).

Lastly, prior to culling, animals were deeply anaesthetised with an intraperitoneal injection of sodium pentobarbitone (60 mg/kg, Virbac, Australia).

2.3 TRANSECTION OF OLIVOCEREBELLAR AXONS

Wistar rats of either sex aged P3, P15, P20 and P30 were anaesthetised with diethyl ether, and climbing fibres from the left hemicerebellum were damaged via a lesion of the inferior cerebellar pathway as follows: the skin over the neck was incised longitudinally and the muscles retracted to expose the atlanto-occipital membrane. Holding the head flexed to 90 degrees, a capsulotomy knife (MSP; 3mm blade for P3 or 5 mm blade for P15-30) was inserted through the membrane into the fourth ventricle parallel to the brainstem and rotated anti-clockwise to cut the left inferior cerebellar peduncle. After full recovery from the anaesthetic in a warm box, the animals were returned to the dam. Sham-operated animals were treated similarly, however the left inferior cerebellar peduncle remained intact. Transection at P15, P20 and P30 are termed Px15, Px20 and Px30.

The pedunculotomy technique described above is difficult to perform with previous studies having only a 66 % success rate (Fournier et al., 2005). This is because the experimenter is blind to the procedure contained within the fourth ventricle (between the cerebellum and the brainstem) and in adolescent and mature animals the temporal bone protrudes into the area of transection. Moreover, the experimenter is unaware of whether the inferior cerebellar peduncle has been completely transected until after the animal is culled and serial sections are inspected under the microscope, which, depending on the experimental design, can be up to 4 months after the initial surgery. In the current study the reproducibility of this technique was low and despite continued efforts to problem solve with Dr Sherrard and constant repeating of experiments, the reproducibility did not improve. Thus, in some areas of this thesis the number of animals included in the study remains low. Therefore, in each chapter at the beginning of the results sections the number of animals used versus the number of animals included is written for clarity.

2.4 CEREBELLAR INJECTIONS

To induce, prevent or identify the alternate olivocerebellar pathway, the cerebellum was injected either with growth factors, BDNF blocker or a retrograde dye, subject to the

experimental design. Adolescent animals (P15 and P20) were anaesthetised with diethyl ether and older animals (P30 onwards) were anaesthetised with an intraperitoneal injection of ketamine/xylazine/acepromazine (as described above).

A longtitudinal skin incision was made over the neck, the left occipitalis muscle removed from the left occipital bone, a craniotomy performed to expose the vermis and left cerebellar hemisphere and the animal placed in a stereotaxic frame (Kopf, USA). The stereotaxic frame earpieces were placed in the ears of adult animals (P30 or older). For animals younger than P30, their head was placed on a mould such that the superior surface of the skull was horizontal and body rested almost vertically so that the dorsum of the brainstem was almost vertical. The 1 µl Hamilton syringe was placed at an angle of 30 degrees vertically, parallel to the longtitudinal axis of the animal and was inserted to a depth of 1.1 mm into the cerebellum. A total volume of 1 µl of the growth factor, blocker or retrograde dye was then slowly injected into the cerebellum in 15 aliquots to maximise exposure to the cerebellar cortex (Fig 2.1). These injections were made between the midline and mid-lateral hemisphere from lobules VIb and lobulus simplex rostrally to lobules VIII copula pyramidis caudally. Immediately after the injections, the syringe was removed, the wound sutured and the animal allowed to recover in a warm box. If the cerebellar injection was performed in animals older than approximately P60, the animals were rehydrated with 0.9 % (w/v) saline (1 ml/kg body weight i.p).

To prevent olivocerebellar reinnervation in neonatal rats 1 µl polyclonal chicken anti-human BDNF antibody (2 – 500 µg/ml: Lot 9140505L, Promega), or 1 µl K252a (0.05 µg/ml: Alomone Labs) was injected into the left hemicerebellum. In all cerebellar injections control animals were treated similarly, however a vehicle (2 or 500 µg/ml non-specific immune chicken serum or 1 µl/ml DMSO) was injected.

The induction of olivocerebellar reinnervation was achieved in chapters 5 and 6 by injecting 1 µl recombinant human BDNF (0.5 - 1 mg/ml: Lot 21075C8, Amgen Inc, CA USA) in 0.1 % (w/v) bovine serum albumin in phosphate buffer (pH 7.4) into the left hemicerebellum. The dose (4 µmol/l) to the cerebellum is that previously shown to be

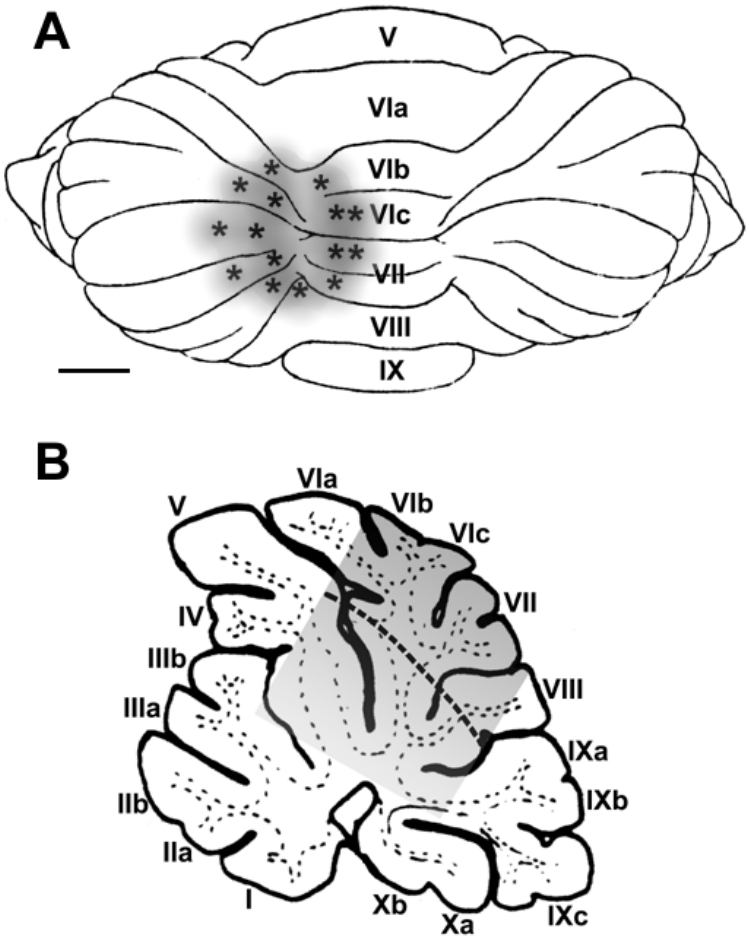


Figure 2.1 Cerebellar injection site
Schematic diagrams (modified from Larsell, 1952) showing the cerebellar injection sites of BDNF or vehicle (cytochrome c).
(A) Dorsal view of the cerebellum indicating location of cerebellar injections. Fifteen injections (*) were made in lobules vermis VIb and lobulus simplex rostrally to lobules VIII and copula pyramidis caudally. The overall area encompassed by the injection is indicated by the grey shading. Bar = 1 mm
(B) A sagittal section view representing the depth (1.1 mm) of the cerebellar injections (dotted line) within the vermis and hemisphere. The gradient of grey shading estimates the concentration of BDNF, which will diffuse around each injection but be greater dorsally due to some reflux up the pipette track.

Key: I–X = vermal lobules I to X.

optimal for inducing transcommissural reinnervation (Sherrard and Bower, 2001). For each age group, the dose was calculated using the left cerebellar hemisphere volume at that age (Heinsen, 1977). Px15 animals received 0.5 mg/ml, Px20 animals received 0.75 mg/ml and Px30 animals received 1 mg/ml. Again, control animals were treated similarly, however a vehicle (cytochrome C, 0.5 – 1 mg/ml: Sigma-Aldrich) with similar physicochemical properties (charge and molecular weight) to BDNF was injected (Caleo et al., 2003).

Additionally, olivocerebellar reinnervation was identified by injecting 1 µl fluorescent retrograde tracer (2 % (w/v) Fast Blue, Illing) into the left hemicerebellum, lateral to the paravermal vein, to ensure no spread of the injection across the midline.

2.5 INFERIOR OLIVE INJECTIONS

To prevent inferior olivary cells from degenerating, or to identify the alternate olivocerebellar pathway, the inferior olivary complex was injected either with growth factors or an anterograde dye, subject to the experimental design. Adolescent animals (P15 and P20) were anaesthetised with diethyl ether and older animals (P30 onwards) were anaesthetised with an intraperitoneal injection of ketamine/xylazine/acepromazine (as described above).

A longtitudinal skin incision was made over the neck, the longtitudinal neck muscles retracted laterally and the atlanto-occipital membrane removed to expose the obex and posterior spinal vein on the dorsal surface of the brainstem. The animal was placed in a stereotaxic frame as described above. The 1 µl Hamilton syringe was placed at an angle of 50 degrees to the vertical and was inserted approximately 1.1 µm into the brainstem along the division of the cuneate and gracile nuclei (approximately 1 mm lateral to the midline in adults). The injections were made according to a weight/depth chart (Sherrard et al., 1986). Three injections were made, the first of which was just caudal to the occipital bone (at the level of the obex), the third just rostral to the cervical C1 vertical arch at the caudal medullo-spinal junction, and the second in between those two points. After injections the syringe was removed immediately, the wound sutured and

the animal allowed to recover in a warm box. If the olive injection was performed in an animal older than approximately P60, the animal was rehydrated with 0.9 % (w/v) saline (1 ml/kg body weight i.p).

The P4 animals in chapter 4 each received 3 injections (into the right inferior olive) of 0.2 µl of BDNF (1 mg/ml or 6.2 mg/ml: Lot 21075C8, Amgen Inc, CA USA) in 0.1 % (w/v) bovine serum albumen in phosphate buffer (pH 7.4) or a vehicle control molecule (cytochrome C, Sigma-Aldrich). Both the BDNF and vehicle solutions contained Fluororuby dye (4 % (w/v) in distilled water; Fluororuby, D-1817 10,000 MW; Molecular Probes, OR USA) to identify the location of the injection site. Depending on the experimental protocol, some animals underwent a second olivary injection at P10, using the same method.

Lastly, the adult rats in chapters 5 and 6 each received 3 injections (into the left inferior olive) of 1 µl of Fluororuby (4 % (w/v) in distilled H_2O; Fluororuby, D-1817 10,000 MW; Molecular Probes, OR USA).

2.6 BEHAVIOURAL TESTS

The behavioural sequelae of animals in chapter 6 was observed from P35 to identify whether climbing fibre reinnervation improved the animals functional recovery. No pre-lesion training was undertaken for any behavioural test and tests were administered during the light portion of the 12 hour light/dark cycle. To reduce observer bias, the tester was blind to the animals' experimental group. Furthermore, since behavioural testing preceded histological verification of the completeness of surgery, the observer was unaware which animals were later to be excluded from the study. Since the cerebellar cortical circuit controls complex motor coordination (Marr, 1969) and the climbing fibre pathway is important for the learning and control of rhythmical synchronisation of sensorimotor skills (Rondi-Reig et al., 1997) the animals motor coordination and sensorimotor skills were assessed. Refer to chapter 6 for behavioural test descriptions.

2.7 TRANSCARDIAC PERFUSIONS AND HISTOLOGICAL PREPARATION

At the end of each experiment tissue fixation was achieved through transcardiac perfusion. Animals were deeply anaesthetised with sodium pentobarbitone, the chest cavity opened, a cannula inserted into the left ventricle and the ascending aorta, and the animal gravity perfused with heparinised saline (5 IU/ml: 20 – 100 ml dependent on animal age) and 4 % (w/v) paraformaldehyde (pH 7.4: 200 – 900 ml dependent on animal age). The cerebellum and brainstem were immediately removed following the perfusion and stored at 4 ^{o}C in 30 % (w/v) sucrose in 4 % (w/v) paraformaldehyde for 5 days.

Tissue was cryoprotected before being frozen in liquid nitrogen and sectioned on a Leica cryostat. Parallel sets of coronal or sagittal 20 – 40 μm serial sections were taken from the medullary-spinal junction through to the midbrain. Sections were cut onto gelatinised slides or into 24-well plates containing 0.1 M phosphate buffered saline (PBS: pH 7.4) containing 0.25 % (v/v) TritonX100 (T-PBS).

For each experiment, one set of slides were stained with methylene blue dye to label nucleic acids and acidic carbohydrates to enable selective staining of neurons and their cytoplasmic extensions. Slides were washed for 8 minutes to remove the cryomatrix, rinsed in 90 % (v/v) ethanol to allow the stain the penetrate the cells, stained with 0.1 % (w/v) methylene blue dye, dehydrated and mounted in DePeX prior to coverslipping with type 1 coverslips.

A second set of sections were used in chapters 4, 5 and 6 to visualise the climbing fibre terminal arbors using immunohistochemistry for the vesicular glutamate transporter VGLUT2, which is localised in both climbing fibre and mossy fibre terminals (Hioki et al., 2003). The VGLUT2 was labelled with guinea pig polyclonal anti-VGLUT2 raised against a synthetic VGLUT2 peptide that has no overlap with VGLUT1 (Chemicon, Hampshire UK; AB5907). To examine the relation of climbing fibre arbors to their target Purkinje cell dendritic tree, Purkinje cells were counterstained using a specific marker, calbindin (Celio, 1990). The monoclonal mouse anti- calbindin antibody is

raised against purified bovine kidney 28kD calbindin, which does not cross-react with other calcium-binding proteins (Sigma-Aldrich; C9848) and has been extensively characterised as a marker for cerebellar Purkinje cells (Doughty et al., 1999). Controls for antibody specificity were abolition of staining following omission of the primary antibody or pre-absorption of the antiserum with the immunogen peptide (Chemicon; AG209). Furthermore, the distributions of both VGLUT2- and calbindin-like immunoreactivity were identical to those previously described in the cerebellum (Doulazmi et al., 2001; Hioki et al., 2003; Kaneko and Fujiyama, 2002; Miyazaki et al., 2003; Zanjani et al., 2004). Floating sections were washed in T-PBS and blocked for 1 hour by incubating in T-PBS containing 0.2 % (w/v) gelatine (T-PBS-G). Slices were then incubated overnight in T-PBS-G containing two primary antibodies, mouse anti-calbindin (dilution 1:200) and guinea pig anti-VGLUT2 (dilution 1:3000). Primary antibodies were incubated for 3 hours with AMCA coumarin-conjugated donkey anti-mouse and either Cy3- or fluoroscein isothiocyanate-conjugated donkey anti-guinea pig secondary antibodies (Jackson ImmunoResearch Laboratories, USA) diluted 1:200 in T-PBS-G. Slices were washed in T-PBS, mounted onto superfrost slides, allowed to air-dry at 4 ^{o}C for 2 hours, coverslipped in citifluor and stored at 4 ^{o}C.

A third set of slides for fluorescence analysis were used in all experiments to identify: the location of the Fluororuby-labelled olivary injection and anterogradely-labelled climbing fibre terminals in chapter 3; inferior olivary cells in chapters 5 and 6: and anterogradely-labelled climbing fibre arbors in chapters 4, 5 and 6. Slides were washed for 8 minutes to remove the cryomatrix, dehydrated and mounted in DePeX (BDH) before being coverslipped with type 1 coverslips

2.8 HISTOLOGICAL ANALYSIS

Slides were analysed on a Nikon eclipse E800 microscope. Complete transection of the left inferior cerebellar peduncle was verified by histological analysis of all (i.e. serial) sections and degeneration of the right inferior olivary complex, where appropriate. Since in adult animals the inferior olivary complex takes up to 60 days to degenerate post axotomy (Buffo et al., 1998) and this is longer than the survival time of animals

with olivocerebellar pathway transection at P30 (in chapters 5 and 6), right olivary degeneration was not included in the successful transection criterion for these animals. Additionally, in animals that underwent behavioural testing in chapter 6, the cerebellum was searched for presence or absence of the left DCN. Animals were excluded from the behavioural analysis if the left DCN was not present, since removal of the DCN removes cerebellar outflow to other motor centres. Also, for animals in chapter 3 that underwent olivary BDNF injections to maintain survival of the right inferior olivary complex, right olivary degeneration was not included in the criterion for complete olivocerebellar transection.

To identify whether BDNF maintained olivary survival in chapter 3, the brainstem of each section was searched to identify the location of the injection site (labelled with 4 % (w/v) Fluororuby), since injections were not always within the right inferior olivary complex. Only those animals with an injection within the right inferior olivary complex and complete transection of the entire left inferior cerebellar peduncle were included in the experiment. In these animals the right olivary nucleus was searched for healthy olivary neurons. These neurons matched previous reports on olivary morphology: round or ovoid cell somata with a nucleus at one end (Cunningham et al., 1999; Lopez-Roman and Armengol, 1994). Although olivary neurons in P8 rats are 16 μm in diameter and in adult rats are 17.4 μm in diameter (Bourrat and Sotelo, 1983), neurons in the axotomised right olivary complex were counted only if they were a similar diameter and morphology to neurons in the intact left inferior olivary complex. The number and location of olivary neurons were mapped in the right inferior olivary complex. Stereology was not used because inferior olivary injections are technically difficult, making the location of the BDNF injection sites variable between animals (as evidenced by the location of the Fluororuby): thus the number of cells counted represents the relative effect of BDNF between groups, rather than the actual number of cells remaining. Additionally, to identify whether BDNF maintained survival of the ipsilateral pathway, the cerebellum was searched for the presence of Fluororuby labelled climbing fibre terminals. The cerebellum of animals was analysed only if the Fluororuby dye was restricted to the right olivary complex.

To indicate the presence or absence of climbing fibre reinnervation in chapters 5 and 6, the cerebellum of animals with fluorescent retrograde tracer injections was checked for restriction of the dye to the left hemicerebellum. Only in those animals in which the dye did not diffuse into the vermis was the inferior olivary complex searched for retrogradely-labelled cells. The number of labelled cells was counted for each group and the mean (± SEM) calculated.

The distribution of reinnervating climbing fibre terminals in chapters 4, 5 and 6 was identified by allocating each VGLUT2- and Fluororuby-labelled arbor in the left hemicerebellum into parasagittal zones 500 μm wide, which started at the midline (zone 1) and progressed laterally to the edge of the lateral hemisphere (zone 10). To assess whether the reinnervating axons in chapters 5 and 6 were normal, the medio-lateral width of parasagittal bands of Fluororuby-filled climbing fibre terminals was measured using an eyepiece graticule on coronal sections. Additionally, arbor size was calculated as follows: in sagittal sections, arborisations that appeared to lay parallel to the section and could be seen individually (i.e. were not overlapped by adjacent arbors) were measured by fitting a rectangle over the arborisation and recording its diagonal.

To identify the location of the anti-BDNF antibody injection site in chapter 4, and to ensure that BDNF-treatment in chapters 5 and 6 did not alter climbing fibre arborisation indirectly by altering cerebellar Purkinje cell growth and granule cell survival, the depth of the molecular layer was measured. Molecular layer depth was measured on coronal sections to ensure that at each site the same medio-lateral position was consistently measured. Measurements were taken at three sites in each experimental and control hemicerebellum and the group mean was calculated.

2.9 PHOTOMICROGRAPHS

The photomicrographs were taken on a Nikon eclipse E800 microscope with a Nikon HC3002i or Photometrics Coolsnap-fx (Roper Scientific Inc, AZ USA) digital camera and were imported into Adobe Photoshop version 5.0.2 prior to cropping and printing.

Where appropriate colour was added and a digital montage of fluorescent labelling made. Selected photomicrographs were taken on a Biorad Confocal microscope using COSMOS software. Blind deconvolution was carried out through AutoDeblur v 9.1 (AutoQuant Imaging Inc., New York, USA) and where appropriate, colour was added and a digital montage of fluorescent labelling made. The behavioural photographs were taken on a Kodak Easy Share CX6330 digital camera and imported into Adobe Photoshop version 5.0.2 prior to cropping and printing.

2.10 STATISTICS

Inter-group comparisons of cell numbers, arbor size and molecular layer depth were made using Mann Whitney-U analysis and the data are expressed as mean (± SEM) for ease of comparison with other studies. Non-parametric analyses were required in the absence of homogeneity of variance (transformation of data did not produce homogeneity). P values smaller than 0.05 were considered significant.

In chapter 6, for each animal the scores of all daily behavioural test trials were averaged to get the mean score per day. Then, the mean daily score obtained by the *n* animals of each group was calculated and plotted on the graphs. Inter-group comparisons for time were made using the Kruskal-Wallis test and post hoc Mann Whitney-U, while intra-group comparisons were by Freidman and post hoc Wilcoxon analyses. Additionally, the ability (percent success) or direction taken was compared between groups using χ^2 test.

In chapter 3, in some groups the number of animals is low (n = 2) due to the pedunculotomies in these groups being incomplete. Although these experiments were repeated to increase the number of animals in each group for statistical purposes, the number of animals remains low in some groups because of an aberrant cerebellar pathology (possibly caused by parvovirus). It should be noted therefore that the data from these groups represents a trend only and statistical measures have been included for ease of discussion.

CHAPTER 3

BRAIN-DERIVED NEUROTROPHIC FACTOR APPLIED TO OLIVARY NEURONS DOES NOT PREVENT THE NATURAL REGRESSION OF THE IPSILATERAL OLIVOCEREBELLAR PATHWAY FOLLOWING NEONATAL AXOTOMY

3.1 INTRODUCTION

After complete unilateral lesions in the developing rodent brain or spinal cord, axons grow from the contralateral uninjured side into the denervated region (Angaut et al., 1982; Angaut et al., 1985; Ballermann and Fouad, 2006; Barth and Stanfield, 1990; Naus et al., 1986; Sherrard et al., 1986; Spear, 1995), which improves the animals' functional recovery (Ballermann and Fouad, 2006; Barth and Stanfield, 1990; Dixon et al., 2005; Weber and Stelzner, 1977). Unfortunately, however, these animals never regain function to levels identical to that of sham-operated control animals and this may be due to the imprecise post-lesion neuronal circuitry formed. An alternative solution is to make use of the supernumerary ipsilateral pathways that regress during development once the normal pathway has been established (Katz and Shatz, 1996; Oppenheim et al., 1992). If the natural regression of these ipsilateral pathways can be prevented and their axons induced to grow across the midline into the denervated region, then it is possible the animals' post-lesion function may improve to levels similar to that of sham-operated control animals, since the side of the brain contralateral to the lesion would once again be controlling the correct side of the body.

While growth factor application can prevent naturally occurring cell death (Oppenheim et al., 1992), it remains unknown whether growth factor application can also prevent the death of neurons belonging to the ipsilateral olivocerebellar pathway. These ipsilaterally projecting neurons begin to degenerate from birth, cease axonal transport by P10 (Bower and Sherrard, 1986) and all but a few fibres regress before the end of cerebellar maturation (Lopez-Roman et al., 1993). Since olivary expression of BDNF, trkB and p75 increases during periods of climbing fibre plasticity (Li et al., 2001; Liu,

2005; Nitz et al., 2001; Sherrard, personal communication), it is possible that BDNF application to the inferior olive may prevent the normal regression of the ipsilateral olivocerebellar pathway. Additionally, target cell availability is important for preventing the natural regression of ipsilateral pathways (Fournier et al., 2005), which is believed to be due to competition for limited amounts of trophic factors secreted from the target cells (Clarke et al., 1998; Ma et al., 1998). Consistent with this proposal, olivocerebellar transection in neonatal rats results in olivary cell death, which is maximal 48 hours after transection (Lopez-Roman and Armengol, 1994), suggesting that elimination of target derived trophic factors is involved in olivary cell death. Since BDNF is synthesised by Purkinje cells in the cerebellum (Maisonpierre et al., 1990; Neveu and Arenas, 1996; Rocamora et al., 1993) when its receptors are expressed in the inferior olive (Merlio et al., 1992; Nitz et al., 2001; Riva-Depaty et al., 1998) and BDNF administration to the cerebellum just beyond the closure of the critical period induces reinnervation into the denervated hemisphere (Sherrard and Bower, 2001), target cell availability may also prevent the natural regression of the ipsilateral pathway.

Therefore, this project examines whether BDNF application to inferior olivary neurons and target cell availability prevents the natural regression of the ipsilateral olivocerebellar pathway to create normal post-lesion neuronal circuitry, albeit through an abnormal route, which may improve the animals' functional recovery.

3.2 METHODS

Twenty three litters of rats were used to investigate whether BDNF maintains olivary neuronal survival and the ipsilateral projection post-lesion. In each experimental animal, the left olivocerebellar pathway was transected at P3 and either immediately or 24 hours later the animal received 3 injections of 200 nl of BDNF (total BDNF = 0.6 – 3.7 μg) or vehicle (total cytochrome C = 0.6 μg) into the right inferior olive with 4 % (w/v) Fluororuby (to identity the location of the injection: refer section 2.5). It was unknown what BDNF concentration would be the most efficacious, and therefore lower (0.6 μg) and higher doses (3.1 – 3.7 μg) of BDNF were used. Furthermore, BDNF was injected either immediately or 24 hours following olivocerebellar transection because

the most efficacious timing of BDNF injection was also unknown, since the first visible signs of olivary cell degeneration appear at 48 hours after transection (Lopez-Roman and Armengol, 1994), although some types of apoptosis in the CNS begin 6 hours following axotomy (Ward et al., 2006). Lastly, some animals that received olivary injections at P4 also received a further 3 olivary injections (200 nl each) at P10 to sustain BDNF availability.

Animals were transcardially perfused (refer section 2.7) at different time points (P8, P12, P15 or P30) to identify whether the BDNF treatment had a time course effect. Since the majority of axotomised inferior olivary cells die within 4 days post-lesion (Lopez-Roman and Armengol, 1994), the first time point observed was 5 days post-lesion to determine the efficacy of the growth factor, and the last time point observed was P30, which was to determine long-term survival.

The brainstem and cerebellum were removed and two parallel sets of 20 µm coronal frozen serial sections of the inferior olive and cerebellum were cut. Sections were 20 µm because olivary neurons are approximately 16 µm in diameter in P8 rats and 17.4 µm in diameter in adult rats (Bourrat and Sotelo, 1983), therefore each section contains whole cells and no single cell will be present in 2 adjacent sections. One set of sections was stained with 0.1 % (w/v) methylene blue for confirming (1) the complete transection of the entire left inferior cerebellar peduncle (Angaut et al., 1982), and (2) mapping the location of olivary cells in the injected right inferior olivary complex. The other set remained unstained to identify the location of the olivary injection site.

In animals that had a complete left unilateral olivocerebellar transection and correct injection of BDNF into the contralateral (right) inferior olivary complex (as identified by Fluororuby labelling), the right olivary nucleus was searched for healthy olivary neurons as described in section 2.8.

Inter-group comparisons of cell numbers were made using Mann Whitney-U analysis and the data are expressed as mean ± SEM for ease of comparison with other studies. In

some groups the number of animals is low due to incomplete inferior cerebellar peduncle transection (despite repeating experiments) and incorrect location of olivary injection (see below). It should be noted, therefore, that the statistical data from these groups represents a trend only.

3.3 RESULTS

The majority of inferior olivary neurons degenerate within 4 days following axotomy in the early postnatal period (Angaut et al., 1985; Cunningham et al., 1999; Lopez-Roman and Armengol, 1994). The effect of BDNF on the number and location of axotomised olivary neurons within the right olivary complex was recorded at different time points (P8, P12, P15 or P30) in animals with a complete unilateral transection of the left inferior cerebellar peduncle on P3.

To examine whether BDNF prevented inferior olivary cell death and thus salvaged the ipsilateral olivocerebellar pathway, 96 rats were used in this study (Vehicle: P8 = 11; Low dose BDNF: P8 = 10, P12 = 11, P15 = 13, P30 = 13; High dose BDNF: P15 = 6, P30 = 2; High dose BDNF + P10 repeat injection: P15 = 4, P30 = 2; Immediate high dose BDNF: P15 = 5, P30 n = 5; Immediate high dose BDNF + P10 repeat injection: P15 = 3, P30 = 1). After histological verification of complete inferior cerebellar peduncle transection and incorrect location of olivary injections, the number of animals to be included in the study was reduced to 29 (Vehicle: P8 = 3; Low dose BDNF: P8 = 8, P12 = 4, P15 = 2, P30 = 4; High dose BDNF: P15 = 4, P30 = 0; High dose BDNF + P10 repeat injection: P15 = 2, P30 = 0; Immediate high dose BDNF: P15 = 0, P30 n = 2; Immediate high dose BDNF + P10 repeat: P15 = 2, P30 = 0).

3.3.1 BDNF delays the death of olivary neurons

To determine the efficacy of BDNF on olive survival, the number of remaining olivary cells after axotomy at P3 were counted in animals with BDNF- or vehicle-treatment and survival to P8, P12, P15 or P30. An injection of lower dose BDNF (0.6 μg) into the olivary nucleus 24 hours following axotomy at P3 salvaged olivary neurons from cell

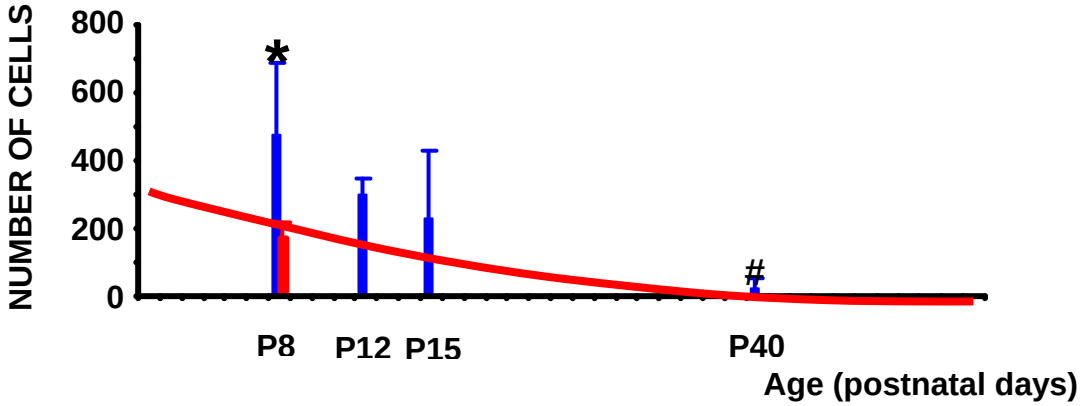
death. However, this effect was only transient as an increase in survival time was inversely proportional to the number of neurons remaining (Fig 3.1A).

In the axotomised inferior olive 5 days following vehicle injection (P8) there were 172 ± 26 scattered olivary cells remaining (mean ± SEM, n = 3: Fig 3.1A&C). The majority of these cells were located in the MAO subnuclei b and c, as well as the ventral lamella of the principal olive (Fig 3.1B). The remainder of the olivary nucleus had degenerated and the area had been invaded by glia. This resembles previous reports of 180 – 271 cells remaining in the olive following axotomy in the neonatal period (Fig 3.1C: Lopez-Roman and Armengol, 1994). Upon this confirmation, it was decided that only P8 was to be measured for vehicle-treated animals, in order to keep animals numbers used to a minimum.

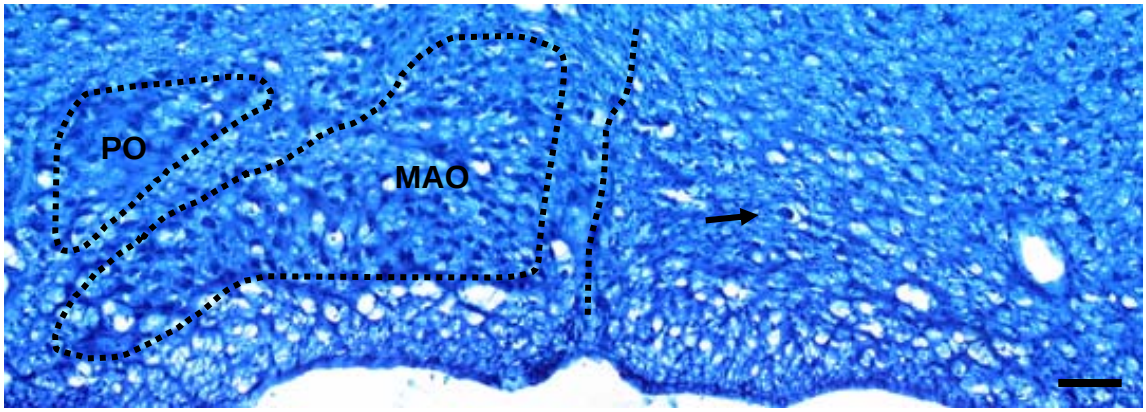
Five days following neonatal transection of the left inferior cerebellar peduncle (P8, n = 8) there was a 240 % increase in the number of olivary cells remaining compared with vehicle-treated control animals (476 and 172 cells, respectively: $p<0.05$: Table 3.1). In BDNF-treated animals, the remaining cells were located within all regions of the MAO, PO and DAO, except for the extreme lateral portion of the DAO where only very few cells were observed. Within the MAO and PO dense patches of cells were observed, but were separated by areas consisting of scattered cells (Fig 3.2A). No obvious rostro-caudal gradient was observed.

By P12 (n = 4), 152 % of olivary cells still remained compared with vehicle-treated lesioned control animals at P8 (301 and 172 cells, respectively: $p<0.05$: Table 3.1). Although this is less than the number of cells remaining in low dose BDNF-treatment at P8 (476), this was not statistically significant. At P12 patches of surviving olivary cells were found amongst apoptotic bodies in the MAO, PO and DAO (except for the far lateral portion: Fig 3.2B).

A



B



C

Treatment Group	Number of Cells
Px1 + Survival to P7	271 ± 30 (Lopez-Roman and Armengol, 1994)
Px3 + Survival to P8 (n = 3)	172 ± 26
Px5 + Survival to P15	180 ± 18 (Lopez-Roman and Armengol, 1994)
Px20 + Survival to P40	26 ± 12 (Lopez-Roman and Armengol, 1994)

Figure 3.1 Effect of low dose BDNF and vehicle on inferior olivary cell survival
(A) Graph showing the number of olivary cells remaining in the axotomised inferior olivary complex following BDNF (0.6 µg: blue bars) or vehicle (0.6 µg: red bar) treatment 24 hours post-axotomy. The red trendline indicates the number of olivary cells remaining following axotomy in the neonatal period from Lopez-Roman and Armengol, 1994.
* $p<0.05$ compared with vehicle-treated group at P8. # $p<0.05$ compared with BDNF-treated group at P8.
(B) Photomicrograph of the ventral brainstem of a vehicle-treated animal 5 days following axotomy. The right inferior olive has degenerated although one olivary neuron remains (arrow). The vertical dotted line indicates the brainstem midline.
(C) Table showing the number of cells (mean ± SEM) remaining in the axotomised inferior olivary complex following vehicle treatment at P8. This is compared with the number of cells remaining in the inferior olivary complex at P7, P15 and P40 following neonatal axotomy (Lopez-Roman and Armengol, 1994).

Key: Bar in **(B)** = 50 µm; MAO = medial accessory olive; PO = principal olive.

Table 3.1 Effect of BDNF (high and low dose) on inferior olivary cell survival

Treatment Group	P8	P12	P15	P30
Low dose BDNF (24 hr)	476 ± 75 (n = 8)	301 ± 23 (n = 4)	228 ± 142 (n = 2)	24 ± 13 * (n = 4)
High dose BDNF (24 hr)	-	-	127 ± 39 * (n = 4)	-
High dose BDNF (24 hr + repeat P10 injections)	-	-	33 ± 17 * (n = 2)	-
High dose BDNF (immediate + repeat P10 injections)	-	-	17 ± 6 * (n = 2)	-

Table shows the number of inferior olivary neurons counted (mean ± SEM) treated with low (0.6 μg) or high (3.1 – 3.7 μg) dose BDNF either immediately or 24 hours following complete transection of the entire left inferior cerebellar peduncle at P3, with or without a repeat BDNF injection at P10. The animals survived to P8, P12, P15 or P30. The number of cells remaining following low dose 24 hr BDNF injection decreases with an increasing survival time and the number of cells remaining at P15 after a high dose BDNF injection 24 hours following olivocerebellar transection is less than that at P8 with a low dose BDNF injection. The trend indicates that the number of cells at P15 after a high dose BDNF injection at 24 hours (with or without repeat injections) is less than the number of cells at P15 with a low dose BDNF 24 hour delayed injection. Also, the trend indicates the number of cells remaining at P15 after an immediate high dose BDNF injection (with repeat injections) is less than that found in the 24 hour delayed high dose BDNF (with repeat injections) group.

Key: * $p<0.05$ compared with 24 hour delayed low dose BDNF injection with survival to P8.

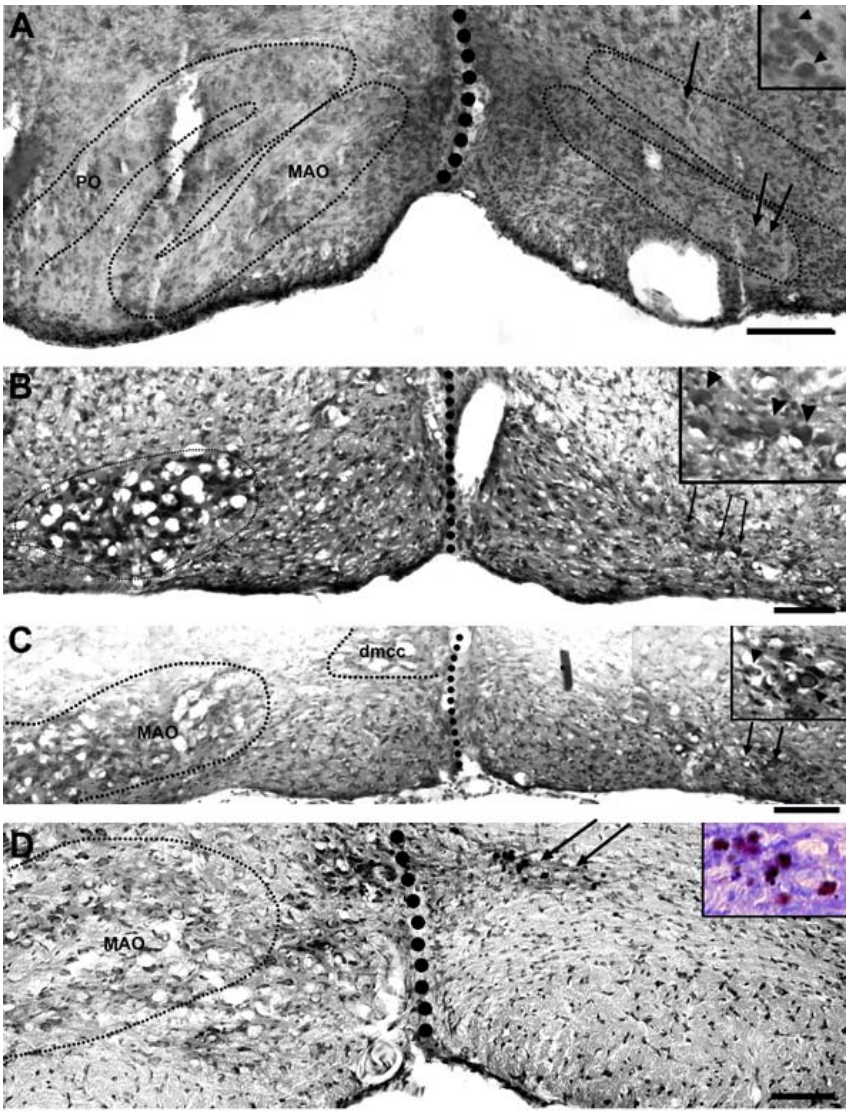


Figure 3.2 Photomicrographs of the inferior olivary complex in BDNF-treated (low dose) animals

(A) Patches of olivary neurons remain at P8 amongst areas of no cells. Inset shows these neurons at higher power, in which the cytoplasm and nucleus can be seen at one end of the cell. Bar = 100 μm.

(B) Patches of neurons remain at P12 (indicated by arrows). Bar = 50 μm.

(C) A few scattered neurons remain in the olivary complex at P15, shown by arrows. Bar = 50 μm

(D) An absence of olivary neurons at P30. The arrows show the remnants of the Fluororuby-labelled olivary injection site (also see inset). Bar = 50 μm.

Key: dmcc = dorsal medial cell column; MAO = medial accessory olive; PO = principal olive. The vertical dotted lines indicate the brainstem midline

Survival to P15 (n = 2) resulted in a reduction in the number of remaining olivary cells compared with survival to P12 (228 and 301 cells, respectively: Table 3.1: 115 % of control lesioned animals), although this was still not statistically less than the number of cells remaining in BDNF-treated animals at P8 (476). At P15 there were fewer patches of surviving neurons and an increase in the number of scattered neurons in the MAO, PO and medial DAO (Fig 3.2C), with no apparent rostro-caudal gradient.

At the longest survival time of 27 days (P30, n = 4) there was a decrease in cell survival: only 24 cells remained, which is 12 % of the number of cells in the vehicle-treated animals at P8 (Table 3.1) and is the first age that the number of neurons counted was statistically less than the number of neurons remaining in the BDNF-treated animals at P8 ($p<0.01$). Additionally, the number of cells remaining at this age is similar to the number of cells remaining following neonatal axotomy in untreated animals in a previous study (P27 – P40: 26 cells: Lopez-Roman and Armengol, 1994). The remaining cells in the current study were scattered throughout the MAO and PO (Fig 3.2D), with no obvious rostro-caudal gradient. At this age most debris appeared to have been scavenged from the site of degeneration and glia had invaded the area (similar size to neurons, except they are filled with central condensations of chromatin or nucleoli: Cunningham et al., 1999; Lopez-Roman and Armengol, 1994).

3.3.2 BDNF applied to the olivary neurons does not salvage the ipsilateral olivocerebellar pathway

Although BDNF appears to provide only transient protection to neonatally axotomised olivary neurons, it was unknown whether the cells salvaged belong to the ipsilateral or contralateral pathways. Therefore, to establish whether the neurons salvaged by BDNF were those of the ipsilateral pathway or axotomised cells of the normal path, the cerebellum of low dose BDNF-treated animals was examined for Fluororuby-labelled climbing fibre terminals and/or axons. Only the cerebellum of rats in which the Fluororuby injection was confined to the axotomised right inferior olive were examined (i.e. the injection did not cross the brainstem midline into the intact left inferior olivary

complex). This was to ensure intact neurons of the left inferior olivary complex were unable to transport dye to the cerebellum, giving false positive results.

In low dose BDNF-treated animals that survived to P8, degenerating Fluororuby labelled axons, but no terminals were found in the right hemicerebellum ipsilateral to the axotomised olivary neurons and Fluororuby injection (Fig 3.3A). These axons were found throughout the right hemicerebellum and did not cross the cerebellar midline into the left hemicerebellum. In contrast, in animals that survived to P12 or P15, Fluororuby labelling was not observed within the cerebellum (Fig 3.3B). Since neurons belonging to the ipsilateral pathway degenerate slowly after birth, such that axonal transport along the ipsilateral projection ceases by P10 (Bower and Sherrard, 1986) the presence of the retrograde transport at P8, but not P12 indicates that BDNF (with available target cells) is unable to prevent the natural regression of the ipsilateral pathway, despite delaying the death of axotomised olivary neurons.

3.3.3 Higher concentration, multiple injections or immediate exposure of olivary neurons to BDNF does not improve olivary survival

Since the lower BDNF dose (0.6 μg) did not appear to salvage neurons of the ipsilateral pathway, the dose was increased (approximately six-fold) and single (P3 only) or multiple (P3 and P10) injections were administered. The concentration of the higher BDNF dose ranged from 3.1 to 3.7 μg because the BDNF was concentrated on 2 different occasions due to repetition of the experiment to increase animal numbers. Animals were allowed to survive until P15.

Animals that received this higher dose of BDNF (127 cells, n = 4: Table 3.1) had 45 % less cells remaining in the caudal MAO and PO dorsal lamellae at P15 (Fig 3.4A) than animals that received a low dose BDNF-treatment (228 cells). Additionally, multiple injections of higher dose BDNF resulted in approximately a four and seven fold reduction of cells remaining at P15 (33 cells, n = 2: Table 3.1 & Fig 3.4B) compared with single injections of high dose (127 cells) or low dose (228 cells) BDNF respectively. Lastly, to identify whether the 24 hour delay of BDNF was after the onset

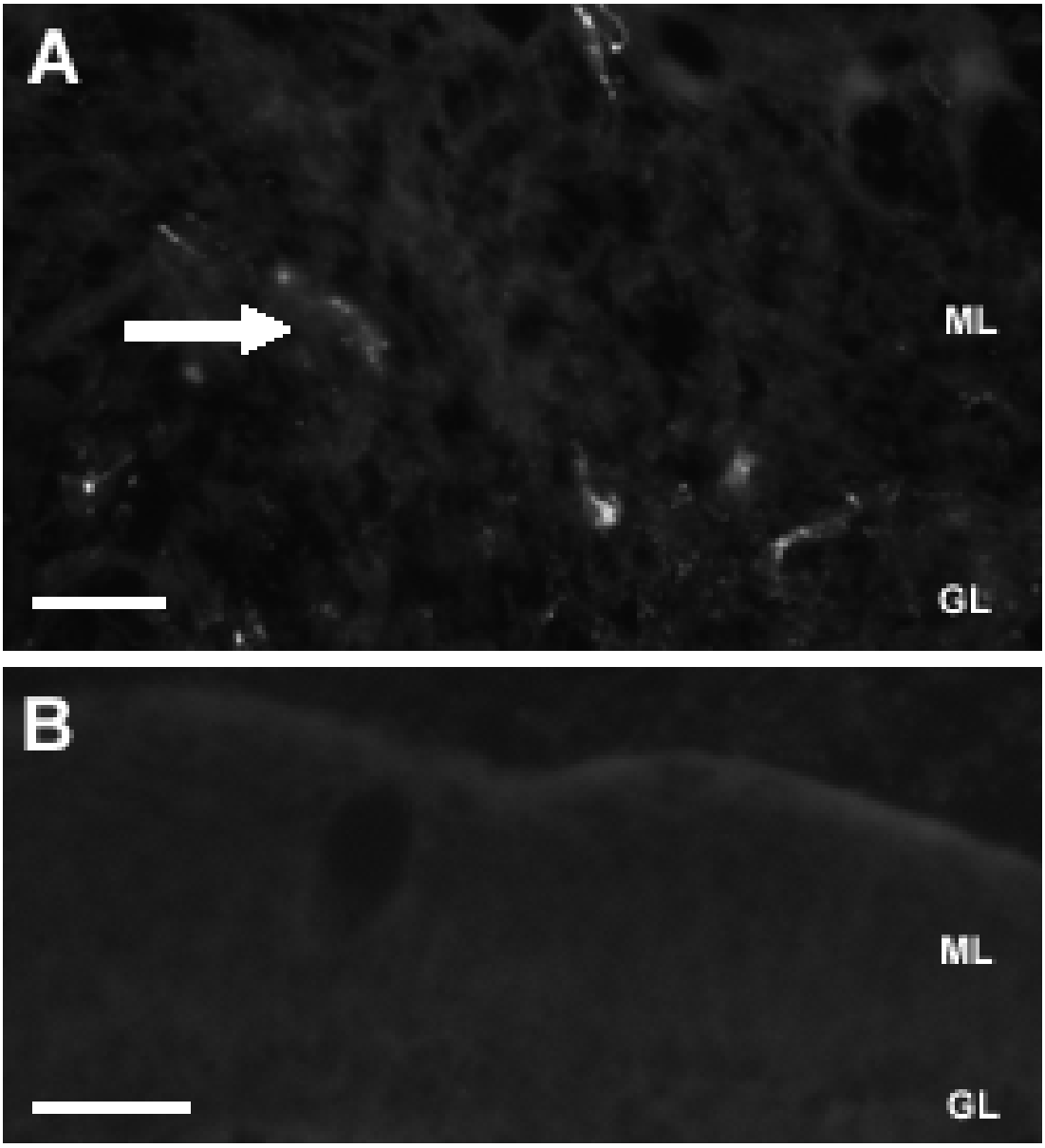


Figure 3.3 Cerebellar cortex of BDNF-treated animals
Photomicrographs of the right hemicerebellum of low dose BDNF-treated animals 24 hours following left unilateral olivocerebellar pathway transection. These photomicrographs show the presence **(A)** or absence **(B)** of Fluororuby-labelled axons at P8 and P12, respectively. Arrow in **(A)** shows an example of a degenerating labelled axon. The absence of Fluororuby labelling in the cerebellar cortex of animals that survived until P12 is similar to the cortex of animals that survived until P15 (data not shown).

Key: Bar in **(A)** = 50 μm; Bar in **(B)** = 100 μm; GL = granular layer; ML = molecular layer.

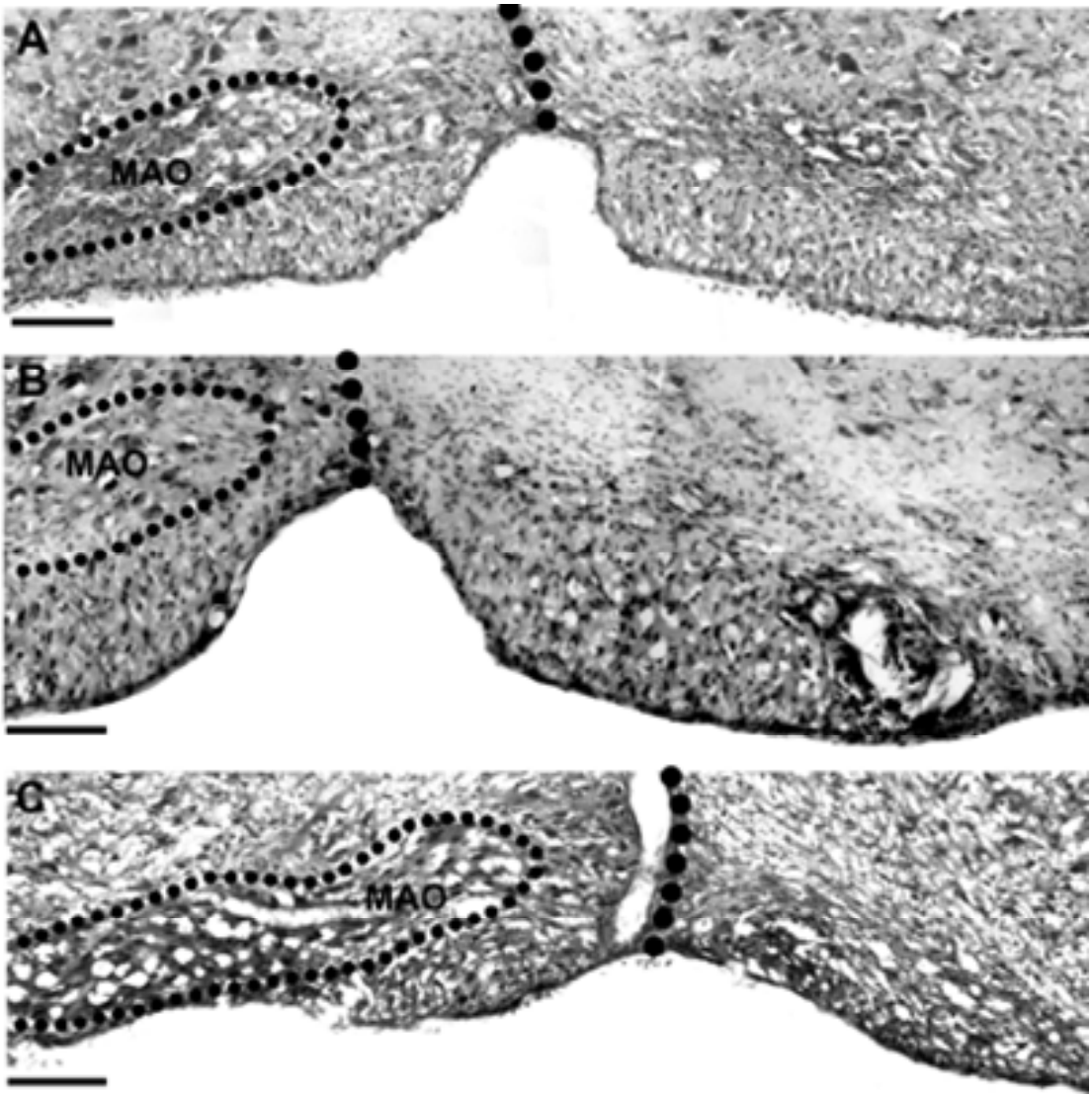


Figure 3.4 Photomicrographs of the inferior olivary complex in BDNF-treated animals (high dose)

Photomicrographs of the inferior olivary complex in P15 animals, treated with high dose BDNF (3.1 – 3.7 μg) immediately or 24 hours after unilateral olivocerebellar pathway transection at P3.

(A) Almost no olivary cells remain within the right olivary complex at P15 following 24 hour delayed BDNF injections at P4.

(B) The right olivary complex remains devoid of almost all olivary cells at P15 following 24 hour delayed BDNF injections at P4 and repeat injections at P10.

(C) The right olivary complex has almost completely degenerated in P15 animals after immediate and repeat injections of high dose BDNF.

Key: Bar = 50 μm; MAO = medial accessory olive; the vertical dotted lines indicate the brainstem midline.

of apoptosis and therefore too late to stop the apoptotic process, BDNF (higher dose) was injected into the right inferior olivary complex immediately following the axotomy, and the animals allowed survive to P15. The number of remaining olivary cells in the animals with immediate and repeat BDNF injections at P10 (17 cells, n = 2; Table 3.1 & Fig 3.4C) was almost half of the number of cells remaining following 24 hour delayed injection with repeat BDNF injections at P10 (33 cells). Unfortunately, these differences were not statistically significant, which is certainly due to the low number of animals in each group (animal numbers not increased, see paragraph below for explanation).

However, the number of cells remaining at P15 in the 24 hour delayed high dose BDNF-treated group with (33 cells) or without (127 cells) repeat injections and in the immediate high dose BDNF-treated group with repeat injections (17 cells) were significantly less than the number of cells remaining at P8 in the low dose BDNF-treated group (427 cells: $p<0.05$: Table 3.1), even though the 24 hour delayed low dose BDNF group at P15 (228 cells) was not less than the 24 hour low dose BDNF group at P8 (476 cells: $p>0.05$: Table 3.1). This suggests that an increased BDNF dose (approximately six fold), sustained exposure of BDNF (through repeat injections), or immediate BDNF injections (compared to 24 hour delayed injections) do not improve olivary survival. As such, the number of animals in this part of the study was not increased and this avenue of investigation was ceased.

3.4 DISCUSSION

This study examined whether increased target cell availability with administration of BDNF around the cell body of inferior olivary neurons prevents the natural regression of the ipsilateral olivocerebellar pathway.

3.4.1 BDNF applied to olivary neurons promotes transient olivary survival

Data from this study confirms previous work that BDNF protects CNS neurons from cell death following axotomy (Pernet and Di Polo, 2006; Sendtner et al., 1992) as shown by an increase in the number of olivary cells remaining at P8 compared with vehicle

treated controls. However, this survival promoting effect is only transient such that the number of cells remaining at the longest survival time point (P30) is less than that of the vehicle control animals at P8, and is consistent with the normal number of neurons found within the inferior olive following neonatal axotomy, but without treatment (Lopez-Roman and Armengol, 1994).

This transient olivary survival is despite the post-lesion upregulation of p75 and trkB receptors in the inferior olivary complex (Nitz et al., 2001; Sherrard, personal communication) and in other areas of the CNS following injury (Hicks et al., 1999; Lu et al., 2002; Venero et al., 2000). One possible explanation is that the delay in BDNF administration was after the onset of apoptosis, which can begin 6 hours post-injury (Ward et al., 2006). However, immediate injections were not able to improve survival rates compared to 24 hour delayed injections; in fact, there was a 2-fold reduction in the number of olivary cells remaining using this regime. Another possible explanation for this transient survival may be receptor availability: BDNF application is known to down-regulate trkB elsewhere in the CNS (Haapasalo et al., 2002; Spalding et al., 2005; Xu et al., 2002), and this may be occurring in the olivocerebellar system. However the number of olivary cells remaining at P12 and P15 suggests that olivary trkB is not completely diminished at these ages (trkB expression was not examined in this study). More likely, however, olivary survival is only transient due to normal receptor availability. While p75 and trkB levels in the olivary complex increase 24 hours post-lesion (Nitz et al., 2001; Sherrard, personal communication), which may aid olivary survival, receptor levels then decrease to normal developmental levels, reaching minimal expression at P15 (Rocamora et al., 1993). Thus, even prolonged availability of this peptide, through repeat injections, was ineffective at prolonging neuronal survival beyond P15, consistent with studies elsewhere in the CNS (Cui and Harvey, 1995).

Additionally, although there was no significant difference between high and low dose BDNF groups at P15 (probably due to the low number of animals creating a large standard error), there was a trend that an increased BDNF dose reduced the number of

olivary cells remaining. This is consistent with previous reports that BDNF can induce glutamate toxicity (Morrison and Mason, 1998) and suggests for the first time that this is dose-dependent.

3.4.2 BDNF applied to olivary neurons does not prevent the natural regression of the ipsilateral olivocerebellar pathway

While growth factor application around the cell body of CNS neurons that have undergone axotomy or deafferentation prevents their death or reverses atrophy (Gillespie et al., 2003; Ruitenberg et al., 2004; Sadakata et al., 2004; Tuszynski et al., 1996) and growth factor application prevents the naturally occurring cell death of motoneurons (Oppenheim et al., 1992), the effect of any growth factor on the natural regression of whole transient developmental pathways had never previously been observed. For the first time this project identifies that BDNF administration to the right inferior olivary complex and the presence of available target cells does not prevent the natural regression of the ipsilateral pathway. This is evidenced by the presence of degenerating Fluororuby-labelled climbing fibre terminals within the right hemicerebellum at P8, and an absence of labelled terminals at P12 and P15 (data not shown for P15), despite no significant reduction in BDNF-treated olivary neurons until P30. This is an interesting finding since it is possible that unilateral olivocerebellar transection during development delays the natural regression of this pathway (Bower and Sherrard, 1986) and increased target cell availability by granule cell removal at P5 maintains the persistence of the ipsilateral olivocerebellar pathway into adulthood (Fournier et al., 2005). There are two potential explanations for the lack of Fluororuby labelling. Firstly, in animals that survived until P12, it is possible Fluororuby-labelled terminals were present in cerebellar sections that were not analysed for fluorescent labelling (i.e. sections stained with methylene blue dye to confirm completeness of surgery), however, the serial sections were cut coronally and thus climbing fibre labelling in parasagittal bands would be detected on many sections and not limited to methylene blue stained sections. Secondly, it is equally possible that at P12 the Fluororuby-labelling in the cerebellum was weak such that it could not be visibly detected from the background auto-fluorescence. However, these animals were allowed

survive 9 days post-anterograde tracer injection, which is both shorter and longer than the survival time in other parallel experiments (chapters 5 and 6), yet these other experiments still had very strong fluorescent labelling within the cerebellum. This suggests that BNDF does not stop ipsilaterally projecting neurons from continuing their natural regression.

Thus, these data suggest that either maintaining BDNF levels within the olive is not a suitable survival factor for ipsilaterally projecting neurons, or the time and/or dose of BDNF delivery was inappropriate. Furthermore, to permanently maintain olivary survival, it is possible that the available target neurons must be in closer proximity to terminals of the ipsilateral pathway, since target populations are known to maintain their innervating neurons (Sherrard and Bower, 1998).

3.5 CONCLUSION

This study extends previous work on the role of BDNF in olivocerebellar plasticity (Dixon and Sherrard, 2006; Lohof et al., 2005; Sherrard and Bower, 2001) by adding that the combination of unilateral olivocerebellar pathway transection and application of BDNF to the cell body does not prevent the natural regression of the ipsilateral olivocerebellar pathway following neonatal axotomy. However, BDNF delays the death of axotomised inferior olivary neurons in the neonatal period and the time-course of survival appears to correspond with olivary receptor expression; thus even prolonged BDNF exposure does not improve survival.

CHAPTER 4

BRAIN-DERIVED NEUROTROPHIC FACTOR REMOVAL INHIBITS POST-LESION OLIVOCEREBELLAR REINNERVATION IN THE NEONATAL RAT CEREBELLUM

4.1 INTRODUCTION

Since BDNF applied to the inferior olivary cell body does not maintain the persistence of the ipsilateral olivocerebellar pathway, it was investigated whether BDNF controls the development of the usual post-lesion alternate pathways that replace those projections that have been destroyed (Zagrebelsky et al., 1997) in order to later manipulate this pathway to promote reinnervation in the mature brain and improve functional outcome (Dixon et al., 2005; Weber and Stelzner, 1977).

As stated earlier, in the normal adult animal olivocerebellar axons enter the cerebellum via the inferior cerebellar peduncle and terminate in climbing fibres that synapse contralaterally on approximately six Purkinje cells, located within a parasagittal microzone (Sugihara et al., 2001). This pathway matures over the first two postnatal weeks (Mariani and Changeux, 1981b; Mason et al., 1990). Following unilateral olivocerebellar transection early in development, the contralateral inferior olive degenerates and new axons, arising from the remaining inferior olive, grow into the denervated hemicerebellum (Zagrebelsky et al., 1997). Within 6 days of the lesion, these transcommissural axons develop normal climbing fibre arborisations within parasagittal microzones and form functional synapses on Purkinje cells (Sugihara et al., 2003; Zagrebelsky et al., 1997).

Since neurotrophins are synthesised in the cerebellum during this time (Maisonpierre et al., 1990; Rocamora et al., 1993) and neurotrophin receptors are expressed in the inferior olive (Nitz et al., 2001; Riva-Depaty et al., 1998), it has been proposed that cerebellar neurotrophins are involved in this reinnervation (Sherrard and Bower, 2002). Furthermore, not only does this plasticity cease by P10 (Sherrard and Bower, 1986), in

parallel with falling olivary receptor expression (Riva-Depaty et al., 1998), but also exogenous BDNF extends the critical period to P12 for transcommissural olivocerebellar reinnervation (Sherrard and Bower, 2001). It still remains unknown, however, whether cerebellar BDNF influences the development of this alternate pathway in the neonatal system.

Therefore this study examines whether removal of cerebellar BDNF or blocking the function of the high affinity receptor for BDNF inhibits the post-lesion climbing fibre reinnervation of denervated Purkinje cells.

4.2 METHODS

Eight litters of rats of either sex aged 3 days were used to determine whether cerebellar BDNF removal or trk receptor blockade (by injecting either anti-BDNF antibody or K252a, which is a known inhibitor of protein kinases (refer Fig 1.1: Ruegg and Burgess, 1989) inhibited development of the alternate olivocerebellar pathway.

Climbing fibres from the left hemicerebellum were damaged via a lesion of the inferior cerebellar pathway at P3, as described in section 2.3. Twenty four hours later 1 µl monoclonal chicken anti-human BDNF IgY (2 – 500 µg/ml: pH 7.4), was injected into the left hemicerebellum and vermis to inhibit any subsequent reinnervation (refer section 2.4 for injection protocol: Fig 2.1). Since 5 and 10 µg/ml anti-BDNF IgY inhibits approximately 75 % and 99 % of BDNF-stimulated trkB phosphorylation, respectively, *in vitro* (Toma and Kaplan, 1997), a range of anti-BDNF IgY concentrations were chosen for the current study (500 µg/ml: n = 11; 100 µg/ml: n = 5; 10 µg/ml: n = 11; or 2 µg/ml: n = 9). Although tests were not performed in the current study to confirm whether *in vivo* application of anti-BDNF IgY inhibits trkB phosphorylation, the term BDNF neutralisation is used in this chapter because the antibody concentrations used are much higher than those used in the previous *in vitro* study (where BDNF was effectively neutralised) and it is therefore presumed that some BDNF will inevitably be neutralised (refer below 4.4.1). As a control, some lesioned

animals received vehicle containing 1 µl non-specific immune chicken serum (2 µg/ml, n = 3; or 500 µg/ml, n = 3).

Additionally, following climbing fibre removal, other animals, that did not receive anti-BDNF IgY, received intracerebellar injections of K252a (dissolved in dimethylsulphoxide: DMSO) to inhibit any reinnervation. Previous studies on the cerebellum *in vitro* used 10 – 100 nM K252a to block granule cell survival (Bhave et al., 1999; Kafitz et al., 1999), therefore, 50 nM (0.05 µg/ml, n = 10) was chosen in an attempt to inhibit protein kinase activation and thus prevent climbing fibre reinnervation (refer below 4.4.1). As a control, some lesioned animals received vehicle containing 1 µl DMSO (0.1 % (v/v), n = 3). To further inhibit trk activation, immediately following intracerebellar K252a injections, some lesioned and sham-operated animals also received 3 injections of 0.2 µl K252a (0.05 µg/ml: Px3, n = 3; sham-operated, n = 1) or vehicle (DMSO: 1 µl/ml; Px3, n = 1) into the inferior olivary complex, as described in section 2.5.

Because climbing fibre reinnervation of the denervated hemisphere takes up to 6 days during the neonatal period (Zagrebelsky et al., 1997), experimental animals were transcardially perfused 10 days after the lesion (P13) (refer section 2.7) to allow enough time for axonal growth to occur. After perfusion, the brainstem and cerebellum were removed and 2 parallel sets of 30 µm coronal frozen serial sections of the inferior olive and cerebellum were cut. One set was stained with 0.5 % (w/v) methylene blue to analyse complete transection of the entire left inferior cerebellar peduncle and complete degeneration of the right inferior olivary complex as described in Angaut et al., 1982. Only those animals with complete transection of the left inferior cerebellar peduncle were included in this study. A second set of sections from animals injected with a total of 2 ng anti-BDNF IgY (2 µg/ml) into the cerebellum, underwent immunohistochemical identification (VGLUT2) of climbing fibre terminals as described in section 2.7.

Since BDNF is a known survival factor for Purkinje and granule cells (Morrison and Mason, 1998; Schwartz et al., 1997) there was a need to assess any non-climbing fibre

effects of BDNF neutralisation, which may in turn affect climbing fibre reinnervation. Qualitative analysis of folia formation and measurement of molecular layer depth were carried out. Measurements were taken at 3 sites in 3 different lobules (lobules VIc, IXb and simplex) of experimental and control hemicerebellum and the group mean was calculated. Only molecular layers that lay parallel (co-planar) to the section were measured.

Additionally, in successfully lesioned animals the distribution of VGLUT2-labelled reinnervating olivocerebellar axons was mapped in the left hemicerebellum. To identify the laterality of reinnervation each arbour was allocated into a parasagittal zone 500 µm wide, as described in section 2.8.

4.3 RESULTS

Although transcommissural olivocerebellar axons are known to reinnervate denervated Purkinje cells following unilateral olivocerebellar transection in the early postnatal period (Zagrebelsky et al., 1997), it was unknown whether BDNF was involved in this reinnervation. Therefore, the distribution of transcommissural climbing fibres was examined in animals treated with intracerebellar anti-BDNF IgY, K252a or vehicle 24 hours following complete unilateral transection of the inferior cerebellar peduncle on P3.

To examine whether BDNF is involved in climbing fibre reinnervation during the neonatal period 65 rats were used in this study (Sham-operated anti-BDNF: 500 µg/ml = 0, 100 µg/ml = 0, 10 µg/ml = 4, 2 µg/ml = 4; Px3 + anti-BDNF: 500 µg/ml = 9, 100 µg/ml = 11, 10 µg/ml = 4, 2 µg/ml = 11; Cbr K252a: sham-operated = 4, Px3 = 10; Cbr DMSO: Sham = 2, Px3 = 1; Olivary K252a: sham = 1, Px3 = 3; Olivary DMSO: Px3 = 1). After histological verification of complete inferior cerebellar peduncle transection, the number of animals to be included in the study was reduced to 52 (Sham-operated anti-BDNF: 500 µg/ml = 6, 100 µg/ml = 9, 10 µg/ml = 4, 2 µg/ml = 4; Px3 + anti-BDNF: 500 µg/ml = 1, 100 µg/ml = 0, 10 µg/ml = 3, 2 µg/ml = 9; Cbr K252a: sham = 4, Px3 = 8; Cbr DMSO: sham = 2, Px3 = 3; Olivary K252a: sham = 1, Px3 = 3; Olivary

DMSO: Px3 = 1). Since, in some animals, the transection procedure missed the entire left inferior cerebellar peduncle and the whole olivocerebellar pathway remained intact (as observed in serial sections), these animals were included in the sham-operated groups to increase the number of animals. Animals were excluded from the study if the left inferior cerebellar peduncle was partially cut.

4.3.1 BDNF neutralisation in the cerebellum induces abnormal cerebellar morphology

Injecting high concentrations of anti-BDNF IgY (500 μg/ml: Px3, n = 1 or sham-operated, n = 6; 100 μg/ml: sham-operated, n = 9), caused large neuronal degeneration within and surrounding the injection site in the left hemicerebellum in all animals (Fig 4.1A-C). This degeneration resulted in a large cavity encompassing the majority of the dorsal aspect of the left hemicerebellum. Any remaining tissue within the left hemicerebellum (lobulus simplex, crus I and crus II) contained an infiltrate of one or more types of inflammatory immune cells (Fig 4.2A): although tests were not done to confirm specific immune cell types. These cells were heavily concentrated around the edges of the injection site, but their density decreased further away from the cavity. Although this pattern of inflammation suggests the degeneration was caused by the presence of an inflammatory response, the vehicle-treated animals (chicken serum: 2 μg/ml, n = 3; 500 μg/ml, n = 3) also had an inflammatory response (Fig 4.2B), although there was an absence of neuronal degeneration. This strongly suggests that the degeneration was a function of the BDNF neutralisation, not the micro-trauma of the injection or the presence of IgY. The neural degeneration observed in this study therefore confirms previous reports that BDNF is a survival factor for cerebellar neurons (Morrison and Mason, 1998; Schwartz et al., 1997). However, the formation of a large cavity within the cerebellum inhibits analysis of climbing fibre reinnervation, therefore lower concentrations of anti-BDNF IgY were analysed. In addition, the number of animals injected with high concentration anti-BDNF IgY with a complete unilateral olivocerebellar pathway transection was not increased.

Injecting lower amounts of anti-BDNF IgY (i.e. at 10 or 2 μg/ml concentrations) had less severe effects on the pathology of the cerebellum (Fig 4.1D-G). These effects

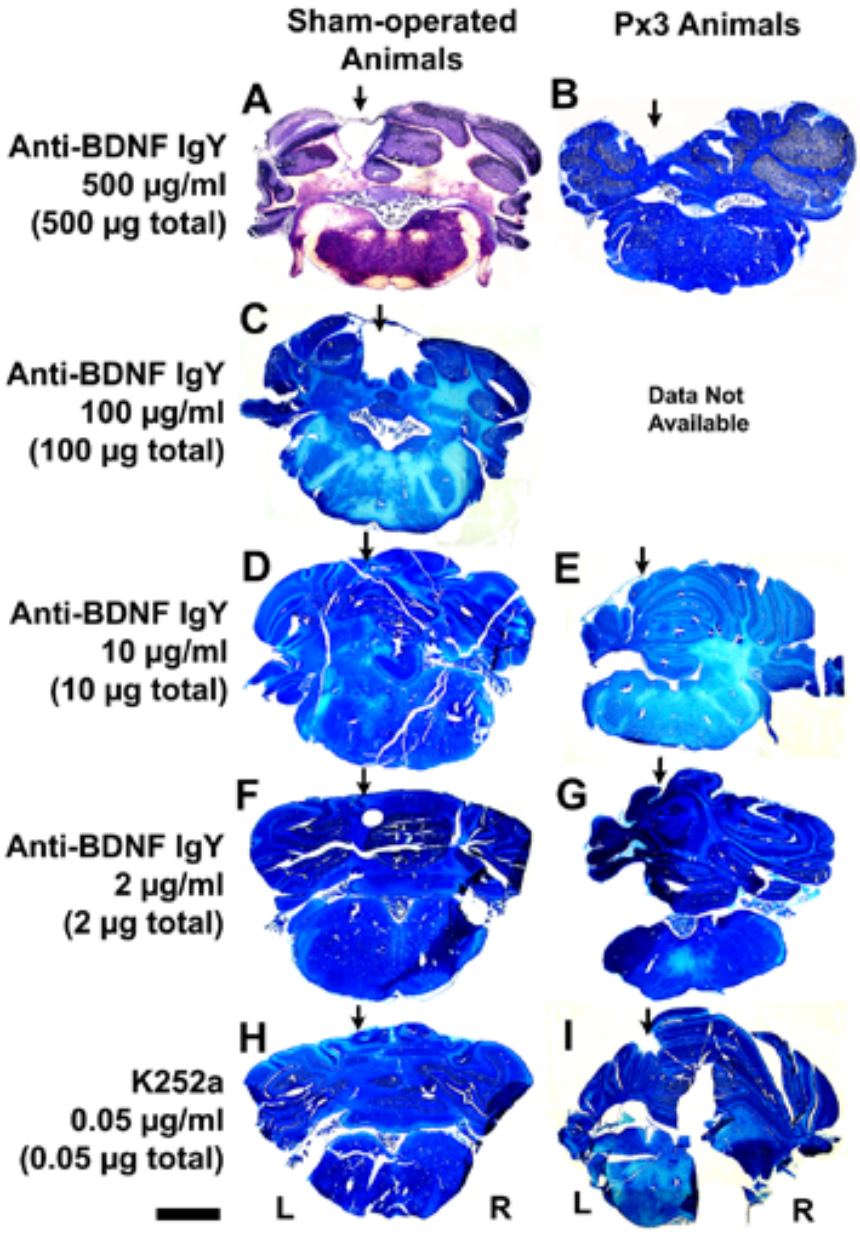


Figure 4.1 Cerebellar morphology following intracerebellar anti-BDNF IgY or K252a administration

A coronal view of sham-operated (A,C,D,F&H) and lesioned (B,E,G&I) cerebella injected with anti-BDNF IgY (500 – 2 µg/ml) or K252a (0.05 µg/ml), stained with methylene blue **(B-I)** or haematoxylin **(A)**.

(A-C) Injection of high dose anti-BDNF IgY caused large neuronal degeneration within and surrounding the injection site resulting in the formation of a large cavity.

(D-G) Injection of low dose anti-BDNF IgY did not cause large neuronal degeneration within or surrounding the injection site.

Key: ↓ = the location of the injection site; Bar = 2 mm; Bregma approx -13.92 mm.

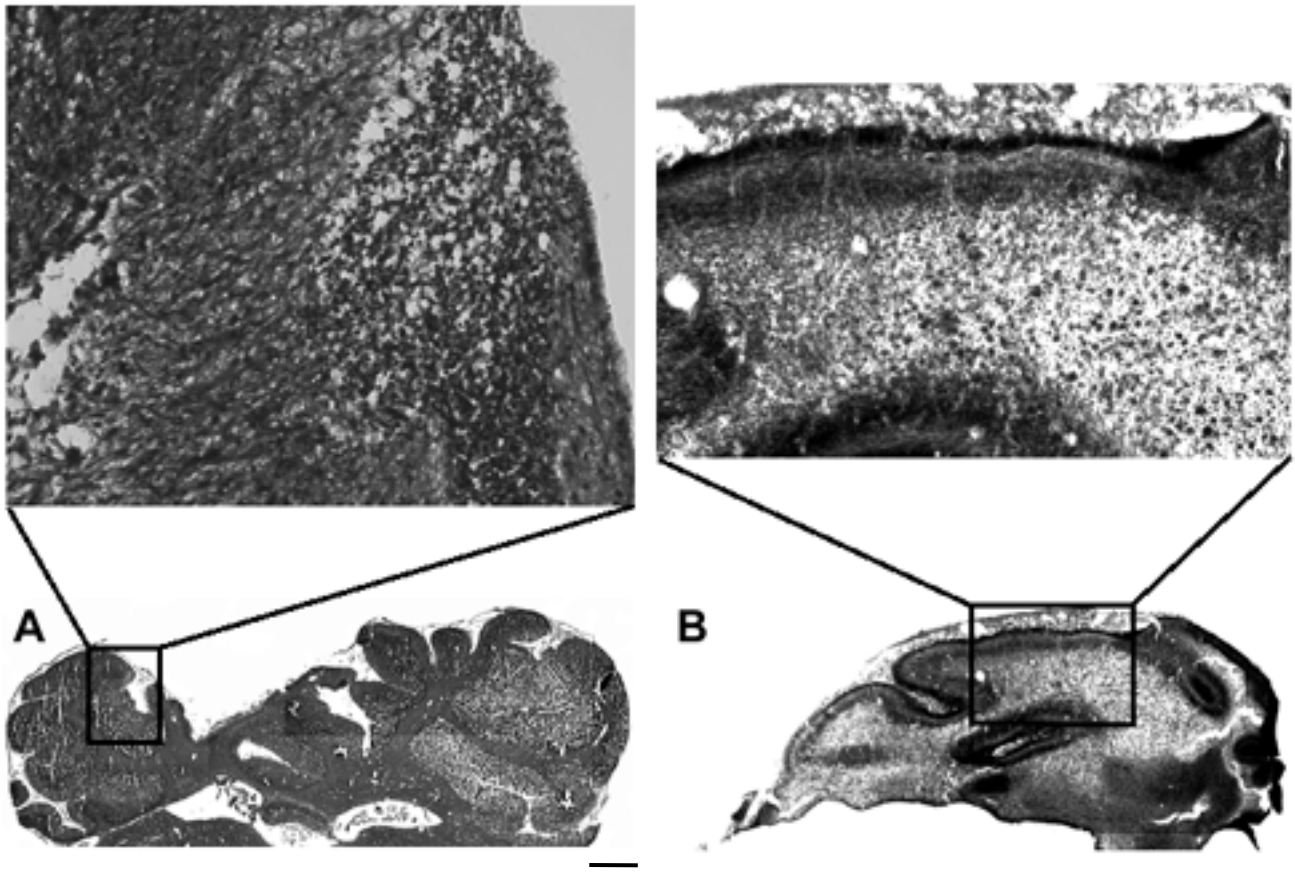


Figure 4.2 Immune cells in the cerebellar cortex of anti-BDNF IgY-treated animals

Coronal sections of the cerebellar cortex of P3 lesioned cerebellum treated with **(A)** anti-BDNF IgY (500 µg/ml) or **(B)** chicken serum (500 µg/ml), stained with methylene blue. Enlarged images show an infiltration of one or more types of inflammatory immune cells.

Key: Bars = 1 mm.

appeared to be dependent on whether or not the left inferior peduncle remained intact. In the cerebellum of sham-operated animals, in which both the olivocerebellar pathways remain intact, administration of 10 ng (10 μg/ml, n = 4) or 2 ng (2 μg/ml, n = 4) anti-BDNF IgY did not cause reduced lobule size or molecular layer depth ($p>0.05$), i.e. the tissue appeared normal. In contrast, injection of both 10 ng (n = 3) and 2 ng (n = 9) anti-BDNF IgY into the climbing fibre deprived hemicerebellum caused reduced size of lobules adjacent to and containing the injection (lobulus simplex and crus I), with subsequent reduction of the presimplex fissure (Fig 4.3). In addition, there was approximately a 28 % reduction in molecular layer depth within lobulus simplex compared with lobulus simplex in the right hemisphere (2 or 10 μg/ml: left = 81.9 – 94.4 μm, right = 114.5 – 127.8 μm: $p<0.05$: Table 4.1 & Fig 4.4). Therefore, BDNF neutralisation had a greater effect when climbing fibres had been removed from the hemisphere.

4.3.2 Protein kinase inhibitor in the cerebellum, but not inferior olive, induces abnormal cerebellar morphology

Similar to anti-BDNF IgY, K252a (0.05 μg/ml) administration also caused morphological changes to the cerebellum. Sham-operated animals that received intracerebellar injections of K252a (n = 4) had a minor reduction in tissue size within the injected left hemicerebellum (Fig 4.1H). In contrast, administration following unilateral olivocerebellar transection (n = 8) lead to extensive neuronal degeneration, often resulting in a large cavity (Fig 4.1I). Since injection of vehicle DMSO (n = 3) alone did not cause any cerebellar morphological defects (data not shown), this suggests that the degeneration, which is exacerbated in the climbing fibre deprived hemicerebellum, was likely due to protein kinase inhibition.

In contrast, in animals that received intraolivary injections of 0.03 ng K252a (0.05 μg/ml: Px3, n = 3; sham-operated, n = 1) or vehicle (DMSO: Px3, n = 1), histological analysis did not reveal histological abnormalities in the inferior olive and cerebellum (Fig 4.5). Thus administration of K252a within the inferior olive does not effect the development of cerebellar or olivary neurons.

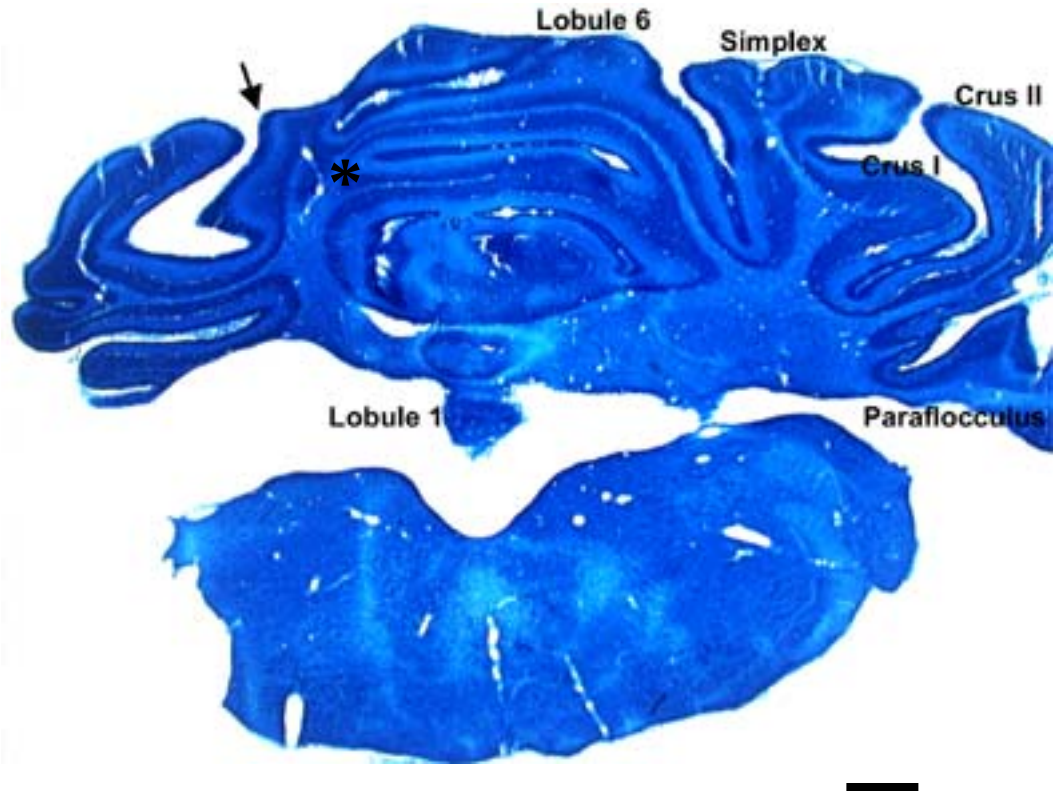


Figure 4.3 Reduction in cerebellar lobule size
Coronal section of the cerebellum and brainstem of a P3 lesioned animal treated with anti-BDNF IgY (2 µg/ml) showing a reduction in size of lobulus simplex and crus I (indicated by arrow), with subsequent reduction of the presimplex fissure (*), compared with control right hemisphere.

Key: Bar = 1 mm.

Table 4.1 Molecular layer depth following anti-BDNF IgY treatment

Treatment Group	Left hemisphere ML Depth		Right hemisphere ML Depth	
	Lobulus Simplex (injection site)	Lobules 9b & 6c	Lobulus Simplex	Lobules 9b & 6c
Sham-operated Animals				
10 µg/ml anti-BDNF IgY	97.2 ± 7.0	116.4 ± 10.6	87.0 ± 7.1	111.7 ± 9.9
2 µg/ml anti-BDNF IgY	87.5 ± 6.1	177.1 ± 21.4	75.9 ± 8.8	211.4 ± 23.6
Px3 Animals				
10 µg/ml anti-BDNF IgY	94.4 ± 0.0 *	128.2 ± 6.7	127.8 ± 3.2	126.4 ± 5.9
2 µg/ml anti-BDNF IgY	81.9 ± 4.7 *	104.3 ± 10.6	114.5 ± 12.3	91.6 ± 5.0

Table shows the depth of the molecular layer in µm (mean ± SEM) within (lobulus simplex) and outside of the injection site (lobules 9b and 6c) in the left and right hemispheres of lesioned and sham-operated animals treated with anti-BDNF IgY (2 or 10 µg/ml).

Key: ML = molecular layer; * p= < 0.05 compared with molecular layer depth in right hemisphere.

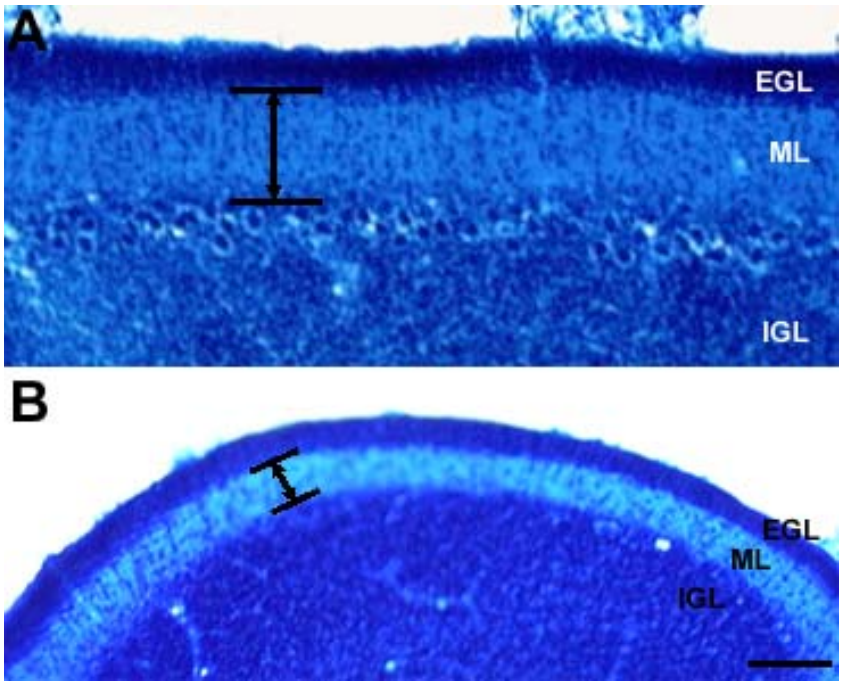


Figure 4.4 Molecular layer depth
Coronal photomicrographs of the cerebellar cortex (lobulus simplex) at the site of injection **(B)** or at the equivalent region in the right hemisphere **(A)**, in an animal treated with 1 µl anti-BDNF IgY (2 µg/ml). The molecular layer within the left hemisphere (climbing fibre deprived hemisphere) is approximately 28 % thinner than the molecular layer within the right hemisphere (control hemisphere).

Key: EGL = external granular layer; GL = granular layer; ML = molecular layer; Purkinje cell = Purkinje cell layer; Bars = 100 µm.

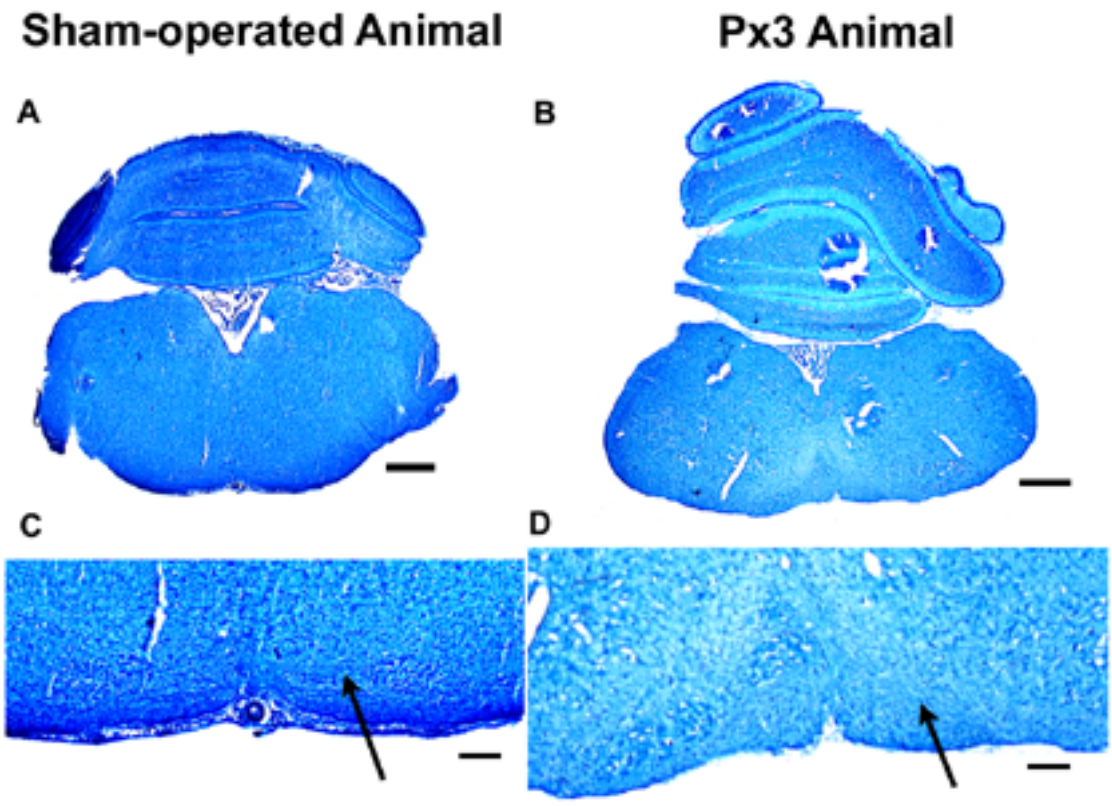


Figure 4.5 Cerebellum and inferior olivary complex following intraolivary K252a administration

Coronal view of a cerebellum and inferior olive from a Px3 lesioned **(A)** and sham-operated animal **(B)** following intraolivary K252a (0.05 mg/ml) treatment (arrows), stained with methylene blue. No morphological abnormalities were observed in the cerebellum or inferior olive. **(C)** and **(D)** show enlarged images of the inferior olivary complex.

Key: Bars in (A) and (B) = 2 mm; Bars in (C) and (D) = 500 μm; Bregma approximately -13.92 mm.

4.3.3 BDNF neutralisation inhibits transcommissural climbing fibre reinnervation

In the control right hemicerebellum of animals injected with a lower concentration of anti-BDNF IgY (2 μg/ml: n = 4) the distribution of VGLUT2-like immunoreactivity confirmed previous studies (Hioki et al., 2003; Kaneko and Fujiyama, 2002). There was VGLUT2 immunolabelling throughout the granular and molecular layers (Fig 4.6A), being in discrete patches in the granular layer and in fine arbors surrounding the Purkinje cell somata, extended into the molecular layer. These patterns of immunoreactivity are consistent with mossy fibre and climbing fibre terminals respectively (Hioki et al., 2003; Kaneko and Fujiyama, 2002).

In the denervated hemicerebellum VGLUT2-like immunoreactivity was again observed in discrete patches in the granular layer (Fig 4.6B), indicative of mossy fibre labelling (Kaneko and Fujiyama, 2002). In contrast, while VGLUT2 labelled terminals were also observed in the molecular layer, none were observed within and immediately surrounding the anti-BDNF IgY injection site (Fig 4.6C). Outside the injection site, VGLUT2 immunoreactivity was observed in a medio-lateral gradient: in all animals there were numerous VGLUT2-like immunoreactive terminals in cerebellar lobules up to 1 mm from the midline (zones 1 – 2: Fig 4.7). Although labelling was less evident further laterally, it was also observed in lobulus simplex and copula pyramidis up to 2 mm (zones 3 – 4) from the midline in some animals. This distribution of immunoreactivity within the lesioned hemicerebellum confirms previous studies that transcommissural climbing fibres densely reinnervate Purkinje cells within the left hemivermis and sparsely reinnervate Purkinje cells in the paravermal hemisphere (Sugihara et al., 2003; Zagrebelsky et al., 1997).

4.4 DISCUSSION

This study examined whether BDNF influences post-lesion olivocerebellar reinnervation of Purkinje cells in the neonatal cerebellum. The data indicate that climbing fibre reinnervation during the neonatal period requires target derived BDNF because neutralisation of endogenous cerebellar BDNF inhibits reinnervation.

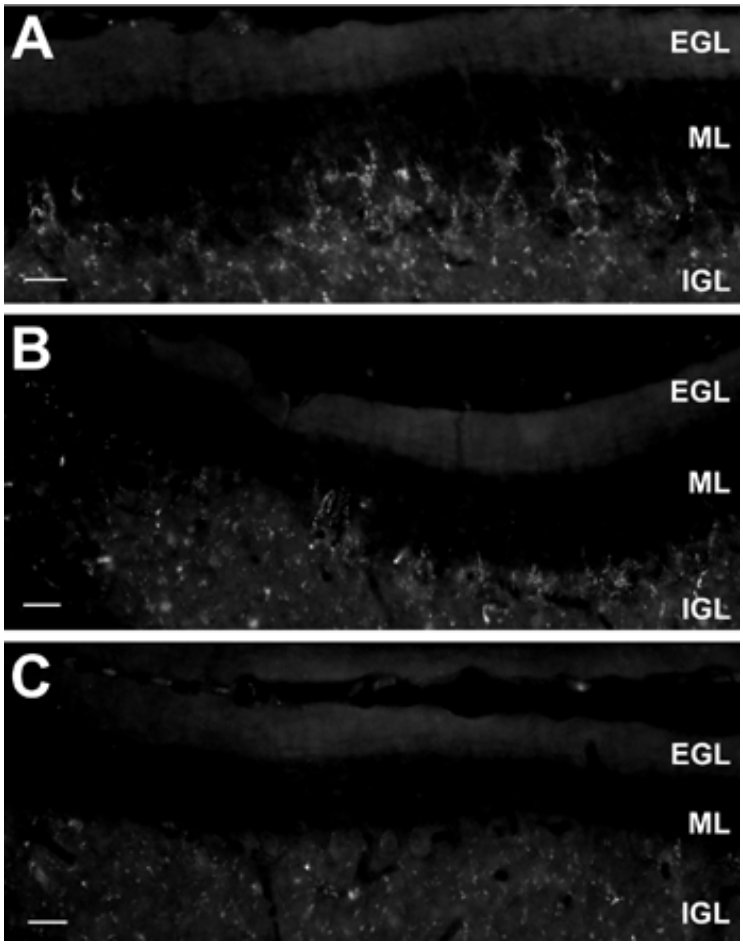


Figure 4.6 VGLUT2-labelled reinnervating climbing fibre terminals
Photomicrographs of the left and right hemicerebella from an anti-BDNF IgY (2 μg/ml) treated lesioned animal showing VGLUT2 labelling of climbing fibre terminals.
(A) Control right hemicerebellum: VGLUT2 labels climbing fibre terminals in the molecular layer (closed arrow) and mossy fibre terminals in the granular layer (open arrow).
(B) Pedunculotomised left hemicerebellum (outside of antibody injection site): VGLUT2 immunoreactivity is present in the molecular and granular layers indicating climbing and mossy fibres respectively.
(C) Pedunculotomised left hemicerebellum (within antibody injection site): VGLUT2 labelling is present in the granular layer indicating mossy fibre labelling. Note, there is an absence of labelling in the molecular layer.

Key: EGL = external granular layer; IGL = internal granular layer; ML = molecular layer; WM = white matter; Bar = 50 μm.

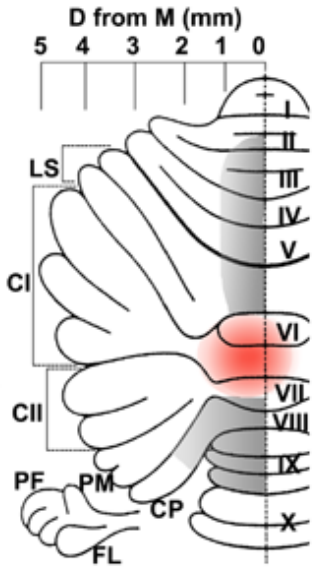


Figure 4.7 Area of VGLUT2-labelled climbing fibre reinnervating terminals
Schematic diagram of the unfolded cerebellar cortex (modified from Buisseret-Delmas and Angaut, 1993) in animals treated with anti-BDNF IgY (2 µg/ml). Grey shading shows the average distribution of VGLUT2-labelled transcommissural climbing fibre terminals. The gradient of grey shading (dark to light) represents the mediolateral gradient of reinnervation density. The red shading illustrates the location of the anti-BDNF IgY injection area. The dotted vertical line indicates the cerebellar midline.

Key: CI = Crus I; CII = Crus II; CP = copula pyramidis; D = distance; FL = flocculus; I – X = vermal lobules I to X; LS = lobulus simplex; M = midline; PF = paraflocculus; PM = paramedian lobule.

4.5 METHODOLOGICAL CONSIDERATIONS

As stated in the methods, tests were not done in the current study to confirm *in vivo* BDNF neutralisation or protein kinase inhibition, following anti-BDNF IgY or K252a administration respectively, thus, neutralisation and inhibition cannot be guaranteed. With regards to the anti-BDNF IgY, application of this antibody does not deplete BDNF levels, rather, this antibody binds to available BDNF and prevents BDNF-stimulated trkB phosphorylation from occurring (Toma and Kaplan, 1997). Therefore, *in vivo* it is extremely difficult to accurately determine what percentage of BDNF has been neutralised and thus how efficacious the antibody is. As such, concentrations used in this study were 50 times stronger than concentrations used *in vitro* (Toma and Kaplan, 1997) in order to provide maximum efficacy. Regarding K252a administration, the K252a concentration used in the current study is within the range used in previous studies in the cerebellum to block granule cell survival *in vitro* (Bhave et al., 1999), thus it is potentially blocking protein kinase inhibition *in vivo*. To determine whether 50 nM K252A (0.05 μg/ml, n = 10) inhibited protein kinase activation, immunohistochemistry is required to identify the location of phosphorylated protein kinase within the cerebellum.

4.5.1 BDNF is required for survival and development of cerebellar neurons

Neutralising BDNF in the cerebellum *in vivo,* by injecting high concentrations of anti-BDNF IgY (500 or 100 μg/ml) resulted in neuronal degeneration within and around the injection site. This confirms previous studies that BDNF is a survival factor for cerebellar granule cells (Minichiello and Klein, 1996; Neveu and Arenas, 1996; Segal et al., 1992) and Purkinje cells (Ernfors et al., 1992; Morrison and Mason, 1998; Rocamora et al., 1993; Shimada et al., 1998), both of which express trkB receptors (Ernfors et al., 1994; Schwartz et al., 1997). In contrast, injecting lower concentrations of anti-BDNF IgY (10 or 2 μg/ml) caused less severe cerebellar morphological changes: reduced molecular layer width and abnormal lobule foliation. This abnormal cerebellar morphology is similar to that observed in the untreated BDNF -/- mutant mice, which

have increased granule cell death and stunted growth of the Purkinje cell dendritic tree (Minichiello and Klein, 1996; Morrison and Mason, 1998; Schwartz et al., 1997; Shimada et al., 1998). Therefore these abnormalities are consistent with a role for BDNF in survival and maturation of cerebellar neurons.

Additionally, the above abnormalities are consistent with the abnormalities observed following intracerebellar K252a treatment, particularly in the climbing fibre deprived hemicerebellum. This confirms previous reports that granule and Purkinje cell survival and Purkinje cell dendritic growth are dependent on trk receptor activation (Schwartz et al., 1997), since trk receptors signal through protein kinase pathways (Heumann, 1994; Kaplan and Cooper, 2001; Schulman and Hyman, 1999).

Within the inferior olivary complex however, BDNF blockade, through protein kinase inhibition, does not appear to affect normal cerebellar development (i.e. normal lobule foliation and correct molecular layer depth), despite trk B protein located within the inferior olivary complex (Yan et al., 1997). This could suggest that either trk activation does not occur in the soma, or that protein kinase cascades in the soma do not affect cascades in the axon terminals. More likely, however, is that a lack of abnormal development following olivary protein kinase inhibition suggests that olivary trk receptors do not play a role in cerebellar development.

4.5.2 Olivocerebellar pathway delivery of BDNF to the cerebellum

The results of this study also suggest that the olivocerebellar pathway delivers BDNF to the cerebellum, as more severe morphological changes are observed following anti-BDNF IgY administration in the climbing fibre deprived hemicerebellum (compare Fig 4.1D with 4.1E). This supports previous proposals that BDNF synthesised in the inferior olivary complex is anterogradely transported to the climbing fibre axon terminals (Lohof et al., 2005; Sherrard and Bower, 2002).

Since BDNF protein accumulates in Purkinje cells during development (Das et al., 2001; Neveu and Arenas, 1996; Schwartz et al., 1997), it has been proposed that BDNF

released from granule cells supports Purkinje cell survival (Mason et al., 1997). However, at the early stages of Purkinje cell dendritogenesis at P7, mature granule cells are few in number with very low levels of BDNF gene expression (Das et al., 2001; Neveu and Arenas, 1996; Schwartz et al., 1997). Alternatively, BDNF and its receptors are synthesised in the inferior olive during this time, when climbing fibres are undergoing growth and synaptogenesis (Nitz et al., 2001; Riva-Depaty et al., 1998; Rocamora et al., 1993), and although no BDNF protein accumulates within the developing inferior olive, this peptide has been localised within the cerebellar white matter tracts (Schwartz et al., 1997), suggesting that BDNF is transported via the olivocerebellar pathways to the cerebellum. Furthermore, climbing fibre input is known to support Purkinje cell dendritogenesis (Lohof et al., 2005; Sotelo and Arsenio-Nunes, 1976), possibly through trkB activation on the Purkinje cells (Mason et al., 1997). Therefore, BDNF may be delivered to the Purkinje cells from the inferior olivary complex via the climbing fibres, as occurs elsewhere in the CNS (Altar et al., 1997; Caleo et al., 2000).

Furthermore, climbing fibre translocation from the Purkinje cell soma to its dendrites is via ionotropic glutamate receptors (Hafidi and Hillman, 1997). Since BDNF aids ionotropic glutamate receptor mobility within the membrane in neurons from other areas of the CNS (Narisawa-Saito et al., 2002), it is proposed that the increase in cerebellar BDNF at this age is afferent derived and regulates the surface expression of Purkinje cell ionotropic glutamate receptors aiding climbing fibre translocation to Purkinje cell primary dendrites.

4.5.3 Cerebellar BDNF promotes neonatal post-lesion transcommissural olivocerebellar reinnervation

Since the morphological abnormalities observed following intracerebellar anti-BDNF IgY (low dose) administration were more evident in and immediately surrounding the injection sites, it is possible that BDNF neutralisation did not occur beyond the area immediately outside of the injection site. Furthermore, the absence of climbing fibre

reinnervation within this area of BDNF neutralisation suggests that cerebellar BDNF is required for olivocerebellar reinnervation.

BDNF promotes axonal elongation during development (Tucker et al., 2001) and in adulthood (Hanamura et al., 2004), modulates neurite fluidity (Horch et al., 1999) and facilitates reinnervation of denervated neurons (Dixon and Sherrard, 2006; McCallister et al., 1999). This is possibly through activation of trkB, which subsequently activates GAP-43 (Fournier et al., 1997; Klocker et al., 2001), a growth associated peptide, involved in climbing fibre development (Console-Bram et al., 1996) and synaptic plasticity (Buffo et al., 1998). Therefore it is proposed that as the olivocerebellar axons elongate into the lesioned hemicerebellum, the removal of available BDNF stops activation of trkB on climbing fibre terminals, which prevents GAP-43 activation within the olive, inhibiting growth into the injection site.

In support of this theory, unilateral climbing fibre transection results in increased expression of p75 within the uninjured inferior olivary complex (Nitz et al., 2001): indicating target derived influences on growth promotion. This increase lasts long enough for the climbing fibre pathway to undergo plasticity, at which time peptide levels revert to normal (Nitz et al., 2001). This is concurrent with other studies of the CNS where p75 is upregulated following injury (Rende et al., 1993; Sebert and Shooter, 1993; Turner and Perez-Polo, 1998).

4.6 CONCLUSION

This study extends previous work on the transcommissural olivocerebellar projection (Angaut et al., 1985; Sugihara et al., 2003; Zagrebelsky et al., 1997) by adding that target-derived BDNF is required for climbing fibre reinnervation in the neonatal system.

Since anatomically appropriate alternate paths are associated with some functional benefit (Coumans et al., 2001; Dixon et al., 2005) and BDNF is widely distributed throughout the central nervous system (Das et al., 2001; Maisonpierre et al., 1990), these data has significance for potential therapeutic strategies after traumatic injury involving

axonal damage: in particular, whether BDNF can induce climbing fibre reinnervation onto mature Purkinje cells and if so, can this provide functional recovery. This is addressed in chapters 5 and 6.

CHAPTER 5

BRAIN-DERIVED NEUROTROPHIC FACTOR INDUCES POST-LESION TRANSCOMMISSURAL GROWTH OF OLIVARY AXONS THAT DEVELOP NORMAL CLIMBING FIBRES ON MATURE PURKINJE CELLS

5.1 INTRODUCTION

Although the results of the previous experiments show that cerebellar BDNF is required for post-lesion neonatal climbing fibre reinnervation, and exogenous BDNF induces reinnervation just beyond the end of the critical period (Sherrard and Bower, 2001) it was unknown whether BDNF could also induce reinnervation onto previously denervated Purkinje cells in the mature cerebellum. Thus, BDNF was injected into the cerebellum of rats 24 hours following unilateral climbing fibre transection at P15 or P30 and climbing fibre reinnervation of the mature cerebellum was assessed.

The study was undertaken for two reasons; first, both the normal olivocerebellar projection (Buisseret-Delmas and Angaut, 1993; Sugihara et al., 2001) and neonatal transcommissural reinnervation (Sugihara et al., 2003) are topographically organised in parasagittal microzones. Since BDNF modulates growth direction (Ming et al., 1997), a focus of high concentration of BDNF following an injection may over-ride normal intra-cerebellar cues that direct target selection (Plagge et al., 2001) and result in reinnervation that does not correctly recreate the normal circuit. Second, climbing fibre synapses must be correctly located on the Purkinje cell dendritic tree for normal function (Ichikawa et al., 2002; Lalouette et al., 2001), but reinnervating axons may not find their appropriate synaptic sites on a complex Purkinje cell dendritic tree that is already fully grown (P30: McKay and Turner, 2005). Therefore it is necessary to examine the organisation and morphology of BDNF-induced climbing fibres in the mature cerebellum in order to characterise the olivocerebellar pathway as a model for extending developmental plasticity to produce circuit re-formation and functional improvement

5.2 METHODS

Four litters of rats of either sex and aged either 15 or 30 days were used to determine whether intracerebellar BDNF injections could induce climbing fibre reinnervation in the mature cerebellum.

Climbing fibres from the left hemicerebellum were damaged via a lesion of the inferior cerebellar pathway at P15 or P30, as described in section 2.3. Twenty four hours later, when the majority of climbing fibre terminals will have degenerated (Cesa et al., 2005; Sotelo et al., 1975), 1 μg recombinant human BDNF (1mg/ml) was injected into the left hemicerebellum and vermis to induce reinnervation (refer section 2.4 for injection protocol). For each age group, the dose (4 μmol/L) was calculated using the left cerebellar hemisphere volume at that age (Heinsen, 1977). Because BDNF only diffuses up to 1 mm through grey matter (Anderson et al., 1995; Kobayashi et al., 1997; Lotto et al., 2001; Reibel et al., 2000; Sobreviela et al., 1996) exposure to the cerebellar cortex was maximised by injecting 15 aliquots between the midline and mid-lateral hemisphere from lobules VIb and lobulus simplex rostrally to lobules VIII and copula pyramidis caudally (Fig 2.1), at a depth of 1.1 mm. Although vehicle injection does not induce transcommissural olivocerebellar reinnervation after lesion on P11 (Sherrard, 1997; Sherrard and Bower, 2001), to confirm the control, some animals lesioned on P15 received 1 μg vehicle containing cytochrome C (1 mg/ml: Sigma-Aldrich), a molecule with similar physiochemical properties to BDNF (Caleo et al., 2003).

Immediately following cerebellar injection of BDNF, some animals received intraolivary injections of 4 % (w/v) Fluororuby, an anterograde neuronal tracer (refer section 2.5 for injection protocol). In each rat there were 2 injections, of 200 nl each, placed in the caudal and mid-rostral regions of the left inferior olive, the areas most commonly involved in transcommissural climbing fibre reinnervation (Angaut et al., 1985; Sherrard et al., 1986; Sherrard and Bower, 2001). Other animals did not receive an olivary injection, but instead underwent intra-cerebellar injection of 2 % (w/v) Fast Blue, a retrograde neuronal tracer, five days after BDNF injection (see section 2.4 for details). Using the same technique as described above for BDNF injection, 1 μl 4 %

(w/v) Fast Blue was injected in 10 aliquots into the left cerebellar hemisphere lateral to the paravermal vein and between the lobulus simplex and the copula pyramidis.

Seven days after Fluororuby injections or two days after Fast Blue injections, experimental animals were deeply anaesthetised with sodium pentobarbitone and transcardially perfused with 4 % (w/v) paraformaldehyde. The brainstem and cerebellum were removed and serial frozen sections of the inferior olive and cerebellum were cut. In animals that received intra-cerebellar Fast Blue, two sets of 30 µm coronal sets were taken: the first was stained with 0.5 % (w/v) methylene blue for histological analysis and the second analysed for restriction of the injection within the left hemicerebellum and the localisation of retrogradely-labelled neurons in the inferior olive. In animals injected with Fluororuby, three parallel sets of 30 µm coronal, or 40 µm parasagittal, sections were taken. One set was stained with 0.5 % (w/v) methylene blue, the second set was analysed for the distribution of Fluororuby labelled olivocerebellar axons, and the third set underwent immunohistochemical identification of climbing fibre terminals and Purkinje cells.

Climbing fibre terminals were visualised in the cerebellum using immunohistochemistry for the glutamate transporter VGLUT2, and Purkinje cells were counterstained using a specific marker, calbindin (refer section 2.8 for immunohistochemical protocol).

In all animals, complete transection of the left inferior cerebellar peduncle was verified by histological analysis of all (i.e. serial) sections, because the axotomised right inferior olive of adolescent and mature animals does not completely degenerate within seven days (Buffo et al., 1998).

In successfully lesioned animals the location of retrogradely labelled olivary neurons and the distribution of reinnervating olivocerebellar axons were mapped in the left inferior olive or hemicerebellum respectively. The analyses were as follows: (1) the laterality of BDNF-induced reinnervation was measured, (2) it was determined whether the reinnervating axons were organised into normal parasagittal zones, (3) the size of the

climbing fibre arbors were calculated and (4) the depth of the molecular layer was measured to ensure that BDNF did not alter climbing fibre arborisation indirectly by altering cerebellar Purkinje cell growth and granule cell survival

5.3 RESULTS

The presence of transcommissural olivocerebellar reinnervation and the distribution and morphology of transcommissural climbing fibres was examined in animals treated with BDNF (B) or vehicle (V) 24 hours following unilateral transection of the left inferior cerebellar peduncle on P15 or P30. In this study 22 rats were used (Px15V = 6; Px15B = 4; Px30B = 12). After histological verification of complete inferior cerebellar peduncle transection, the number of animals to be included in the study was reduced to 15 (Px15V = 3; Px15B = 4; Px30B = 8).

5.3.1 BDNF induces transcommissural olivocerebellar reinnervation

To establish whether BDNF successfully induced transcommissural axonal growth through the cerebellar white matter, which is usually not permissive to olivocerebellar axonal growth (Bravin et al., 1997), inferior olivary labelling was examined following left cerebellar hemisphere injection of Fast Blue in rats successfully lesioned on P30 and treated with intra-cerebellar BDNF.

In BDNF-treated animals, there were no labelled neurons in the right inferior olive, consistent with complete transection of the left inferior cerebellar peduncle. In contrast, the left inferior olive contained a few scattered, 1-11 (mean ± SEM; 6.0 ± 2.8; n = 3), retrogradely labelled neurons (Fig 5.1). These neurons displayed the histological features of olivary neurons with an ovoid soma, a large unlabelled nucleus and a few processes filled with fluorescent dye (Fig 5.1). This labelling indicates reinnervation to the left hemicerebellum lateral to the vermis. In addition, the paucity of retrogradely labelled cells is consistent with sparse reinnervation to the hemisphere previously

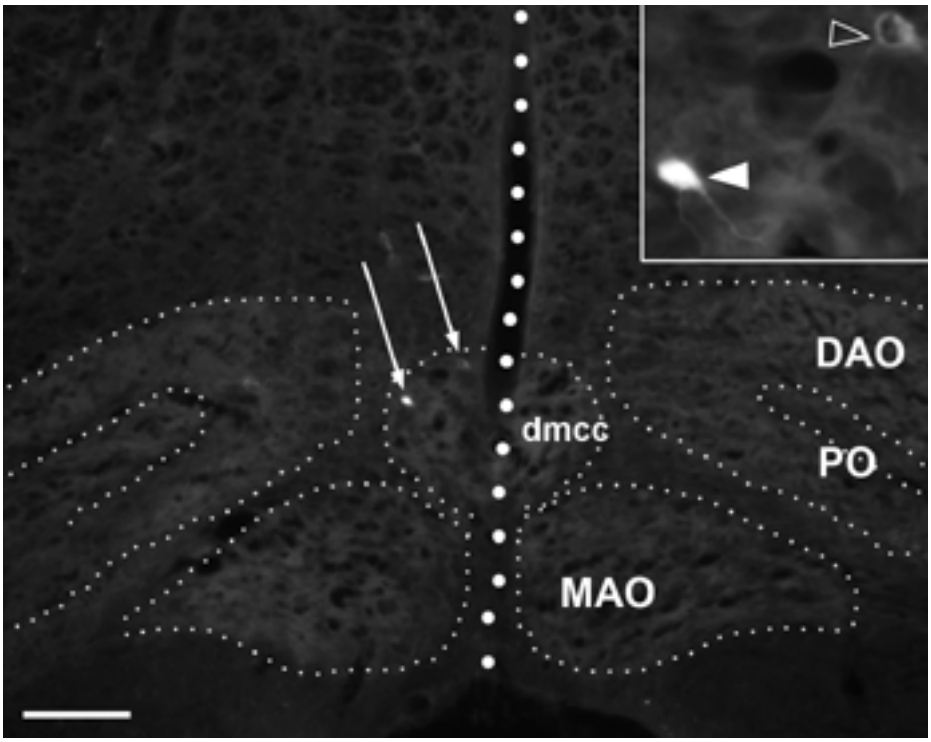


Figure 5.1 Fast Blue-labelled neurons in left inferior olivary complex
Photomicrograph of the ventral brainstem showing two Fast Blue retrogradely labelled neurons (arrowed) in the mid-rostral left inferior olive. Inset shows these neurons at higher power, in which an unlabelled nucleus (open arrowhead) and filled neurites (filled arrowhead) are visible. Note that the right inferior olive is devoid of labelled neurons, consistent with transection of the left inferior cerebellar peduncle. The vertical dotted line indicates the midline.

Key: Bar = 100µm; DAO = dorsal accessory olive; dmcc = dorsal medial cell column; MAO = medial accessory olive; PO = principal olive.

documented in the neonate (Angaut et al., 1985; Sugihara et al., 2003) and young juvenile animal (Sherrard and Bower, 2001; Sherrard and Bower, 2003).

5.3.2 Distribution of transcommissural climbing fibre reinnervation

To identify whether the olivocerebellar reinnervation observed above displayed similar organisation to that which develops following neonatal lesion (Sugihara et al., 2003; Zagrebelsky et al., 1997), or represented axonal growth only to the BDNF injection site, the organisation and morphology of transcommissural reinnervating axons was examined by anterograde tracing and VGLUT2 immunohistochemistry.

In lesioned BDNF-treated animals (P15, n = 4; P30, n = 5), anterograde tracing revealed Fluororuby-filled axons in the cerebellar white matter of both left and right hemicerebella (Fig 5.2A). These labelled axons could be followed as they ascended through the lobular white matter and granular layer to form climbing fibre arbors in the molecular layer. Immunohistochemistry for VGLUT2 confirmed the reinnervation, with VGLUT2-like immunoreactive terminals in the granular and molecular layers of the denervated left hemicerebellum (Fig 5.2D). These immunoreactive terminals had the same structural characteristics as those seen in the control right side (Fig 5.2B): discrete patches in the granular layer and fine branched arbors in the molecular layer, which are consistent with mossy fibre and climbing fibre terminals respectively, as described elsewhere (Kaneko and Fujiyama, 2002). In contrast within the denervated left hemicerebellum of vehicle-injected animals (P15, n = 3), VGLUT2-like immunoreactive terminals were present only in the granular layer, but never in the molecular layer (Fig 5.2C), indicating the presence of mossy fibres but the absence of climbing fibres.

The distribution of reinnervating climbing fibres was also examined: VGLUT2 immunohistochemistry was used because anterograde tracing revealed only a small number of climbing fibre terminals, consistent with the few reinnervating neurons being widely scattered in the olive (Fig 5.1: Angaut et al., 1985; Sherrard and Bower, 2001; Sherrard and Bower, 2003), such that an olivary injection will label only some of these neurons and thus a proportion of transcommissural axons. The distribution of

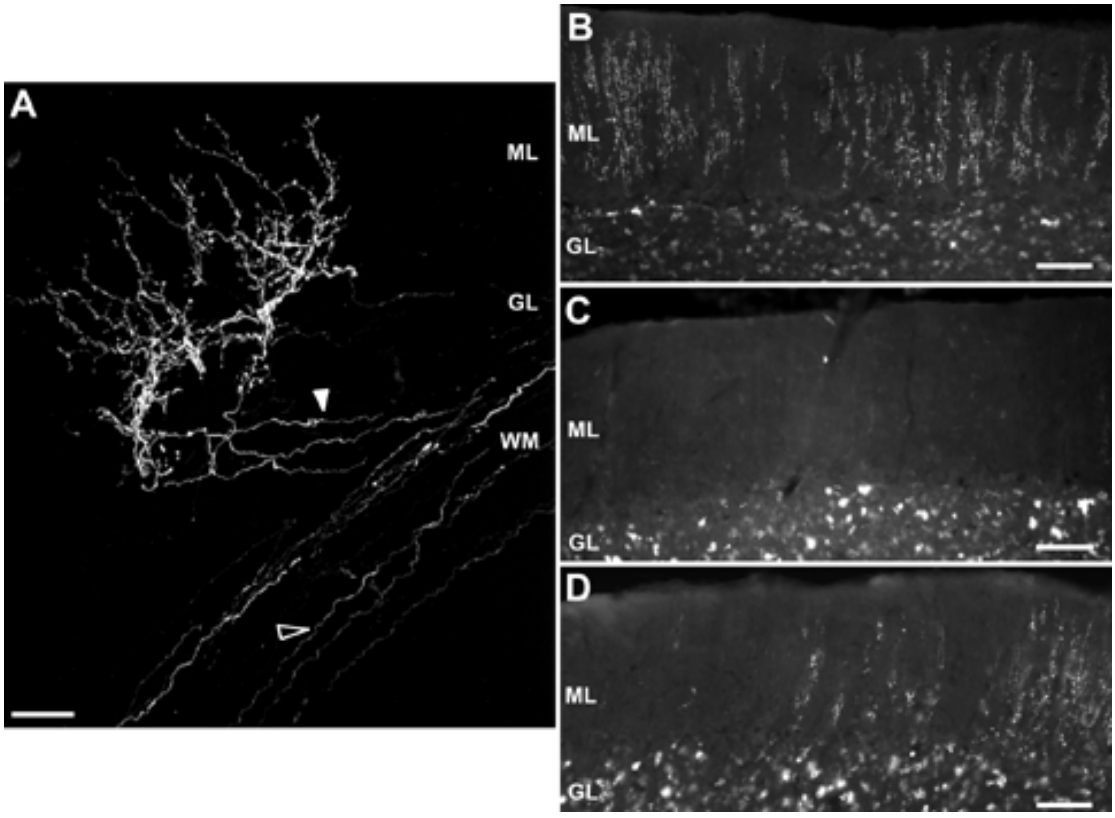


Figure 5.2 Fluororuby- and VGLUT2-labelled reinnervating axons and terminals
Photomicrographs of labelled olivocerebellar axons and climbing fibres in the reinnervated **(A and D)** or control **(B)** hemicerebella.
(A) Fluororuby filled olivocerebellar axons and climbing fibre arbors in the reinnervated left hemicerebellum from a P15-lesioned animal treated with BDNF. Labelled axons are present in the lobular white matter (open arrowhead), of which three (filled arrowhead) can be followed through the granular layer to the molecular layer where they form terminal arbors on Purkinje cells. Bar = 25 μm.
(B) A photomicrograph of the paramedian lobule of the control right hemicerebellum. VGLUT2 labels climbing fibre terminals in the molecular layer and mossy fibre terminals in the granular layer. Bar = 50 μm.
(C) In the left denervated hemicerebellum (lobule IX) of a vehicle-injected animal, VGLUT2 labelling was only in the granular layer indicating mossy fibre labelling. Bar = 50 μm.
(D) In the left hemicerebellum (lobule VII) of a BDNF injected animal VGLUT2 immunoreactivity is present in the molecular and granular layers indicating climbing and mossy fibres respectively. Bar = 50 μm.

Key: GL = granular layer; ML = molecular layer; WM = white matter.

VGLUT2-like immunoreactivity was similar in both P15 and P30 lesion groups, but was not uniform throughout the left hemicerebellum. Qualitatively, the VGLUT2-like immunoreactivity in the molecular layer was not as dense as in the control right side: in each lobule labelling was interspersed with small patches of unlabelled molecular layer (Fig 5.2D). Moreover, the labelled terminals also appeared to be more densely packed in those lobules into which BDNF was injected, i.e. dorsally located vermal lobules VIb – VIII and the medial part of their associated hemispheric regions (Fig 2.1), compared with the ventral lobules II - V and IX. In addition, in all lobules, irrespective of their location within or outside of the injection sites, there was a medio-lateral gradient of labelling within the reinnervated hemicerebella (Fig 5.3). There were numerous densely packed VGLUT2-like immunoreactive terminals in cerebellar lobules up to 1.5 mm from the midline (zones 1 - 3) of all animals. Although labelling was less evident further laterally and varied between animals (Fig 5.4), scattered clusters of 1 – 3 immunolabelled arbors were also observed in lobulus simplex, crus I, paramedian lobule and copula pyramidis up to 3.5 mm (zones 4 – 7) from the midline in a few animals. This distribution confirms the data showing a small number of neurons retrogradely labelled by hemispheric tracer injections. Thus, BDNF induces transcommissural olivocerebellar reinnervation in both the juvenile and mature cerebellum.

5.3.3 Parasagittal distribution of transcommissural olivocerebellar axons

In order to examine whether the discontinuous pattern of VGLUT2-like labelling of BDNF-induced reinnervation indicated that these transcommissural olivocerebellar axons followed a parasagittal organisation similar to the normal path, axons labelled by anterograde tracing of Fluororuby from the left inferior olive were examined. As expected, within the control right hemicerebellum Fluororuby labelled axons were distributed in parasagittal bands (Fig 5.5). Labelling was present in the vermis and lobulus simplex, crura and paramedian lobule, in accordance with injection into the caudal and mid-rostral olive and the known olivocerebellar topography (Buisseret-Delmas and Angaut, 1993; Sugihara and Shinoda, 2004).

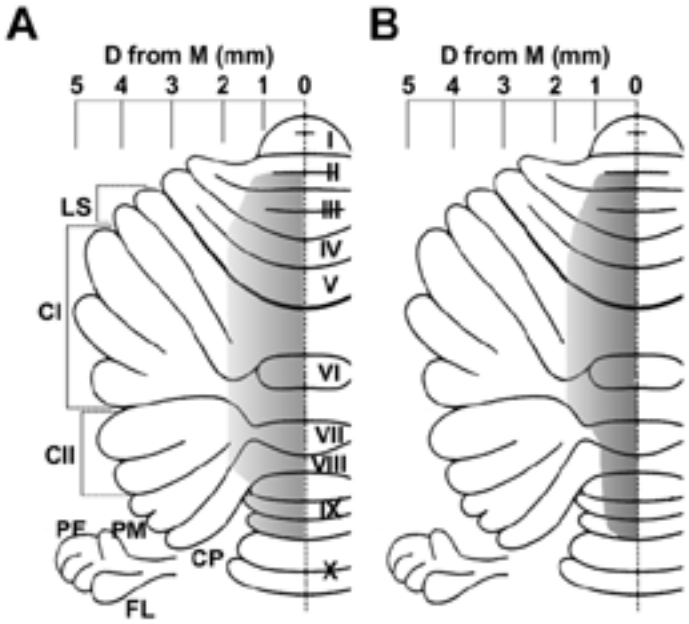


Figure 5.3 Area of VGLUT2-labelled reinnervating terminals (unfolded cerebellum)

Diagrams illustrating the distribution of transcommissural climbing fibre reinnervation induced by BDNF.

(A & B) Schematic diagrams of the unfolded cerebellar cortex (modified from Buisseret-Delmas and Angaut, 1993) showing the average distribution of VGLUT2 labelling in each animal group injected with BDNF; **(A)** P15-lesion group, or **(B)** P30-lesion group. The gradient of shading (dark to light) represents the mediolateral gradient of reinnervation density. The dotted vertical line indicates the cerebellar midline.

Key: CI = Crus I; CII = Crus II; CP = copula pyramidis; D = distance; FL = flocculus; I – X = vermal lobules I to X; LS = lobulus simplex; M = midline; PF = paraflocculus; PM = paramedian lobule.

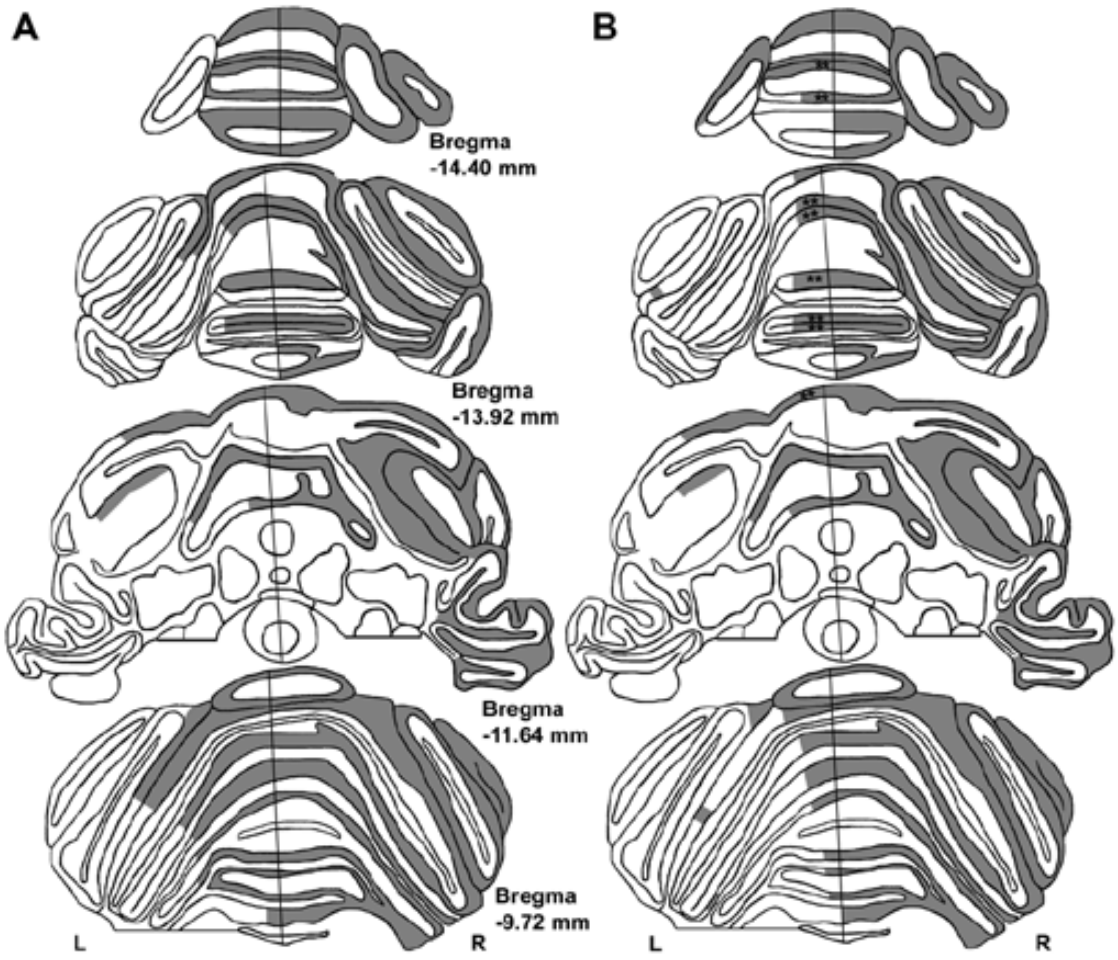


Figure 5.4 Area of VGLUT2-labelled reinnervating terminals (coronal sections)
Diagrams illustrating the distribution of transcommissural climbing fibre reinnervation induced by BDNF.
(A) Camera lucida drawing of coronal cerebellar sections from a P15-lesioned animal showing the location of climbing fibre terminals: areas shaded in grey represent the location of VGLUT2-labelling. Each outline represents the summation of labelling from 5 adjacent sections.
(B) Camera lucida drawing of cerebellar sections as in **(A)** above, showing the distribution of climbing fibre reinnervation following lesion at P30 revealed by VGLUT2 immunohistochemistry. Asterisks indicate the location of Fluororuby-labelled climbing fibre terminals in the left hemicerebellum of the same animal.

Key: L = left; R = right.

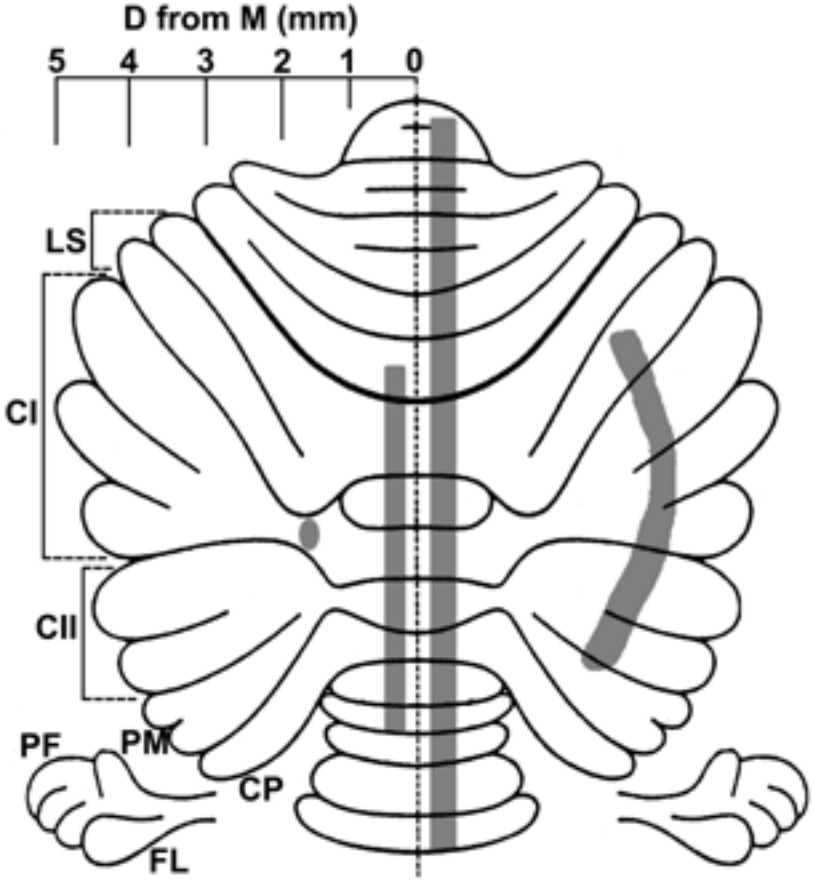


Figure 5.5 Parasagittal distribution of Fluororuby-labelled terminals
A diagram of the unfolded cerebellar cortex (modified from Buisseret-Delmas and Angaut, 1993) showing the distribution of Fluororuby filled climbing fibre terminals in the cerebellar cortex of BDNF injected animals. In the right hemisphere Fluororuby labelling is organised in parasagittal stripes (grey shaded area). Labelled transcommissural reinnervating axons in the left hemivermis are less numerous than on the control right side, but are organised almost symmetrically about the midline.

Key: Dotted vertical line indicates the cerebellar midline. CI = Crus I; CII = Crus II; CP = copula pyramidis; D = distance; FL = flocculus; LS = lobulus simplex; M = midline; PF = paraflocculus; PM = paramedian lobule.

In the lesioned left hemicerebellum of BDNF-treated animals, Fluororuby-filled axons were also organised into parasagittal stripes (Figs 5.4B & 5.5), as in the right hemicerebellum. Although inter-animal differences were observed, within the vermis these stripes were almost symmetrical about the midline to those on the right side (Fig 5.5), suggesting that the BDNF-induced olivocerebellar reinnervation followed an appropriate topographic distribution.

To examine whether the sagittal banding of reinnervating axons conformed to the normal olivocerebellar microzone, that is 250 - 500 μm wide (Fukuda et al., 2001; Sugihara et al., 2001), the medio-lateral breadth of labelled bands was measured. In the right hemicerebellum, Fluororuby labelled axon terminals were contained within zones 194 – 349 μm wide (269.5 μm ± 12.2 μm; mean ± SEM; n = 39 zones). In the reinnervated left hemicerebellum the Fluororuby labelling was also organised in narrow bands, however these were 48 – 101 μm wide (69.6 μm ± 11.1 μm; mean ± SEM; n = 4 zones).

5.3.4 Reinnervating climbing fibre arbor morphology

Although transcommissural olivocerebellar axons develop normal climbing fibre arbors after unilateral pedunculotomy in the early neonatal period (Sugihara et al., 2003; Zagrebelsky et al., 1997), the arbor morphology of growth factor-induced transcommissural olivocerebellar axons has not been previously described.

In the denervated BDNF-treated hemicerebellum most reinnervating climbing fibre terminal arborisations were normal (Fig 5.6A-G), branching along Purkinje cell primary and secondary dendrites (Fig 5.6C&F: closed arrow head) and in most cases gave descending perisomatic branches (Fig 5.6H). However, closer observation revealed some differences between the groups.

In animals lesioned on P15, the reinnervating climbing fibre arbor ramified through the molecular layer sending fine tendrils almost to the pial surface (Fig 5.7B), whereas in the control right hemicerebellum the arbors only appeared to ramify within the lower

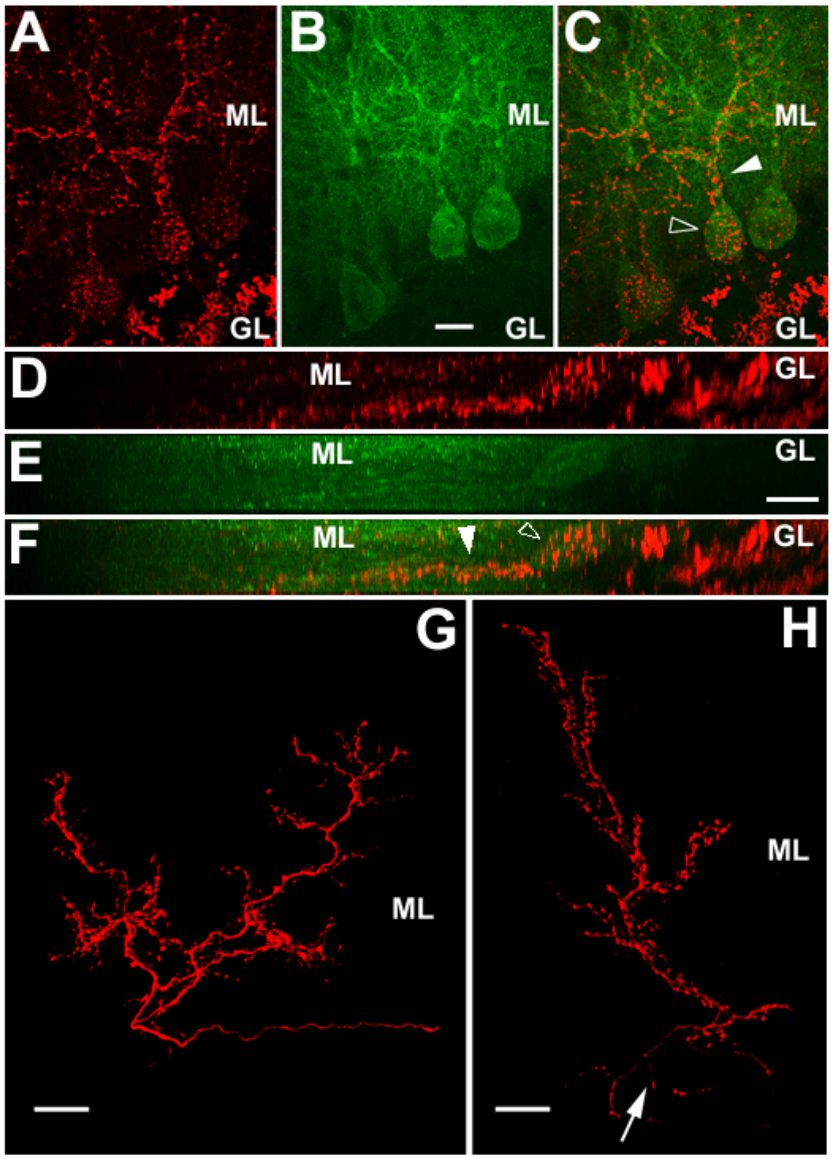


Figure 5.6 Morphology of transcommissural reinnervating terminal arbors
(A-F) Double-labelled sections following left unilateral olivocerebellar transection on P15 showing localisation of VGLUT2 (red) in mossy and climbing fibre terminals and calbindin (green) in Purkinje cells. Labelling for VGLUT2 **(A,D)** and calbindin **(B,E)** are shown separately and merged **(C,F)**. **(A-C)** show Purkinje cells in the sagittal plane, while **(D-F)** are rotated confocal images showing the centre Purkinje cell from **(C)** in the coronal plane. **(C&F)** show that the climbing fibre terminals are present on and around the Purkinje cell soma (open arrowheads in C and F) and ramifying along and through the dendritic tree (white arrowheads in C and F).
(G) Following transection of the left olivocerebellar path on P30, an anterogradely-labelled olivocerebellar axon ascends obliquely through the granular layer and develops an extensive arbor within the molecular layer;
(H) An example of an abnormal climbing fibre arbor in a P30-lesioned left hemicerebellum. It is less branched in the molecular layer, but sends thin descending terminals (arrow) to the Purkinje cell soma.
Key: Bars = 15 µm; GL = granular layer; ML = molecular layer.

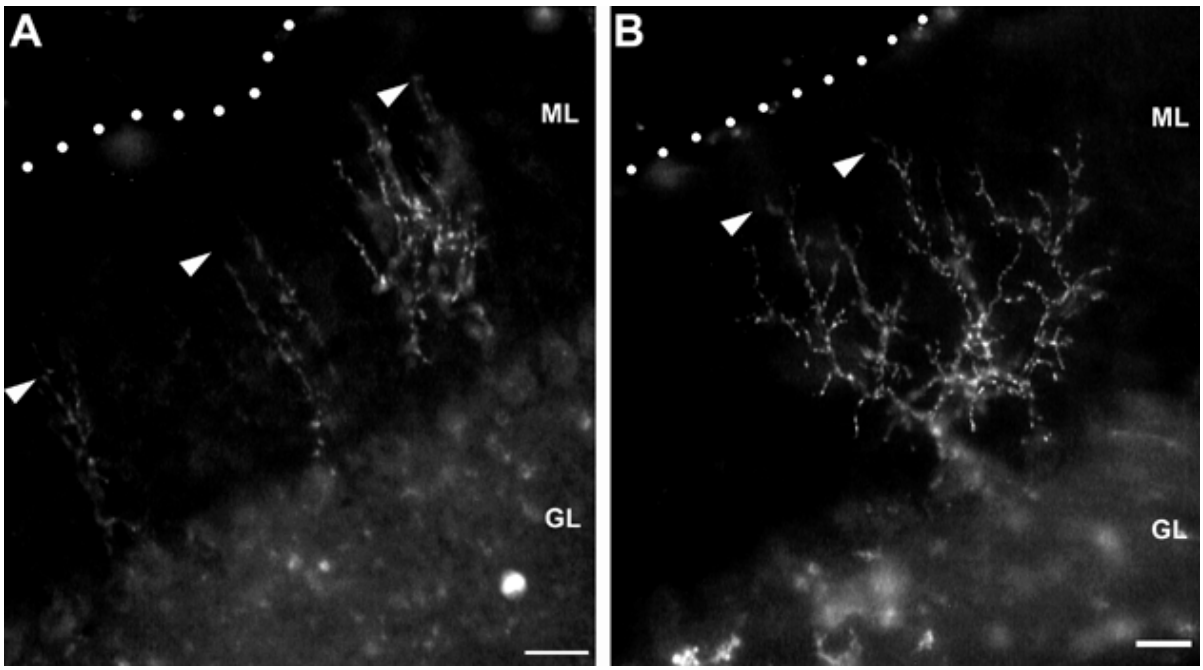


Figure 5.7 Reinnervating and control VGLUT-2-labelled climbing fibre terminals
Photomicrographs showing the extension of VGLUT2-labelled climbing fibre terminals through the depth of the molecular layer in a BDNF treated P15-lesioned animal. The dotted line indicates the pial surface of the folium.
(A) In the control right hemicerebellum climbing fibre terminals do not extend into the upper one third of the molecular layer.
(B) Two overlapping climbing fibre arbors in the left hemicerebellum. Reinnervating climbing fibre tendrils (arrowheads) can be observed approaching the edge of the molecular layer.

Key: Bars = 25 μm; GL = granular layer; ML = molecular layer.

two thirds (Fig 5.7A), consistent with the developmental stage of climbing fibre-Purkinje cell arborisations at P23 (McKay and Turner, 2005; Morris et al., 1985). Quantitatively, reinnervating arbors were larger than those in the control right hemicerebellum: the mean diagonal of a rectangle fitting the terminal arborisation on a single Purkinje cell was 155.7 ± 8.6 μm (mean ± SEM: n = 16) in the left hemisphere compared to 126.8 ± 6.6 μm (mean ± SEM; n = 12) in the right hemisphere (Mann Whitney-U $p<0.02$).

This contrasts with the P30 group in which climbing fibre arborisations were the same size in both the reinnervated and control hemispheres: all climbing fibres ascended through the depth of the molecular layer and the mean diagonal of a rectangle fitting the reinnervating climbing fibre arbors was the same length as for the control arbors (mean ± SEM: reinnervating arbors = 144.0 ± 7.2 μm, n = 38; control arbors = 142.9 ± 13.2 μm, n = 7: Mann Whitney-U $p>0.3$). In addition, 10.5 % (4 of 38) of reinnervating climbing fibre arbors in P30-lesioned animals were either less branched (Fig 5.6H), or sent a branch through the molecular layer to form a secondary arbor on an adjacent Purkinje cell. These few secondary arbors were located in the same parasagittal plane as the primary arbors, but were uncharacteristically small and therefore not measured.

To verify that the larger arbors of reinnervating climbing fibres in the P15 BDNF-treated animals did not reflect an indirect trophic effect of BDNF on Purkinje cell dendritic growth (Morrison and Mason, 1998) or granule cell and their parallel fibre axon survival (Bonthius et al., 2003; Neveu and Arenas, 1996) resulting in a deeper molecular layer, the depth of the molecular layer in each hemicerebellum was measured. In both P15- and P30-lesioned groups the depth of the molecular layer of the BDNF-injected hemisphere was the same as the control non-injected hemisphere (mean ± SEM: P15 injected left hemisphere = 320.5 μm ± 52.3 μm, n = 3; P15 control right hemisphere = 390.8 μm ± 27.7 μm n = 3; P30 injected left hemisphere = 461.7 μm ± 115.6 μm, n = 3; P30 control right hemisphere = 428.4 μm ± 125.6 μm n = 3: Mann Whitney-U $p>0.1$).

In summary the majority of BDNF-induced reinnervating arbors were normal, although in the P15 group the arbors extended further through the molecular layer and thus were larger than control arbors.

5.4 DISCUSSION

This study examined whether BDNF reproduces developmental transcommissural reinnervation to recreate the olivocerebellar circuit in a maturing cerebellum when Purkinje cells have developed complex dendritic trees. These results extend previous studies which show olivocerebellar axonal growth into the denervated mature hemicerebellum (Sherrard and Bower, 2003) to add that exogenous BDNF induces reinnervation, with axons that develop normal climbing fibre terminal arbors organised in discrete parasagittal bands. Since the distribution of transcommissural reinnervation mirrors that seen during development (Angaut et al., 1985; Sugihara et al., 2003), data from this study indicates that exogenous BDNF may recreate in the mature cerebellum the same post-lesion plasticity that occurs in the developing system.

5.4.1 BDNF induces transcommissural olivocerebellar reinnervation

The results of this study show that, at an age when the cellular milieu is myelinated (Reynolds and Wilkin, 1991), exogenous BDNF can induce transcommissural olivocerebellar axonal growth. The absence of transcommissural reinnervation in vehicle-treated animals confirms that the extension of developmental plasticity was a function of the BDNF, not the micro-trauma of the injection or the presence of a basic molecule.

BDNF-induced axonal growth has been demonstrated in other areas of the nervous system during development (Tucker et al., 2001), in myelinated projection pathways (Lu et al., 2004) and facilitating reinnervation of denervated neurons (McCallister et al., 1999). Since BDNF increases intra-neuronal cAMP (Cai et al., 2001; Lu et al., 2004), which in turn facilitates axonal outgrowth from mature neurons (Cai et al., 2001; Shen et al., 1999), it may be hypothesised that BDNF stimulates transcommissural sprouting by

recreating those cellular dynamics of immature neurons (i.e. high cAMP), which allows axonal outgrowth over myelin (Bandtlow and Loschinger, 1997).

In addition, the data from this study suggest that BDNF induces this transcommissural reinnervation by reproducing the mechanisms of neonatal plasticity. Evidence for this comes from three areas. First, the distribution of reinnervating climbing fibres is similar to that observed following unilateral pedunculotomy in the neonatal rat (Angaut et al., 1985; Sugihara et al., 2003) and is *not* confined to the BDNF injection sites. In the reinnervated hemivermis, climbing fibres were observed throughout its rostro-caudal extent, i.e. outside the BDNF injections that were concentrated in dorsal lobules (compare Fig 2.1 with Figs 5.3 & 5.4) and beyond the distance that BDNF is known to diffuse (Kobayashi et al., 1997; Lotto et al., 2001; Reibel et al., 2000; Sobreviela et al., 1996). Furthermore the density of reinnervation decreased laterally so that there were few climbing fibres in the lateral hemisphere; despite BDNF injections into those lobules. Again this distribution of transcommissural reinnervation parallels that which occurs in the neonatal period (Sugihara et al., 2003), suggesting that similar mechanisms are involved. Second, the rate of transcommissural reinnervation is similar to that which occurs following pedunculotomy in the neonatal period, in which axonal growth begins at 24 hours and climbing fibres reach the lateral vermis in 4 days (Lohof et al., 2005; Zagrebelsky et al., 1997). This rate of axonal elongation is routinely demonstrated by central nervous system neurons during normal retinal ganglion cell development (Lund and Bunt, 1976), innervation of graft tissue (Harvey and Lund, 1984), reinnervation of the denervated tectum (Sabel and Schneider, 1988), regeneration into peripheral nerve grafts (Cho and So, 1987; Maki et al., 2003), and *in vitro* (Gomez and Spitzer, 1999). Again this suggests similar underlying mechanisms facilitating climbing fibre reinnervation in the neonatal and BDNF treated cerebella. Third, BDNF is thought to be implicated in olivocerebellar development (Lindholm et al., 1997; Sherrard and Bower, 2002), because it is synthesised in the immature cerebellum (Das et al., 2001; Maisonpierre et al., 1990) when its receptors are expressed in the inferior olive (Rocamora et al., 1993). BDNF also activates GAP-43 (Fournier et al., 1997; Klocker et al., 2001) which is a growth associated peptide involved in climbing fibre development

(Console-Bram et al., 1996) and synaptic plasticity (Buffo et al., 1998). Thus delivery of exogenous BDNF is consistent with reactivation of normal olivocerebellar developmental processes.

Data from this study show that BDNF can induce transcommissural olivocerebellar axonal growth through a growth-inhibitory environment. It also suggests that the distribution of reinnervating climbing fibres is possibly due to recapitulating the mechanisms of developmental plasticity.

5.4.2 Transcommissural axons are distributed in parasagittal bands

The results of this study also showed that BDNF-induced reinnervating climbing fibres are organised into parasagittal zones. This distribution is similar to that seen in the normal adult olivocerebellar pathway (Buisseret-Delmas and Angaut, 1993; Sugihara and Shinoda, 2004) and in reinnervating climbing fibres that develop following neonatal pedunculotomy (Sugihara et al., 2003; Zagrebelsky et al., 1997), or partial olivary destruction by 3-acetylpyridine (Rossi et al., 1991b). However, the BDNF-induced transcommissural axons of this study were aligned in bands that are narrower than normal olivocerebellar microzones (Fukuda et al., 2001). Whilst this narrower banding may reflect inhibitory factors in the cellular environment (Reynolds and Wilkin, 1991) restricting terminal axonal growth, it is also consistent with a small number of axons being labelled by the anterograde tracing since an olivary injection will label only a proportion of transcommissural axons whose cells of origin are widely dispersed throughout the nucleus (see Fig 5.1: Angaut et al., 1985; Sherrard and Bower, 2001; Sherrard and Bower, 2003). Nevertheless, these axons are still contained within a normal microzone indicating that the large number of denervated Purkinje cells in adjacent areas, with their associated growth-attractant signals, did not over-ride the cues that guide normal olivocerebellar parasagittal organisation. Since narrow parasagittal zones are defined early in development (Fournier et al., 2005; Sugihara, 2005), the microzonal organisation of the transcommissural axons suggests that the same guidance cues are inherent in both immature and mature cerebella and that they are not disrupted by positive growth-promoting signals from the BDNF injection sites. Again this

parasagittal distribution of BDNF-induced reinnervation is consistent with normal developmental processes and supports the proposal that BDNF can reproduce to a great extent the mechanisms of developmental plasticity.

5.4.3 Reinnervating climbing fibres develop normal terminal arbors on mature Purkinje cells

In addition to demonstrating a parasagittal organisation, the majority of reinnervating climbing fibres also develop normal terminal arbors, similar to those that reinnervate target cells following either neonatal (Sugihara et al., 2003) or adult (Rossi et al., 1991b) climbing fibre lesion. A small percentage of arbors (10.5 %) however, were not normal: either being small or projecting a thick branch through the molecular layer to an adjacent Purkinje cell within the same parasagittal plane – abnormalities that have also been observed following pedunculotomy on P3 (Sugihara et al., 2003) or 3-acetylpyridine lesion (Rossi et al., 1991b). The structural similarities of reinnervating climbing fibres in our study and other experimental paradigms confirm the central role of the Purkinje cell in the growth and maintenance of climbing fibre arbors (Rossi et al., 1993; Rossi et al., 1995).

This study also suggests, that the size of reinnervating climbing fibre terminal arbors varies with the age at which they are induced to grow: reinnervating arbors that develop following unilateral climbing fibre transection on P30 are the same size as those within the control right hemisphere, while arbors that form after lesion on P15 are larger than those within the control side. The larger size of individual arbors that develop in the P15-lesioned animals is in accord with climbing fibre terminals extending to the distal molecular layer (Fig 5.7B), which contrasts with arbors in the right hemicerebellum that are restricted lower in the molecular layer (compare Fig 5.7B with Fig 5.7A). Since the molecular layer was the same depth in both BDNF-injected and control hemicerebella and Purkinje cell dendrites extend through the molecular layer to the external granular layer (Lohof et al., 2005; McKay and Turner, 2005), the larger climbing fibre arbor cannot be due to their growth over a larger Purkinje cell dendritic tree.

This larger arbor is, however, consistent with the powerful tropic effect of denervated neurons on afferent axons (Strata et al., 1997; Zuddas et al., 1991), in particular the growth-promoting effect of Purkinje cells on climbing fibre terminals (Strata and Rossi, 1994) because the larger size of reinnervating arbors appears to parallel the degree of Purkinje cell deafferentation. The transcommissural climbing fibre arbors observed in this study (145 μm and 155 μm, respectively) are considerably smaller than those which develop following pedunculotomy in the neonatal period (approximately 250 μm: Sugihara et al., 2003), although direct comparison cannot be made due to different tracer protocols between the two studies. Climbing fibre ablation at P3 removes most Purkinje cell afferents and reinnervating climbing fibres develop large arbors (Sugihara et al., 2003). By P15, when each Purkinje cell receives parallel fibre input (Altman, 1972b), climbing fibre removal partially denervates the cell and the reinnervating climbing fibre arbor is still larger than in the age-matched control. In contrast, by P30 each Purkinje cell receives a very large number of parallel fibres (Altman, 1972b), thus climbing fibre deafferentation only removes a small percentage of total input and there is no associated increase in arbor size of the reinnervating climbing fibre. This direct relation of reinnervating climbing fibre arbor size to the degree of Purkinje cell deafferentation is consistent with Purkinje cell adaptation that tries to maintain a constant afferent contact area (Chen and Hillman, 1985; Hillman and Chen, 1981). Thus, the data from this study indicate that BDNF-induced transcommissural olivocerebellar axons can respond appropriately to their target Purkinje cell's tropic cues.

5.5 CONCLUSION

This study extends previous work on the transcommissural olivocerebellar projection (Angaut et al., 1985; Sugihara et al., 2003; Zagrebelsky et al., 1997) by adding that exogenous BDNF extends the critical period for developmental plasticity to maturity. It also suggests that recreating the immature cellular milieu with target-derived growth-promoting agents permits the development of an alternate axonal projection which has appropriate structure and organisation.

Since anatomically appropriate alternate paths are associated with some functional benefit (Coumans et al., 2001; Dixon et al., 2005) the next chapter of this thesis analyses the functional recovery of animals with climbing fibre reinnervation of mature Purkinje cells.

CHAPTER 6

POST-LESION TRANSCOMMISSURAL OLIVOCEREBELLAR REINNERVATION IMPROVES MOTOR FUNCTION IN THE MATURE CEREBELLUM OF WISTAR RATS

6.1 INTRODUCTION

Post-lesion transcommissural olivocerebellar reinnervation during the neonatal period is organised into parasagittal microzones with normal synaptic function (Sugihara et al., 2003), and improves the animals functional recovery (Dixon et al., 2005). Likewise, previous chapters in this thesis show that BDNF-induced reinnervation in the mature cerebellum is also organised into parasagittal microzones with normal terminal arbor morphology. However, this reinnervation occurs at a time when the Purkinje cell dendritic tree is already fully grown (P30: McKay and Turner, 2005) and the area of reinnervation is not identical to normal (i.e. dense medially but sparse laterally). Since climbing fibre synapses must be correctly located on the Purkinje cell dendritic tree for normal function (Ichikawa et al., 2002; Lalouette et al., 2001), it was unknown whether BDNF-induced reinnervation on mature Purkinje cells would also improve the functional recovery. Therefore the present study assessed the behavioural sequale associated with climbing fibre reinnervation on mature Purkinje cells.

6.2 METHODS

Twenty one litters of rats of either sex were used to determine whether reinnervation onto mature Purkinje cells improved the animals' functional recovery after unilateral climbing fibre removal.

6.2.1 Induction of reinnervation

Climbing fibres from the left hemicerebellum were damaged via a lesion of the inferior cerebellar pathway at P15, P20 or P30 and 24 hours later 1 µl human recombinant BDNF (0.5 – 1 µg/ml) or 1 µl vehicle containing cytochrome C (0.5 - 1 µg/ml) was injected into the left hemicerebellum and vermis (refer section 2.3 for details on transection and injection procedures). All sham-operated animals also received 1 µl

intracerebellar BDNF (0.5 - 1 μg/ml) injections to ensure the presence of BDNF did not disrupt the normal cortical circuit.

6.2.2 Behavioural testing

The animals were subjected to a series of behavioural tests to determine the function of the post-lesion transcommissural olivocerebellar pathway.

6.2.2.1 Tests of motor coordination

The cerebellar cortical circuit controls complex motor coordination (Marr, 1969), therefore, the animals' motor coordination skills were assessed. These were divided into 3 categories: dynamic postural adjustment, gait and complex motor skills. With the exception of the footprint gait test, these tasks were attempted 3 times a day for 5 days, with an inter-trial interval of at least 3 minutes. The upper time limit (UTL) of the observation period for each test was 180 seconds, except for the vestibular drop, which had an upper time limit of 30 seconds (Dixon et al., 2005). This is sufficient extra time to assess whether animals with reduced motor performance could complete each test (Dixon et al., 2005). The footprint test was attempted only twice at the end of the behavioural testing.

Dynamic postural adjustments

The dynamic postural adjustments (integrity of the vestibular reflexes) were tested through the righting reflex and vestibular drop. *Righting reflex*: the rats were placed on their backs with the dorsal surface of their head and trunk in contact with the table (Fig 6.1A). After the observer moved their hands away, the rats turned their body onto all 4 limbs (reflex action). Presence or absence and directionality of response were recorded. *Vestibular drop*: this procedure also tests truncal muscular strength as well as vestibular reflex. The rats were suspended by their tail and tested on their ability to bring their head up to tail level by arching their body (Fig 6.1B). Presence, absence and directionality of the response were noted, as was time taken.

Quadruped locomotion (gait) and Muscular Tone

The gait patterns and muscular tone of the animals were tested through a bridge-crossing task, foot print analysis and cliff avoidance test. *Crossing a narrow bridge*: the animals were placed in the start position at one end of a narrow bridge 3 cm wide, 60 cm long

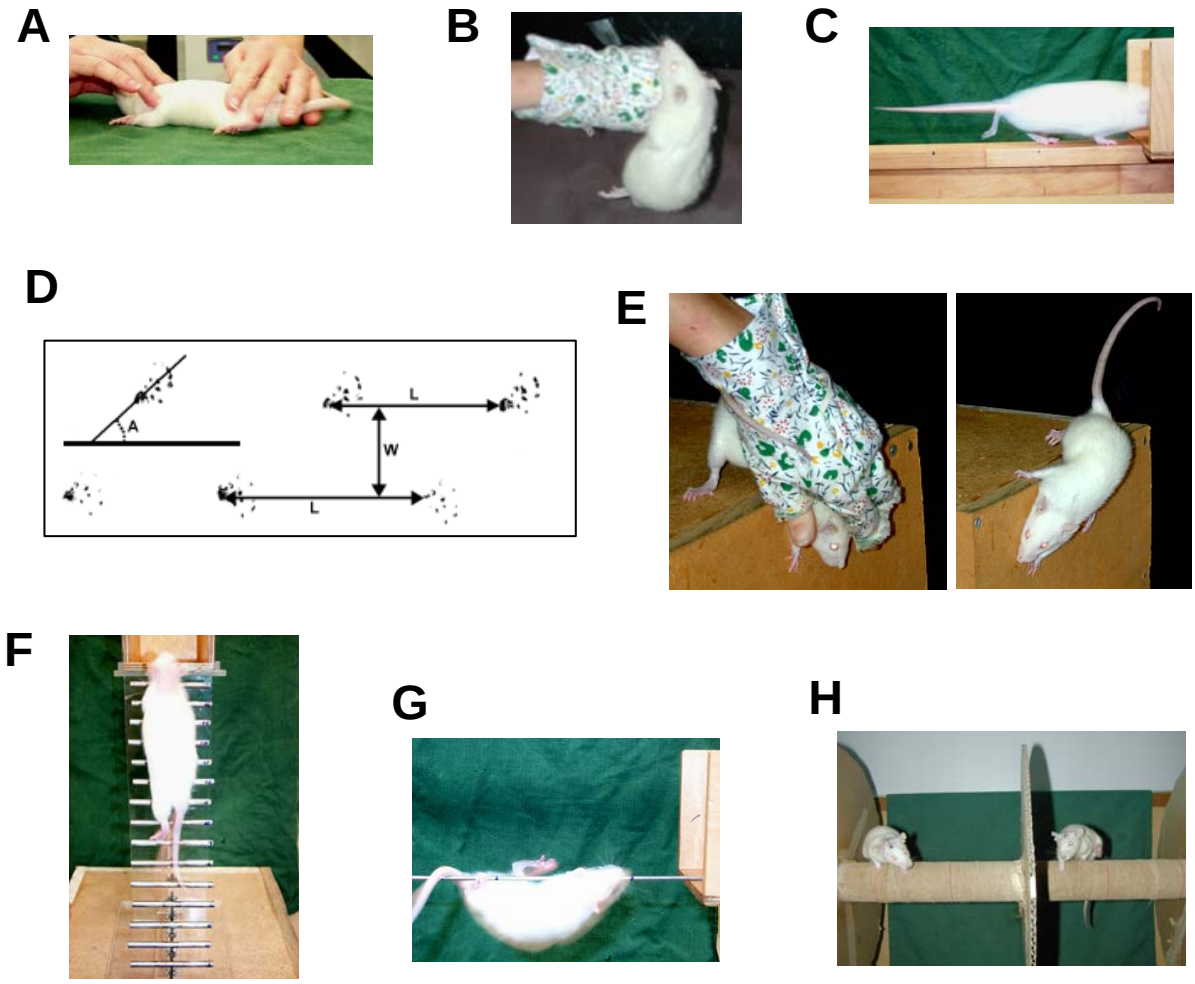


Figure 6.1 Behavioural tests
Photographs show sham-operated control animals on each behavioural apparatus.
(A) Righting reflex test: rats were placed on their back with their dorsal surface in contact with the table and observed turning their body onto all 4 limbs.
(B) Vestibular drop test: rats were suspended by their tail and tested on their ability to bring their head up to tail level by arching their body.
(C) Bridge test: the rats were placed at the end of a narrow bridge, which had a platform at the opposite end (seen on the right hand side of the photograph) and the rat was observed crossing the bridge.
(D) Footprint test: the rats' hindlimb footprint patterns were measured. A = hindlimb rotation angle; L = stride length; W = stride width.
(E) Cliff Avoidance test: the rats' heads and forelimbs were placed over the edge of a wooden box and the retraction time and direction of response were observed.
(F) Ladder test: animals were placed at the bottom of a steel ladder and observed climbing into the box at the top.
(G) Wire test: the rats were suspended by their forelimbs at the middle of a horizontal wire and observed climbing along the wire into an escape platform situated at either end
(H) Rotarod test: the animals were placed on top of a rotating rod turning at 10, 20, or 30 rpm and their total forward walking time was recorded.

and 60 cm high, which had a platform at the opposite end (Fig 6.1C). A littermate was placed in the opposite goal platform to entice the rat under observation to cross the bridge. The ability of the animal to traverse the bridge and the time taken to cross the bridge were recorded. *Footprint test*: this test was attempted twice at the end of behavioural testing and observed the rat's hindlimb footprint patterns (Fig 6.1D) by dipping their hind paws in black ink and observed the rats to run or walk along a white paper runway (15 cm wide and 90 cm long) to a dark escape box. The clearest (most easily identifiable) of the two traces was used for the following measurements: stride width (left-right), stride length (left-left and right-right) and angle of hindlimb rotation from direction of progression. For each animal, linear strides of 5 or more matched footprints were used for these analyses. *Cliff avoidance*: the rats were placed on a wooden edge (30 cm high, 20 cm wide), with their forelimbs and nose just over the edge (Fig 6.1E). Retraction time from the cliff edge was noted, as was directionality of response.

Complex motor skills

All of these tests assess the animals' motor coordination skills. *Ascending a ladder*: one animal at a time was placed at the bottom of a steel ladder (8 cm wide, 35 cm high, with 18 steps 2 cm apart at an angle of 25 degrees) with the top in contact with a platform containing a littermate. Successful arrivals at the top platform and time on ladder were recorded (Fig 6.1F). Pausing or freezing time was recorded and removed from the total time. *Suspension from a wire*: this procedure also tested the animal's muscular strength and limb motility. The rats were suspended by their forelimbs at the middle of a horizontal wire (3 mm thick, 60 cm long and 60 cm above thick foam) with an escape platform at each end (Fig 6.1G). Successful arrivals at the escape platform and time on the wire were recorded. Pausing or freezing time was again recorded and removed from the total time.

6.2.2.2 Motor synchronisation task

The climbing fibre pathway is important for the learning and control of rhythmical synchronisation of sensorimotor skills (Rondi-Reig et al., 1997). Therefore, the sensorimotor skills of the animals were assessed by walking on a rotating rod (rotarod:

Fig 6.1H). The rotarod is a horizontal cylinder 5 cm in diameter and 50 cm long, covered in sticking plaster to increase grip, which is mounted 50 cm above foam to cushion any falls. The cylinder rotates at 10, 20 and 30 rpm and the animal must walk on top of the cylinder, opposite to the direction of rotation, facing away from the observer.

The animals were subjected to 10 trials a day for 7 days, with at least a 3 min interval between each trial. For each trial the total amount of time spent walking on the rotarod was recorded, as was time in error - the rat walking backwards towards the observer or clinging to the rotarod and being passively rotated. The trial ended either when the rat fell off the rotarod onto the foam, or when the UTL of 180 s was reached. The UTL was arbitrarily fixed to 180 seconds because a rat which can walk on the rotarod for this period can maintain its equilibrium for a much longer time (15 – 20 minutes: Auvray et al., 1989). In these conditions, to wait for the rat to fall spontaneously would make the rats tired, thus the next trial of the day does not represent their ability to synchronise their gait – it represents how much the animals have recovered from the previous trial. Thus an UTL was created. However, it should be acknowledged that in setting this UTL, there is an inaccurate indication of ability and learning when comparing some groups (i.e. when one of the groups has reached the UTL and another has not: Auvray et al., 1989).

6.2.3 Anatomical analysis

To identify any reinnervation at the end of behavioural testing, half of the animals in each group received an anterograde tracer (4 % (w/v) Fluororuby) injection into the left inferior olive (refer section 2.5) and the other half of the animals received a retrograde tracer (2 % (w/v) Fast Blue) injection into the cerebellum (refer section 2.4). Thirteen days following Fluororuby injection or 4 days after Fast Blue injection experimental animals were perfused (refer section 2.7) and the brainstem and cerebellum were removed. In animals that received intra-cerebellar Fast Blue, two sets of 30 µm coronal sets were taken: the first was stained with 0.5 % (w/v) methylene blue for histological analysis and the second analysed for restriction of the injection within the left

hemicerebellum and the localisation of retrogradely-labelled neurons in the inferior olive. In animals injected with intra-olivary Fluororuby, three parallel sets of 30 µm coronal, or 40 µm parasagittal, sections were taken. One set was stained with 0.5 % (w/v) methylene blue, the second set was analysed for the distribution of Fluororuby labelled olivocerebellar axons, and the third set underwent immunohistochemical identification of climbing fibre terminals (VGLUT2) and Purkinje cells (calbindin) as described in section 2.7.

In all animals, complete transection of the entire left inferior cerebellar peduncle was verified by histological analysis of all (i.e. serial) sections, because the axotomised right inferior olive of mature animals does not completely degenerate within one month (Buffo et al., 1998). Additionally, animals were excluded from this study if the left DCN was not present, since removal of the DCN removes cerebellar outflow to other motor centres.

In successfully lesioned animals the laterality of reinnervating axons were mapped in the left hemicerebellum, the parasagittal organisation measured and arbor morphology identified, as described in chapter 5 (section 5.2). Also, the total area of reinnervation was measured using Image J software and compared between groups.

6.2.4 Behavioural data analysis

Although non-parametric analyses were required in the absence of homogeneity of variance (transformation of data did not produce homogeneity), the data are expressed as mean (± SEM) or percentage for ease of comparison with other behavioural studies. Thus, for each animal the scores of all daily trials were averaged to get the mean score per day. For all motor tests except the rotarod, the mean scores obtained by the *n* animals of each group were used on the last day to compare between groups, however for the rotarod test the mean daily scores were calculated, plotted on the graphs and used for subsequent analysis.

The ability (percent success) or direction taken was compared between groups using χ^2 test. Inter-group comparisons for time were made using the Kruskal–Wallis test and post hoc Mann–Whitney U, while intra-group comparisons were by Friedman and post hoc Wilcoxon analyses.

6.3 RESULTS

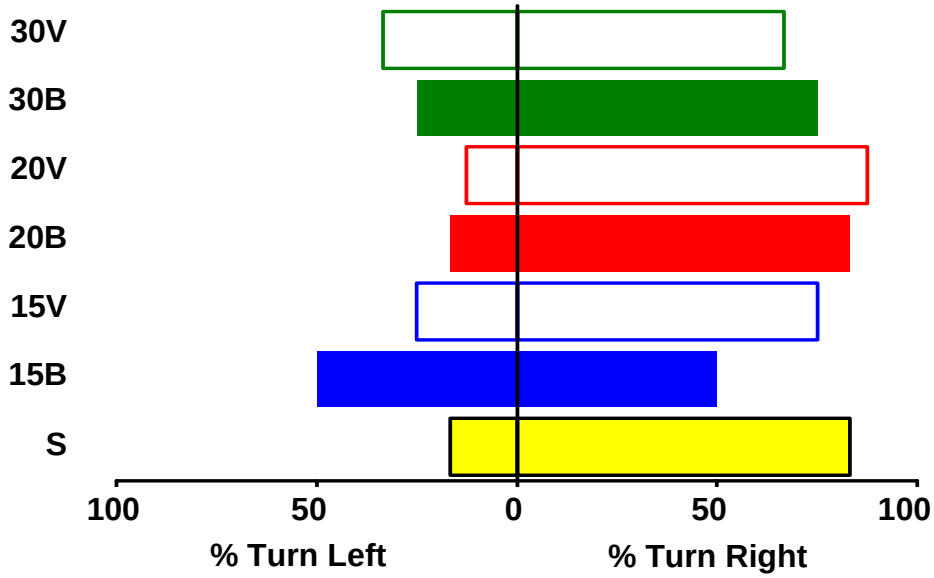
To determine whether climbing fibre reinnervation beyond the end of the critical period improved the animals functional recovery, the behavioural sequelae of 107 rats was examined, which had been treated with BDNF (B) or vehicle (V) 24 hours following unilateral olivocerebellar transection on P15, P20 or P30 (Px15B, n = 22; Px15V, n = 12; Px20B, n = 17; Px20V, n = 14; Px30B, n = 20; Px30V, n = 16; sham-operated, n = 6). After histological verification of complete inferior cerebellar peduncle transection and presence of left DCN, the number of animals to be included in the study was reduced to 43 (Px15B, n = 8; Px15V, n = 4; P20B, n = 6; Px20V, n = 8; P30B, n = 8; Px30V, n = 3; sham-operated, n = 6).

6.3.1 Dynamic postural adjustments are not dependent on climbing fibre reinnervation

Dynamic postural adjustment was assessed first to ensure that the lesion did not have any adverse effects on the animals' other motor circuits e.g. vestibulo-spinal reflexes. In the righting reflex task, all animals rapidly corrected their posture from supine to prone position, although, there was a trend for animals to turn to their right side, no group showed any directional bias different to the sham-operated control group ($p>0.05$: Fig 6.2). The postural adjustment made when animals are suspended by their tails (vestibular drop) is more complex, requiring truncal muscle strength and coordination in order to raise the body up to the level of the tail. All animals in each group were able to perform the vestibular drop task with equal time and ability and there was no directional bias ($p>0.05$: Fig 6.3).

Therefore, unilateral transection of the olivocerebellar pathway in the adolescent and mature cerebellum does not affect dynamic postural adjustments.

A

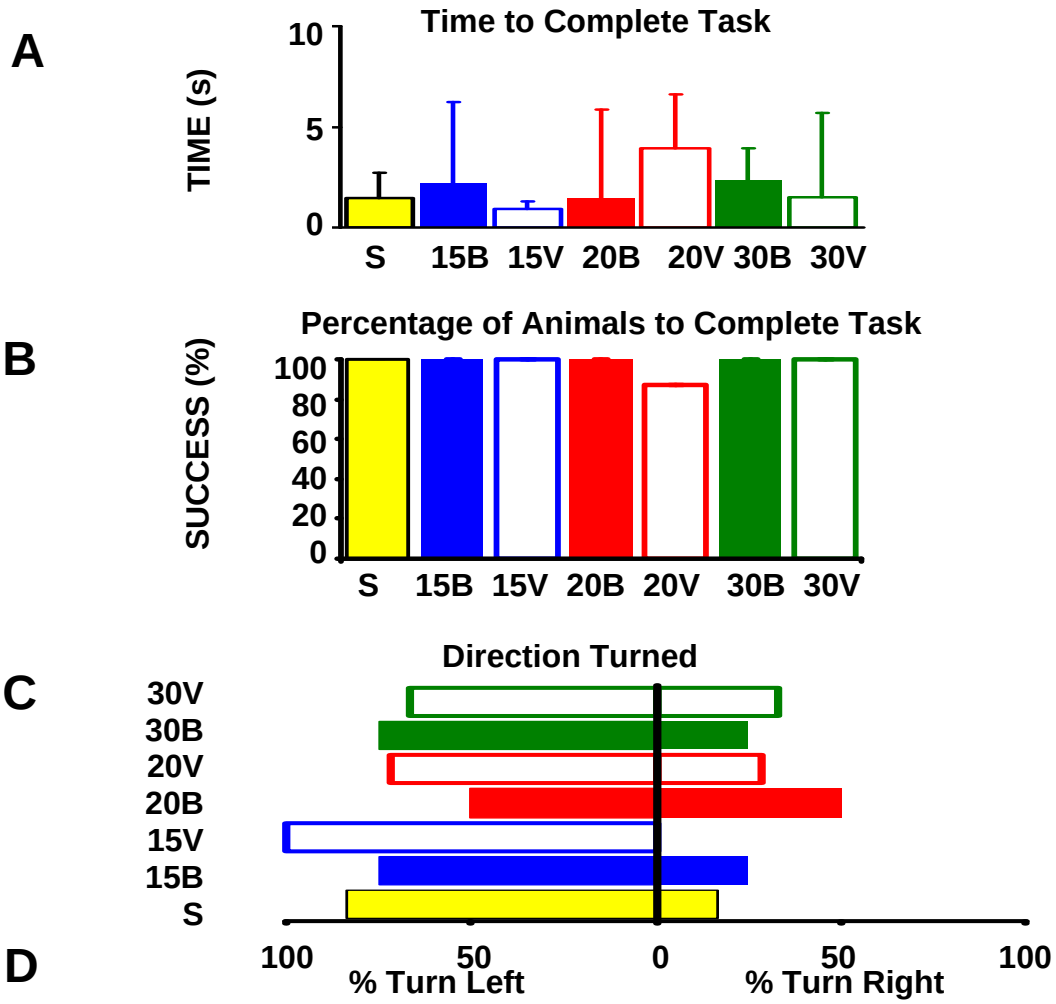


B

Treatment Group	Direction (% turn right)
Sham-operated Control	83
Px15 BDNF	50
Px15 Vehicle	75
Px20 BDNF	83
Px20 Vehicle	87
Px30 BDNF	75
Px30 Vehicle	67

Figure 6.2 Righting reflex
(A) Graph showing the percent of animals in each group for the righting reflex that turn to the left and right. The sham-operated control animals have a directional bias towards the right and there are no significant differences between any groups.
(B) Table of data for each group showing the percentage of animals to turn right.

Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle.



Treatment Group	Time (s)	Ability (% succeed)	Direction (% turn right)
Sham-operated Control	1.5 (± 0.4)	100	17
Px15 BDNF	2.2 (± 1.2)	100	25
Px15 Vehicle	0.9 (± 0.3)	100	0
Px20 BDNF	1.5 (± 0.5)	87	50
Px20 Vehicle	3.9 (± 2.3)	100	29
Px30 BDNF	2.3 (± 0.9)	100	25
Px30 Vehicle	1.5 (± 0.8)	100	33

Figure 6.3 Vestibular drop
(A-C) Graphs showing the time taken (s: mean ± SEM) to complete the vestibular drop test, the number of successful animals to complete the task and any directional bias. There are no significant differences between any groups for this test.
(D) Table showing data for the vestibular drop test.

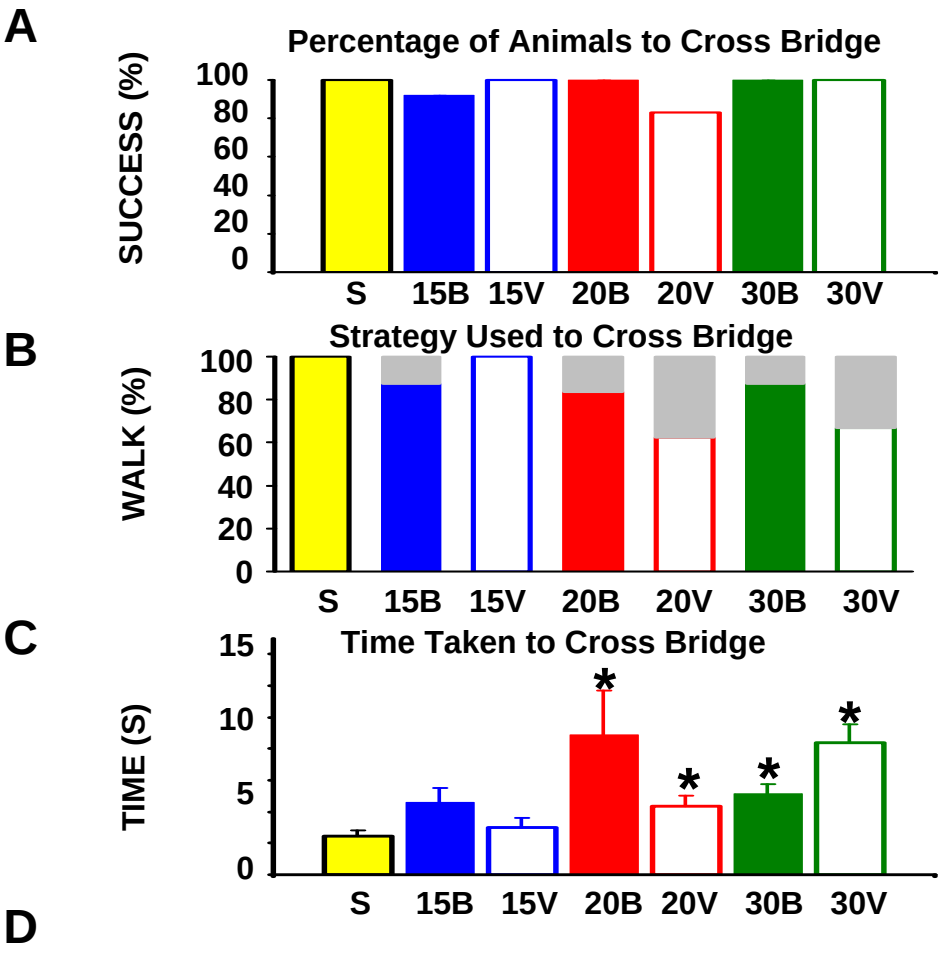
Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle.

6.3.2 Early climbing fibre reinnervation aids normal gait

Climbing fibre removal from the cerebellum results in the development of abnormal gait patterns (Dixon et al., 2005; Rondi-Reig et al., 1997), therefore this study tested the animals' gait by observing their ability to walk across a narrow bridge, as well as measuring hindlimb footprint patterns. Since Wistar rats can walk by P12 (Westerga and Gramsbergen, 1990) most animals in this study were able to successfully walk across the bridge to reach the platform ($p>0.05$: Fig 6.4A&B), although the time taken to reach the end platform varied between groups. While the Px15B and Px15V groups (4.6 s and 3.0 s, respectively) were both as fast as the sham-operated controls (2.4 s), the Px20B, Px20V, Px30B and Px30V groups were slower ($p<0.05$: Fig 6.4C). Therefore, climbing fibre reinnervation in the later adolescent or mature cerebellum does not improve the abnormal gait induced by the lesion.

Since all animals appeared to be equally mobile around their cages and each group used the same strategy to cross the bridge (walking: $p>0.05$: Fig 6.4B), their footprint patterns were examined to determine why some groups were slower at crossing the bridge. All groups had a normal hindlimb rotation and stride length compared to the sham-operated controls ($p>0.05$: Fig 6.5A&B), a sign of normal gait. While the Px15B, Px15V, Px30B and Px30V groups displayed no differences in stride width between their left and right hindlimbs ($p>0.05$), both the Px20B (4.3 cm) and Px20V (3.9 cm) groups had a wider stride width than the sham controls (3.1 cm: $p<0.01$ and $p<0.05$, respectively), so that their base was much wider than the narrow bridge (Fig 6.5C).

Additionally, the animals' directionality on the cliff avoidance test was also analysed to determine if muscular tone on both sides of their body was even. The Px30B and Px30V groups were the only groups to have a directional bias when retracting their bodies from a cliff edge (Sham-operated control animals = 50 % turn right; Px30B: 87.5 %; Px30V = 100 %: $p<0.01$ and $p<0.05$ respectively: Fig 6.6). This indicates that these animals are weaker on one side of their body (unilateral hypotonia) and as a result take a longer time to cross the bridge.



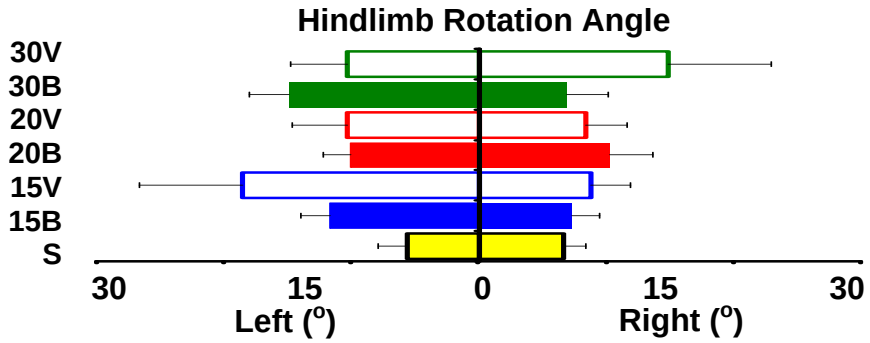
D

Treatment Group	Success to Cross (%)	Walk (%)	Time (s)
Sham-operated	100	100	2.4 (± 0.4)
Px15 BDNF	92	87	4.6 (± 0.9)
Px15 Vehicle	100	100	3.0 (± 0.6)
Px20 BDNF	100	83	8.9 (± 2.8) *
Px20 Vehicle	83	62	4.3 (± 0.7) *
Px30 BDNF	100	87	5.1 (± 0.6) *
Px30 Vehicle	100	67	8.4 (± 1.1) *

Figure 6.4 Bridge test
Graphs showing **(A)** the percent of successful animals to cross the bridge, **(B)** the strategy used (crawling or walking) and **(C)** the time taken. There are no significant differences in the percentage of animals to successfully cross the bridge, nor the strategy used (walking: coloured bars; crawling: grey bars) although the trend suggests the BDNF-treated groups and the Px20V and Px30V groups are not able to walk for as long as the sham-operated control animals. All Px20 and Px30 groups take longer to cross the bridge than sham-operated control animals. **(D)** Table shows the data for the bridge tests (s: mean ± SEM).

Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle; * = $p<0.05$ compared to sham-operated vehicles.

A



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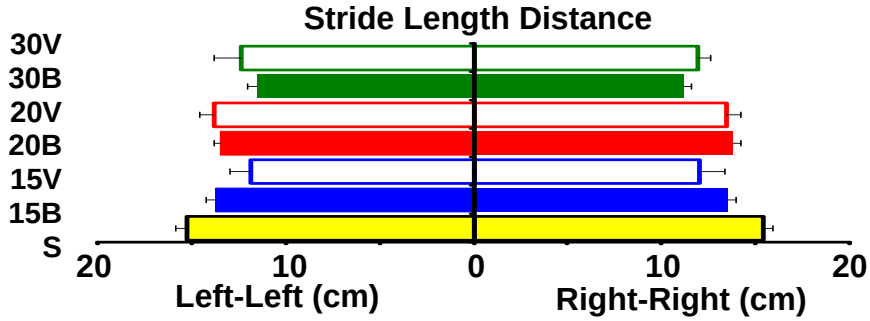


C

D

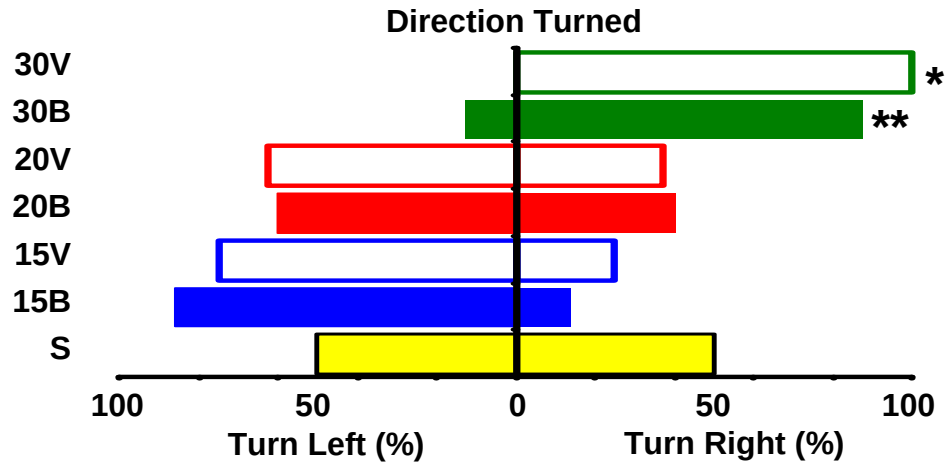
Treatment Group	Hindlimb Rotation (°)		Stride Length (cm)		Stride Width (cm)
	Left	Right	Left-Left	Right-Right	
Sham-operated	5.6 (± 2.2)	6.6 (± 1.8)	15.2 (± 0.6)	15.4 (± 0.5)	3.1 (± 0.1)
Px15 BDNF	11.6 (± 2.4)	7.3 (± 2.2)	13.7 (± 0.5)	13.5 (± 0.5)	3.7 (± 0.3)
Px15 Vehicle	18.6 (± 8.1)	8.8 (± 3.1)	11.8 (± 1.1)	12.0 (± 1.3)	4.5 (± 0.9)
Px20 BDNF	10.1 (± 2.2)	10.3 (± 3.4)	13.4 (± 0.4)	13.8 (± 0.5)	4.3 (± 0.1) *
Px20 Vehicle	10.3 (± 4.3)	8.4 (± 3.2)	13.8 (± 0.7)	13.5 (± 0.8)	3.9 (± 0.3) *
Px30 BDNF	14.9 (± 3.0)	7.0 (± 3.1)	11.5 (± 0.5)	11.1 (± 0.4)	3.3 (± 0.3)
Px30 Vehicle	10.3 (± 4.4)	14.9 (± 8.1)	12.4 (± 1.4)	12.0 (± 0.7)	3.6 (± 0.3)

Figure 6.5 Footprint test

(A-C) Graphs showing the animals' hindlimb rotation angle, stride length and stride width. The dotted line on **(C)** indicates the width of the bridge (3 cm). There are no significant differences in hindlimb rotation or stride length between groups. However, the Px20B and Px20V groups have a wider stride width than sham-operated control animals. **(D)** Table showing the data for the footprint test: amount of hindlimb rotation, stride length and stride width (mean ± SEM).

Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle; * $p<0.05$, ** $p<0.01$ compared with sham-operated control animals.

A



B

Treatment Group	Direction (% Turn Right)
Sham-operated Control	50
Px15 BDNF	14
Px15 Vehicle	25
Px20 BDNF	40
Px20 Vehicle	38
Px30 BDNF	88 **
Px30 Vehicle	100 *

Figure 6.6 Cliff avoidance test
(A) Graph showing the percentage of animals that turn left and right in each group in the cliff avoidance test. The Px30B and Px30V groups were the only groups to turn right more than the sham-operated control animals.
(B) Table showing the direction turned on the cliff avoidance tests

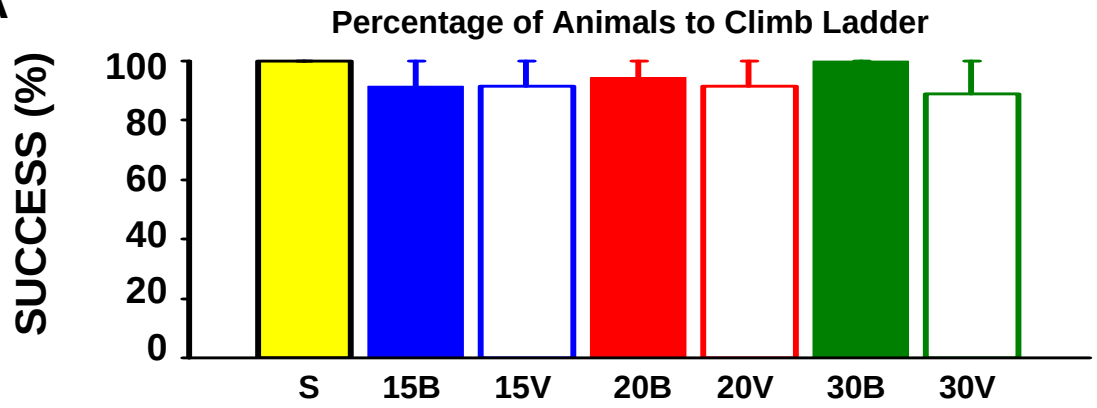
Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle; * $p<0.05$, ** $p<0.01$ compared with sham-operated control animals.

6.3.3 BDNF-treated animals under-perform vehicle-treated counterparts in limb coordination tasks

As a further measure of motor control, the ability to complete the complex tasks of climbing a ladder and progressing along a wire was assessed. Although climbing a ladder involves muscular strength, rats try to avoid falling from the ladder by using their hindlimbs to propel their body weight up the ladder to get to the escape box at the top. This task requires co-ordination of all 4 limbs. All groups were able to climb the ladder successfully ($p>0.05$: Fig 6.7A-C), although some groups were slower than others. While the Px15B group (3.1 s) was able to climb the ladder in the same time as the sham-operated controls (2.6 s), the Px15V animals (1.7 s) were faster than the Px15B group ($p<0.05$). Likewise, both the Px20V and Px30V groups were not significantly slower than the sham-operated controls (3.7 s and 4.9 s, respectively), while the Px20B and Px30B groups (5.3 s and 4.6 s, respectively) were slower than the sham-operated controls ($p<0.05$).

Similar to the ladder, rats try to avoid falling from the wire by using their hindlimbs to support their weight and to progress along the wire to its junction with the upright (Altman and Sudarshan, 1975), where an escape box is located. This complex task requires coordination of all 4 limbs with truncal muscles to enable the hindlimbs to be lifted to the wire and propel the animal along it. Control rats can easily perform this task by P25 (Dixon et al., 2005), therefore all sham-operated animals are able to reach the box at the end of the wire successfully (Fig 6.8A-C). Likewise, there were no statistically significant differences between any of the treatment groups in their ability to reach the platform at the end of the wire, nor the time taken to get there ($p>0.05$). However, the trend suggests that the Px20B and PX20V groups were less able to coordinate their limbs as fewer animals reached the box at the end (61 – 63 %) compared with the other groups (87.5 – 100 %). Additionally, the trend suggests that the Px20B group (19.0 ± 4.9 s) were slower than all other groups (7.2 – 12.0 s).

A



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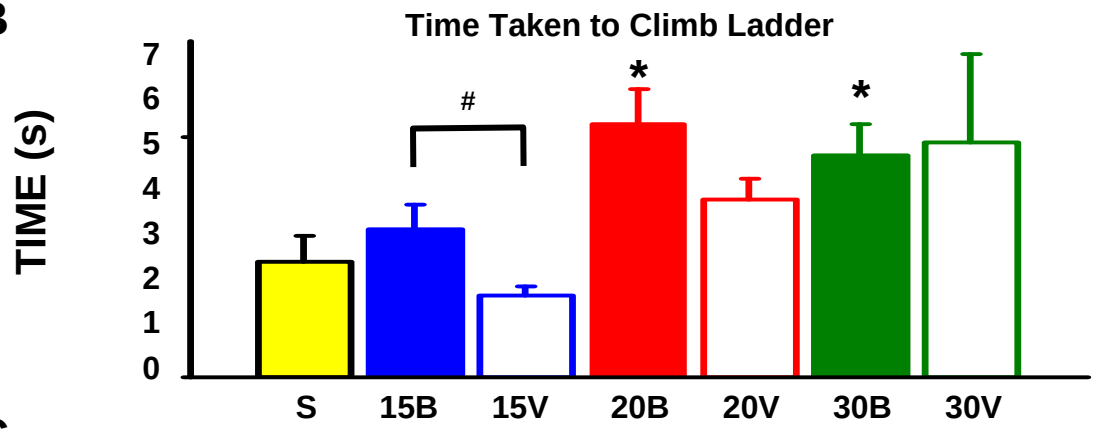
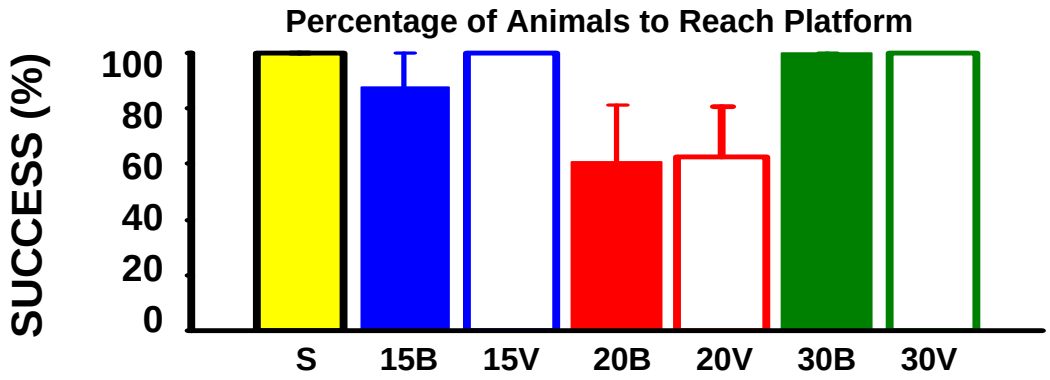


C

Treatment Group	Ability (%)	Time (s)
Sham-operated	100	2.6 (± 0.4)
Px15 BDNF	92	3.1 (± 0.5) #
Px15 Vehicle	92	1.7 (± 0.2)
Px20 BDNF	94	5.3 (± 0.7) *
Px20 Vehicle	91	3.7 (± 0.4)
Px30 BDNF	100	4.6 (± 0.6) *
Px30 Vehicle	89	4.9 (± 1.8)

Figure 6.7 Ladder test
(A&B) Graphs showing the percentage of animals in each group successful at climbing the ladder and the time taken to reach the end platform. There are no significant differences in the percentage of animals to successfully climb the ladder, although the Px15, Px20 and Px30 BDNF-treated groups were all slower than the sham-operated control animals. Also, the Px15B group was slower than the Px15V group.
(C) Table showing the data for the percentage of animals to climb the ladder and the time taken (s: mean ± SEM) to reach the end platform.
Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle; * $p<0.05$ compared with sham-operated control animals; # $p<0.05$ compared with Px15V group.

A



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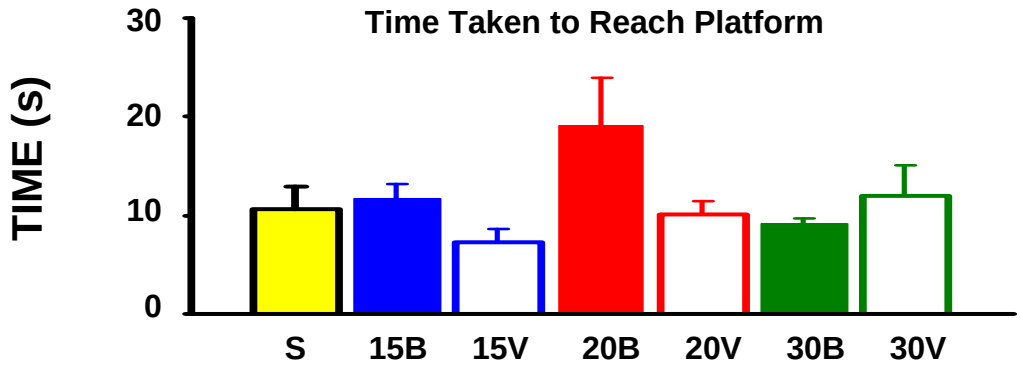


C

Treatment Group	Ability (%)	Time (s)
Sham-operated Control	100	10.6 (± 2.3)
Px15 BDNF	87.5	11.7 (± 3.6)
Px15 Vehicle	100	7.2 (± 1.3)
Px20 BDNF	61	19.0 (± 4.9)
Px20 Vehicle	63	10.0 (± 1.4)
Px30 BDNF	100	9.1 (± 1.5)
Px30 Vehicle	100	12.0 (± 3.0)

Figure 6.8 Wire test
(A&B) Graphs showing the percentage of animals in each group to successfully climb along the wire and reach the end platform, and the time taken to reach the box. There were no significant differences between any groups, although the trend suggests that the Px20 groups were less able to reach the end platform, and subsequently the Px20B group took a longer time.
(C) Table showing the data for the percentage of animals to successfully complete the task and the time taken (s: mean ± SEM).

Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle.

Therefore, the BDNF-treated groups were less able to coordinate their limbs than the control vehicle groups, and as a result were slower on complex tasks. Furthermore, reinnervation in the later stages of the adolescent cerebellum (Px20) renders the animal less able to co-ordinate their limbs than reinnervation in the early stages of the adolescent cerebellum (Px15).

6.3.4 Vehicle-treated animals walk for longer on the rotarod than BDNF-treated animals

Having assessed basic cerebellar postural and locomotory function, a more detailed analysis of the transcommissural climbing fibre reinnervation was made by testing an animal's ability to walk on the rotarod, since synchronisation of gait and learning of this skill are primarily controlled by the olivocerebellar pathway (Rondi-Reig et al., 1997).

At 10 rpm on the last day of testing the sham-operated control group was able to walk on the rotarod for the maximum time (180 s: Fig 6.9). The total walking time of all groups, except Px20B, was not significantly less than the sham-operated controls ($p>0.05$ and $p<0.05$, respectively). An increase in speed to 20 rpm revealed that both the Px20 groups and the Px15B group were unable to walk for as long as the sham-operated control group ($p<0.01$ and $p<0.05$, respectively), while the Px15V and both Px30 groups did not walk significantly less than the sham-operated controls. A further increase in speed to 30 rpm revealed that no experimental groups were able to walk for as long as the sham-operated control group ($p<0.05$). Also the Px20B animals become worse than their aged-matched vehicle-treated counterparts ($p<0.05$). Therefore, in the adolescent-lesioned cerebellum (Px15 or Px20), earlier lesions or vehicle-treated animals have better synchronisation of gait (compared with later lesions and BDNF-treated animals), although in the mature-lesioned cerebellum (Px30) both BDNF- and vehicle-treatment provide the same functional outcome.

In addition to controlling gait synchronisation, the olivocerebellar pathway is involved in learning motor patterns. Therefore, the differences between groups may result from motor skill deficiencies due to the lesion, an inability to learn the task due to climbing fibre dysfunction or a combination of both. This can be assessed by comparing the

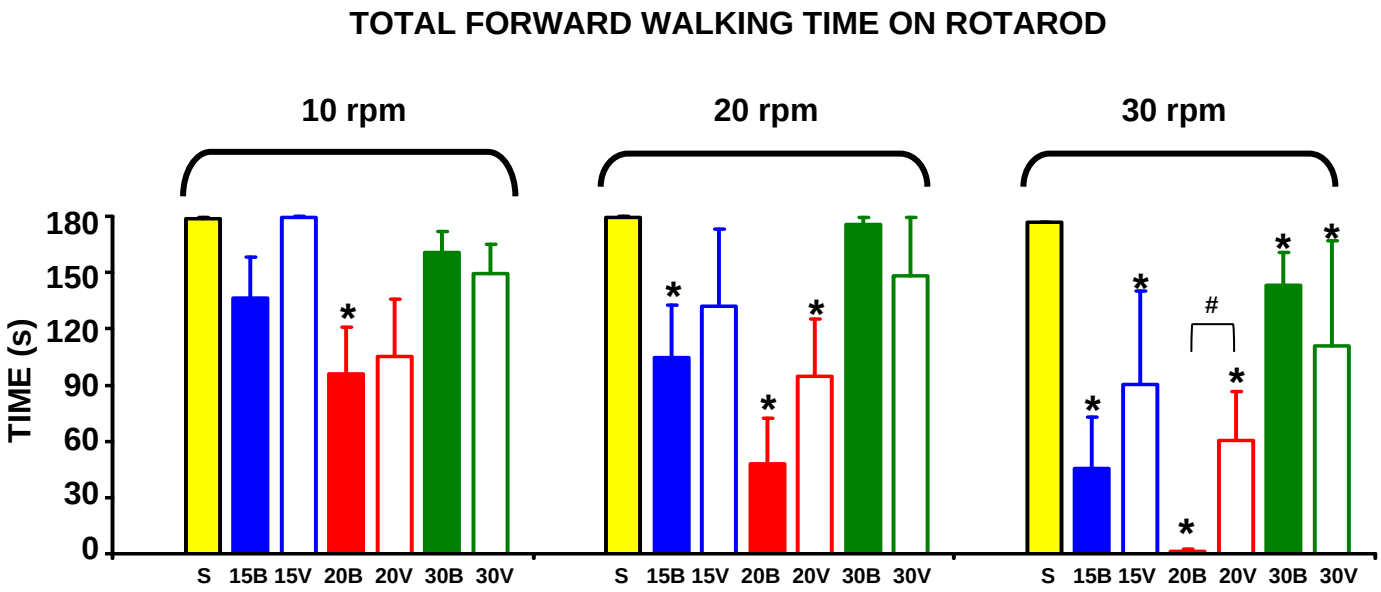


Figure 6.9 Forward walking time of animals on the rotarod
The graph shows the forward walking time of each group on the rotarod on the last day of training at 10, 20 and 30 rpm. At 10rpm the Px20B group walk for less time than the sham-operated controls ($p<0.05$). At 20 rpm the Px15B and both Px20 groups also walk for less time than sham-control animals ($p<0.05$). When the speed increases to 30 rpm the Px15V and both Px30 groups walk for less than sham-operated controls ($p<0.05$), and the Px20B group walked for less time then the Px20V group.

Key: S = sham-operated control animals; 15B = Px15 BDNF; 15V = Px15 Vehicle; 20B = Px20 BDNF; 20V = Px20 Vehicle; 30B = Px30 BDNF; 30V = Px30 Vehicle; * $p<0.05$ compared to sham-operated controls; # $p<0.05$ compared to Px20 BDNF group; rpm = revolutions per minute.

relative differences in total walking time in untrained animals (first day of testing), when any between-group differences will be due only to motor impairment, with trained animals (seventh day of testing) whose performances will reflect both motor and learning abilities (Rondi-Reig et al., 1997).

At 10 rpm sham-operated control animals easily learned the task ($p<0.05$) and post-hoc analysis indicated this had occurred by the second day. At this speed, the Px15V group also learned the task quickly, improving their total walking time from 127 ± 19 s to 179 ± 1 s by the third day of testing (Fig 6.10). As stated above, however, learning is more clearly revealed by comparing the relative motor deficit ({control time – treatment group time}/control time) in untrained and trained animals. Comparison of total walking time with sham-operated controls revealed the Px15V animals had a relative motor deficit of 22 % on day 1 (untrained, {163-127}/163 = 0.22) in comparison with a 0 % deficit after 7 days of training ({179-179}/179 = 0): an improvement that indicates learning (intragroup statistics did not confirm this improvement: $p>0.05$). Likewise, the Px15 BDNF group also improved their total walking time from 96 ± 25 s to 137 ± 21 s, so that their relative motor deficit compared with the sham-operated controls also improved from a 41 % deficit on day 1 to a 23 % deficit after 7 days of training. This is also indicative of learning and occurred by the end of the second day of testing (intragroup analysis between days 1 and 2: $p<0.05$). A comparison of learning between the Px15 groups reveals that the Px15B and Px15V groups learn similarly, as the Px15B group has a relative motor deficit compared to Px15V of 24 % on day 1 and 23 % on day 7. However, as stated in the methods, this is an erroneous comparison between groups, as the Px15V group reaches the UTL by the third day of testing, while the Px15B group never reaches the UTL.

Neither of the Px20 groups were able to reach the maximum speed at 10 rpm (Fig 6.11). At the start of testing the Px20V animals walked on the rotarod for less time than the sham-operated controls ($p<0.05$), but did not walk for significantly less time from the fourth day of testing (Friedman analysis indicates learning occurred between days 1 and 2: $p<0.05$). The Px20B group never walked for as long as the sham-operated control

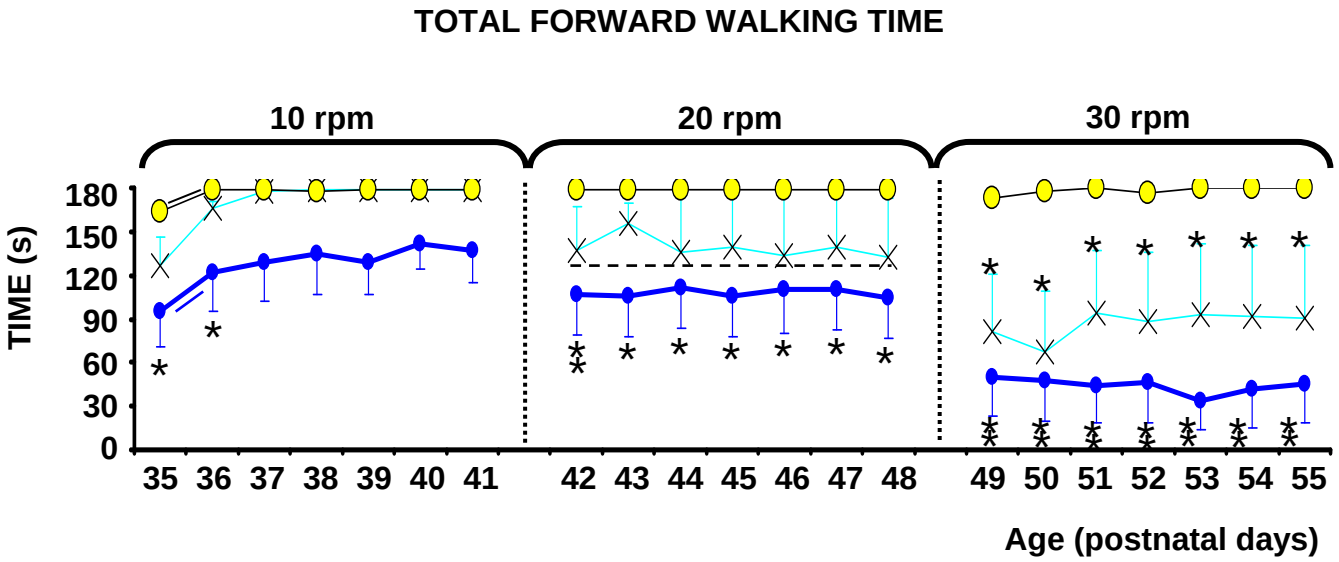


Figure 6.10 Effect of training on the rotarod of the Px15 groups
The graph shows the forward walking time of the Px15 groups on the rotarod at all speeds. The sham-operated group (yellow line) quickly learn the task and walk for the maximal time on all rotation speeds. At the slowest speed (10 rpm) the Px15V (light blue) group quickly learn the task to reach 180 s by the third day of testing, while the Px15B (dark blue) group undergo learning from the first to the second day of testing, but they walk for significantly less time than the sham-operated control group until the third day of testing. At 20 rpm the Px15B group are significantly worse than the sham-operated animals, although the Px15V group are not significantly worse. At 30 rpm however, both groups are now significantly worse. The double line between some data points indicates learning ($p<0.05$) between consecutive days. For comparison, the horizontal dotted line has been added to indicate the total walking time of rats with a total lesion of the inferior olive by 3-acetylpyridine at P15 (Jones et al., 1995).

Key: —•— = BDNF group; —×— = vehicle group; * $p<0.05$ compared with sham-operated control animals; ** $p<0.01$ compared with sham-operated control animals; rpm = revolutions per minute.

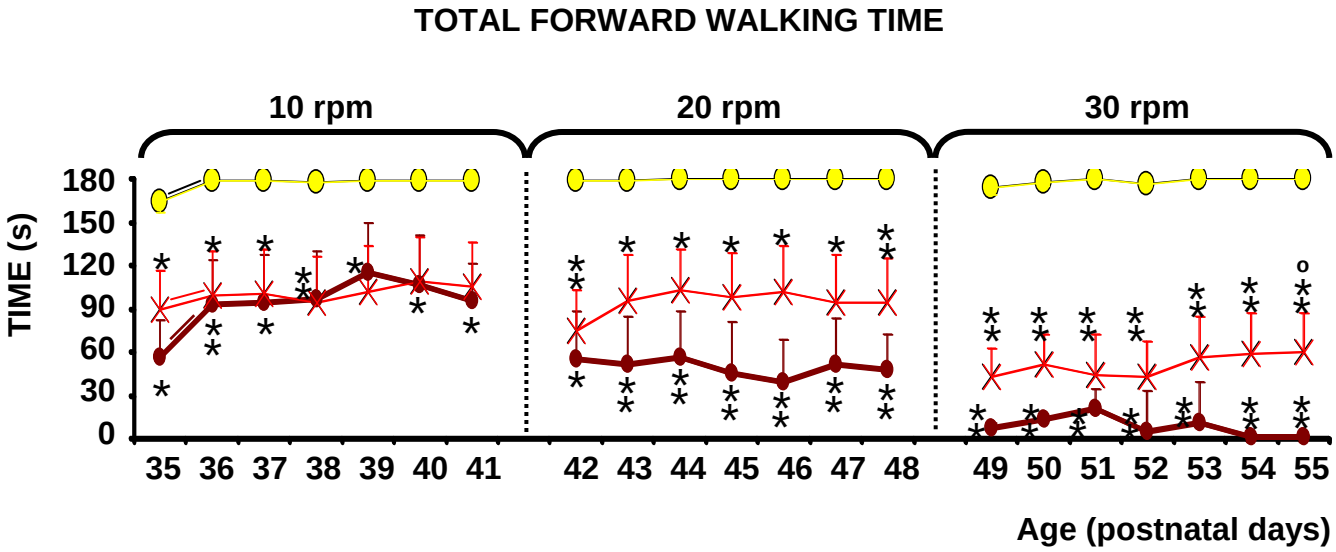


Figure 6.11 Effect of training on the rotarod of the Px20 groups
The graph shows the forward walking time of the Px20 groups on the rotarod on each day at each speed. The sham-operated group (yellow line) quickly learn the task and walk for the maximal time on all rotation speeds. The Px20V (light red) group also learn the task between the first and second day of testing, but walk for significantly less time than the sham-operated controls until the fourth day of testing. The Px20B (dark red) group also learn between the first and second day at 10rpm, but always take significantly less time to walk than the sham control animals. At 20 and 30 rpm both groups walk for less than the sham-operated animals. The double line between some data points indicates learning ($p<0.05$) between consecutive days.

Key: —•— = BDNF group; —×— = vehicle group; * $p<0.05$ compared with sham-operated control animals; ** $p<0.01$ compared with sham-operated control animals; rpm = revolutions per minute.

animals ($p<0.05$), however the Px20B improved their total walking time between days 1 and 2 ($p<0.05$). The relative motor deficit of the Px20 groups compared with the sham-operated control group reveals that while both groups learn the task, the BDNF group learn more than the vehicle group: the BDNF group had a deficit of 65 % on day 1 and 46 % on day 7, while the vehicle group had a deficit of 45 % on day 1 and 41 % on day 7. This is confirmed by comparing the relative motor deficit of the Px20B group to the Px20V group: 36 % on day 1 of training and 8 % on the last day of training. Thus while the Px20V group undergo learning, the Px20B group have a faster rate of learning.

Lastly, the Px30 groups also have some degree of learning at 10 rpm (Fig 6.12). The Px30B group achieve their maximal walking time on the sixth day of testing, can walk for as long as the sham-operated controls on the third day of testing ($p<0.01$) and improve their total walking time between days 1 and 2 ($p<0.05$). In contrast, the vehicle animals begin significantly worse than both the Px30B and sham-operated groups, with a total walking time of 4.3 ± 1.3 s on the first day, but by the seventh day of testing the Px30V animals have increased their total walking time (149.6 ± 15.2 s) to that of the sham-operated controls (178.7 ± 0.8 s: $p>0.05$), despite no statistical improvement between any two consecutive days. A comparison of relative motor deficits with these groups reveals that both groups learn, although the Px30V group learn more than the Px30B group. The relative motor deficit of the Px30B group compared with the sham-operated controls is 59 % on day 1 of training and 10 % on the last day of testing: indicative of learning. Similarly, the Px30V group have a motor deficit of 97 % on day 1 and 16 % on day 7: also indicative of learning. Comparing the relative motor deficits of the Px30B and Px30V groups confirms that the vehicle animals learn more than the BDNF group: the vehicle group has a relative motor deficit of 94 % on day 1 and 7 % on the last day of training at this speed.

Furthermore, injury to the mature cerebellum does not hinder performance at higher speeds, thus the Px30 groups continue to improve their total walking time on the rotarod at 20 and 30 rpm. At 20 rpm the Px30B group increased their total walking time with a

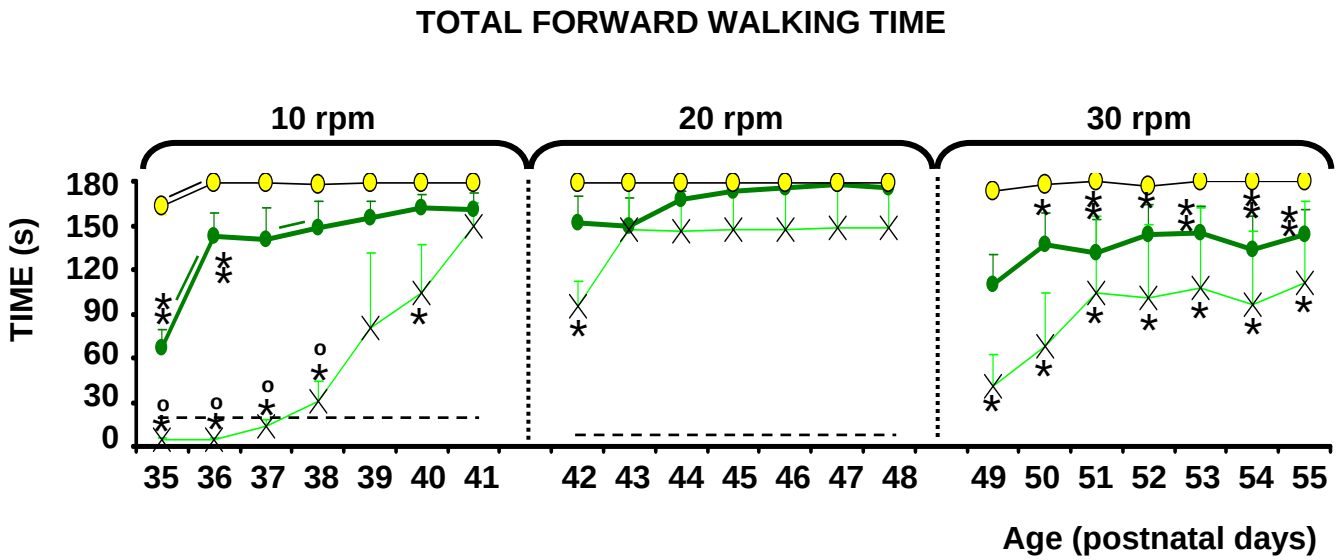


Figure 6.12 Effect of training on the rotarod of the Px30 groups
The graph shows the forward walking time of the Px30 groups on the rotarod on each day at each speed. The sham-operated group (yellow line) quickly learn the task and walk for the maximal time on all rotation speeds. The Px30B (dark green) group quickly improve their total walking time at 10 rpm so that by the third day of testing this group is not walking for less time than the sham control group. Despite the Px30V (light green) group never improving their total walking time between any individual days, the Px30V group walk for significantly less time than the Px30B and sham-operated groups until the fifth day of testing at 10 rpm. At 20 rpm neither group is significantly worse than the sham-operated animals (except Px20V on day 1), however when the speed increases to 30 rpm both groups become significantly worse than the sham-operated control animals. The double line between some data points indicates learning ($p<0.05$) between consecutive days. For comparison, the horizontal dotted lines have been included to indicate the total walking time of rats with a total lesion of the inferior olive by 3-acetylpyridine in the adult rat (Rondi-Reig et al., 1997).

Key: —•— = BDNF group; —×— = vehicle group; * $p<0.05$ compared with sham-operated control animals; ** $p<0.01$ compared with sham-operated control animals; ^{o} $p<0.05$ compared with age-matched BDNF group; rpm = revolutions per minute.

relative deficit compared to sham-operated controls from 14 % on day 1 to 2 % on day 7 and at 30 rpm improved from 37 % on the first day to 20 % on the last day. Likewise the vehicle animals improved their total walking time on both 20 rpm and 30 rpm, with a relative deficit compared to sham controls of 47 % on day 1 of 20 rpm and 17 % on day 7, and from 76 % on day 1 of 30 rpm to 38 % on the last day. This is indicative of ongoing learning. When comparing the relative motor deficit of the Px30V to the Px30B animals, at 20 rpm the vehicle animals have a relative motor deficit of 37 % on day 1, which decreases to 16 % by the seventh day of testing. When the speed increases to 30 rpm this relative motor deficit widens to 62 % on the first day at this speed, but is again reduced to less than half by the end of testing, reaching 22 % on day 7, indicating that the vehicle injected animals learn gait synchronisation more than the BDNF injected animals.

Therefore, in the adolescent cerebellum (Px15 and Px20) vehicle-treated animals have the best functional outcome on the rotarod and the earlier the lesion the better the function. In the mature cerebellum (Px30), reinnervation provides better functional improvement on the rotarod than no reinnervation (compare Px30V with Px30B on the rotarod at 10 rpm on day 1: Fig 6.12), but both BDNF- and vehicle-treated animals are not statistically different at the end of testing on the rotarod. The behavioural results of all tests in this study are summarised in Tables 6.1 & 6.2.

6.3.5 BDNF induces transcommissural climbing fibre reinnervation

To confirm whether BDNF successfully induced transcommissural axonal growth, in half of the animals, inferior olivary labelling was examined following injection of Fast Blue into the left hemicerebellum in rats with complete inferior cerebellar peduncle transection on P15, P20 and P30 and treated with intra-cerebellar BDNF 24 hours later.

In all BDNF-treated animals, scattered Fast Blue labelled neurons were observed in the inferior olivary complex, which indicates reinnervation of the left hemicerebellum has occurred since the entire left inferior cerebellar peduncle was transected. All neurons displayed the histological features of olivary neurons with an ovoid soma, a large

Table 6.1 Motor test summary

Treatment Group	Dynamic Postural Adjustments	Gait & Muscular Tone	Limb Coordination
	Righting Reflex (RR) Vestibular Drop (VD)	Bridge, Footprint, Cliff Avoidance	Ladder, Wire
Sham Controls	RR = right bias VD = normal	Normal	Normal
Px15 BDNF	As controls	Bridge success = controls Bridge time = controls Hindlimb stride length L=R Hindlimb stride width = controls Cliff avoidance = controls	Ladder success = controls Ladder time = controls Wire success = controls Wire time = controls
Px15 Vehicle	As controls	Bridge success = controls Bridge time = controls Hindlimb stride length L=R Hindlimb stride width = controls Cliff avoidance = controls	Ladder success = controls Ladder time = controls Wire success = controls Wire time = controls
Px20 BDNF	As controls	Bridge success = controls Bridge time > controls * Hindlimb stride length L=R Hindlimb stride width > controls ** Cliff avoidance = controls	Ladder success = controls Ladder time > controls * Wire success = controls ^ Wire time > controls ^
Px20 Vehicle	As controls	Bridge success = controls Bridge time > controls * Hindlimb stride length L=R Hindlimb stride width > controls * Cliff avoidance = controls	Ladder success = controls Ladder time = controls Wire success = controls ^ Wire time = controls
Px30 BDNF	As controls	Bridge success = controls Bridge time > controls * Hindlimb stride length L=R Hindlimb stride width = controls Cliff avoidance direction ≠ controls **	Ladder success = controls Ladder time > controls * Wire success = controls Wire time = controls
Px30 Vehicle	As controls	Bridge success = controls Bridge time > controls * Hindlimb stride length L=R Hindlimb stride width = controls Cliff avoidance direction ≠ controls *	Ladder success = controls Ladder time = controls Wire success = controls Wire time = controls

Summary table showing results of the Px15, Px20 and Px30 BDNF- and vehicle-treated animals on the motor tests. The Px20 and Px30 groups have an abnormal gait, the Px20 groups are slower on the complex tasks and the Px30 groups have a side preference in the cliff avoidance test.

Key: Righting Reflex = RR; VD = vestibular drop; * $p<0.05$ compared with sham-operated control animals; ** $p<0.01$ compared with sham-operated control animals; ^ trend indicates performance is less than sham-operated control group.

Table 6.2 Rotarod test summary

Treatment Group	Rotarod 10 rpm (last day)	Rotarod 20 rpm (last day)	Rotarod 30 rpm (last day)	
Sham Controls	UTL	UTL	UTL	
Px15 BDNF	Time = controls	Time < controls *	Time < controls *	
Px15 Vehicle	Time = controls	Time = controls	Time < controls *	
Px20 BDNF	Time < controls *	Time < controls *	Time < controls *	BDNF < Vehicle #
Px20 Vehicle	Time = controls	Time < controls *	Time < controls *	
Px30 BDNF	Time = controls	Time = controls	Time < controls *	
Px30 Vehicle	Time = controls	Time = controls	Time < controls *	

Summary table showing results of the Px15, Px20 and Px30 BDNF- and vehicle-treated animals on the rotarod. The Px15 and Px20 vehicle-treated groups walk for as long as the sham-operated control group, while the Px15 and Px20 BDNF-treated groups can not walk for as long (i.e. they are significanatly worse than the sham-operated control group). The Px30 vehicle- and BDNF-treated groups perform similarly, but the vehicle group show delayed learning (10 rpm: not on table).

Key: UTL = upper time limit; * $p<0.05$ compared with sham-operated control animals; # $p<0.05$ compared with BDNF group.

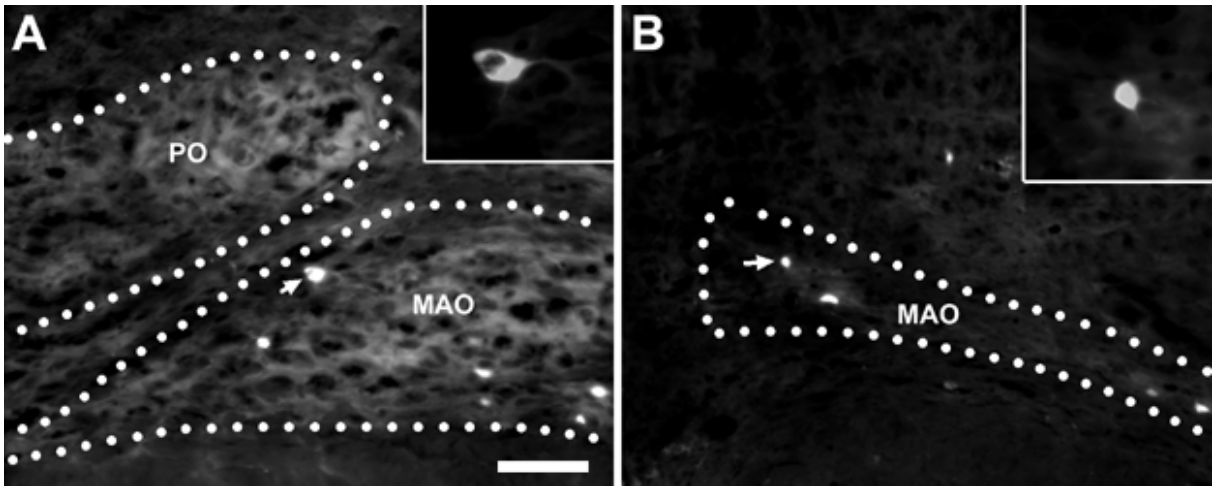
unlabelled nucleus and a few processes filled with fluorescent dye. In the Px15 (n = 2) and Px20 (n = 2) animals, labelled cells were found in both the left and right olives (1 – 26 labelled cells: Fig 6.13A-C), while in the Px30 animals (n = 4) labelled neurons were observed in the right olivary complex only (15 cells: Fig 6.13C). This is in contrast to previous studies in the cerebellum, which did not find Fast Blue-labelled neurons in the right olivary complex contralateral to the lesion (Dixon and Sherrard, 2006; Sherrard and Bower, 2003).

6.3.6 Distribution of BDNF- and exercise-induced transcommissural climbing fibre reinnervation

In the other half of the animals, the organisation and morphology of transcommissural reinnervating axons was examined by anterograde tracing (Fluororuby) and VGLUT2 immunohistochemistry.

In the control right hemicerebellum of all BDNF- and vehicle-treated animals, the distribution of VGLUT2-like immunoreactivity confirmed the results of chapter 5: there was extensive immunolabelling throughout the granular and molecular layers (Fig 6.14A), localised in discrete patches in the granular layer and in fine arbors that surrounded the Purkinje cell soma and extended along the primary dendrite into the molecular layer. Likewise, in animals treated with either BDNF or vehicle (Px15B, n = 5; Px15V, n = 4; Px20B, n = 2; Px20V, n = 3; Px30B, n = 3) VGLUT2-like immunoreactive terminals were observed in both the granular and molecular layers of the denervated left hemicerebellum (Fig 6.14B&C). This is in contrast to the results of chapter 5, in which the vehicle-treated animals did not have any climbing fibre reinnervation. Thus, exercise also induces climbing fibre reinnervation into the denervated hemicerebellum.

In BDNF injected animals VGLUT2-like labelling was similar to that observed in chapter 5: intense labelling in those lobules into which BDNF was injected (Fig 6.15A, C&E). Similarly, there was a medio-lateral gradient of labelling within the reinnervated hemicerebella. However, the reinnervation was more dense further away from the



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	Left Inferior Olive (mean number of cells)	Right Inferior Olive (mean number of cells)
Px15 (n = 2)	26	14
Px20 (n = 2)	1	6
Px30 (n = 4)	0	15

Figure 6.13 Fast Blue-labelled cells in the left and right inferior olivary complex after extensive exercise
(A&B) Photomicrographs of the ventral brainstem of Px15 animals showing Fast Blue retrogradely labelled neurons in the mid-rostral left **(A)** and right **(B)** inferior olives. Insets show arrowed neurons at higher power, in which an unlabelled nucleus **(A)** and labelled neurites **(A&B)** are visible.
(C) Table showing the mean number of Fast Blue-labelled cells within the inferior olivary complex in animals with complete transection of the left inferior cerebellar peduncle on P15, P20 or P30, followed by behavioural observations.

Key: Bars = 50 μm; MAO = medial accessory olive; PO = principal olive.

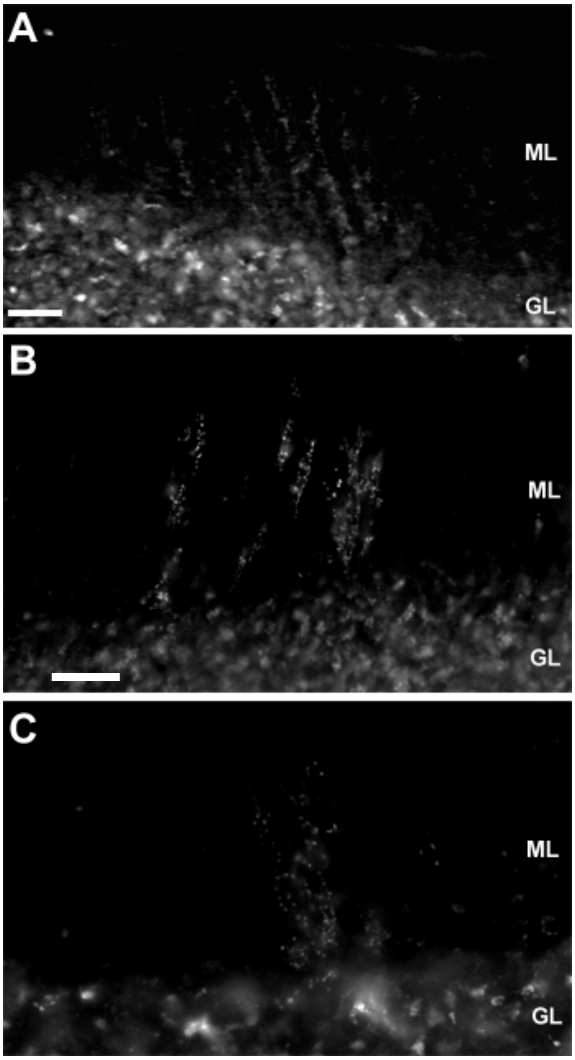


Figure 6.14 VGLUT2-labelled reinnervating climbing fibre terminals after extensive exercise

Photomicrographs of VGLUT2 labelled climbing fibre terminals in the reinnervated and control hemicerebella.

(A) Control right hemicerebellum of a Px15 vehicle-treated animal. VGLUT2 labels climbing fibre terminals in the molecular layer and mossy fibre terminals in the granular layer.

(B) Left denervated hemicerebellum of a Px15 BDNF-treated animal. VGLUT2-labelled climbing fibre terminals are visible in the molecular layer, as well as mossy fibre terminals in the granular layer.

(C) Left denervated hemicerebellum of a Px20 vehicle-injected animal. VGLUT2 labelling was also present in the granular and molecular layers indicating mossy and climbing fibre labelling respectively.

Key: Bars = 50 μm; GL = granular layer; ML = molecular layer.

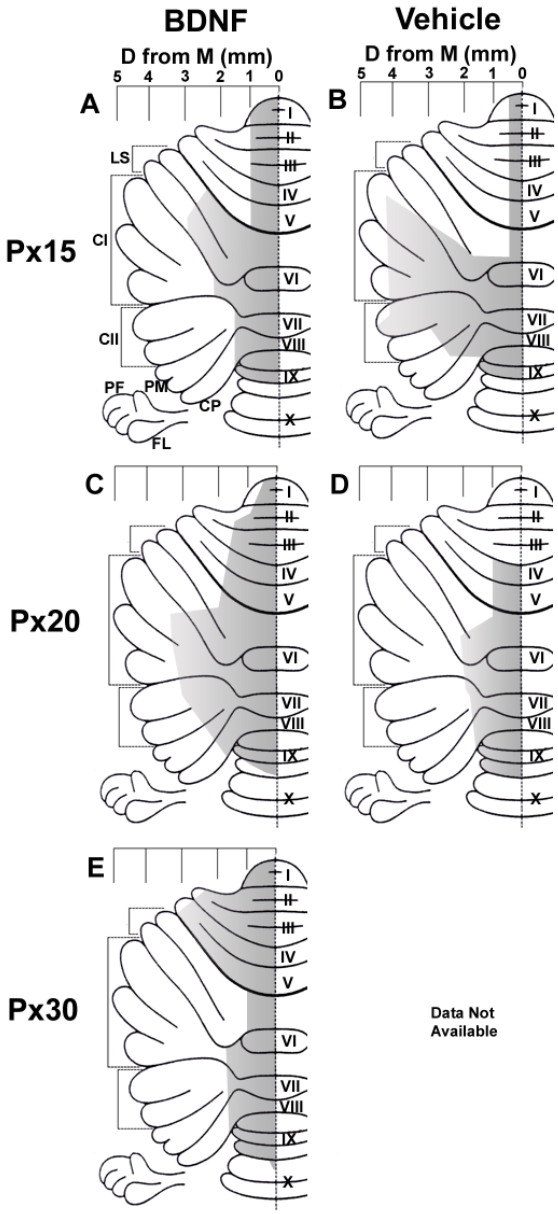


Figure 6.15 Area of VGLUT2-labelled reinnervating climbing fibre terminals after extensive exercise (unfolded cerebellum)
Schematic diagrams of the unfolded cerebellar cortex (modified from Buisseret-Delmas and Angaut, 1993) showing a summary of the distribution of VGLUT2-labelled transcommissural climbing fibre reinnervation induced by BDNF and exercise: **(A)** P15-lesion BDNF-treated group, **(B)** P15-lesion vehicle-treated group, **(C)** P20-lesion BDNF-treated group, **(D)** P20-lesion vehicle-treated group, **(E)** P30-lesion BDNF-treated group. The gradient of shading (dark to light) represents the mediolateral gradient of reinnervation density. The dotted vertical line indicates the cerebellar midline.

Key: CI = Crus I; CII = Crus II; CP = copula pyramidis; D = distance; FL = flocculus; I – X = vermal lobules I to X; LS = lobulus simplex; M = midline; PF = paraflocculus; PM = paramedian lobule.

midline in BDNF-treated animals that underwent extensive exercise, compared with BDNF-treated animals in chapter 5 (not subjected to extensive exercise): numerous VGLUT2-like immunoreactive terminals were observed up to 2 mm from the midline (zones 1 – 4) after exercise compared with those observed only 1.5 mm in BDNF-treated animals. Additionally, a few terminals were also observed in lobules II – V, lobulus simplex, crura, paramedian lobule and copula pyramidis up to 3.5 mm (zones 4 – 7) from the midline. Therefore, the exercise these animals were subjected to induced more reinnervation further into the denervated hemisphere.

In the vehicle injected animals VGLUT2-like immunolabelling in the molecular layer of the deafferented hemicerebellum was not concentrated within the injection site and appeared to be spread out through the rostro-caudal axis of the left hemicerebellum with equal density (Fig 6.15B&D). There was still a medio-lateral gradient of labelling within the reinnervated hemicerebella, but unlike the BDNF treated animals, the extent of labelling laterally was dependent on the age of the lesion. While both Px15 and Px20 groups had numerous VGLUT2-like immunoreactive terminals up to 2 mm from the midline (zones 1 – 4), labelling was also observed up to 5 mm from the midline (zones 4 – 10) in crus I, crus II, paramedian lobule and copula pyramidis of the Px15 animals (Fig 6.15B).

Thus, in animals subjected to extensive exercise, reinnervation was observed in both BDNF and vehicle-treated groups, but with different distances of laterality (Fig 6.15A-E). To identify whether these differences in laterality correlated with an increased area of reinnervation (which may in turn correlate with improve functional recovery), the total area of reinnervation was measured in each group. Statistical analysis revealed no differences in the total area of reinnervation between groups (mean total area 6.75 – 15.93 mm^2: $p>0.05$: Table 6.3). Therefore, any differences in motor function between groups are not dependent on the area of reinnervation.

Table 6.3 Area of VGLUT2-labelled reinnervating climbing fibre terminals after extensive exercise

Treatment Group	Total Area of Reinnervation (mm^2: mean ± SEM)
Px15 BDNF	6.95 (± 1.8)
Px15 Vehicle	13.25 (± 3.5)
Px20 BDNF	15.93 (± 3.2)
Px20 Vehicle	6.75 (± 0.5)
Px30 BDNF	10.88 (± 1.9)
Px30 Vehicle	data not available

Table shows the total area of VGLUT-2-labelled reinnervating climbing fibre terminals in the deafferented hemicerebellum, after extensive exercise. There were no significant differences identified between groups ($p>0.05$).

6.3.7 Parasagittal distribution and arbor morphology of reinnervating olivocerebellar axons

Since functional improvement depends on correct olivocerebellar circuit re-formation, the normal parasagittal organisation is essential for behavioural effect. As expected, within the control right hemicerebellum Fluororuby labelled axons were distributed in parasagittal bands similar to chapter 5 (Fig 6.16A). Also, distribution in the reinnervated hemisphere was observed in BDNF- and vehicle-injected animals. Fluororuby filled axons were present up to 1.5 mm left of the midline organised into parasagittal stripes. Within the vermis these stripes were almost symmetrical about the midline to those on the right side, confirming that BDNF-induced reinnervating terminals, and suggesting that the exercise-induced reinnervating terminals followed normal topographical distribution. Additionally, the distribution of VGLUT2 immunoreactivity in the molecular layer of some sections confirmed this result: dense VGLUT2-like labelling was found right of the midline, however left of the midline there was only sparse labelling in parasagittal stripes (Fig 6.16B).

As indicated in chapter 5, normal sagittal banding of reinnervating axons is in microzones 250 – 500 μm wide (Fukuda et al., 2001; Sugihara et al., 2001). In the reinnervated left hemicerebellum of Px15 BDNF-treated animals subjected to exercise, the Fluororuby labelling was also organised in narrow bands, however these were 92 – 130 μm wide (114.5 ± 9.1 μm; mean ± SEM; n = 4), which is significantly wider than BDNF-induced banding in Px30 animals without exercise in chapter 5 (69.6 ± 11.1 μm: $p<0.05$).

Finally, in the denervated BDNF-treated hemicerebellum most reinnervating climbing fibre terminal arborisations were normal (Fig 6.17A), and identical to those described in chapter 5 (section 5.3.3). However, some reinnervating arbors were less branched (Fig 6.17B): 33 % and 44 % in the Px15 and Px30 groups, respectively. Despite this, the reinnervating arbors in both Px15 and Px30 BDNF-treated groups were the same size (measured diagonally) as control arbors in the right hemisphere (Fig 6.17C: $p>0.05$).

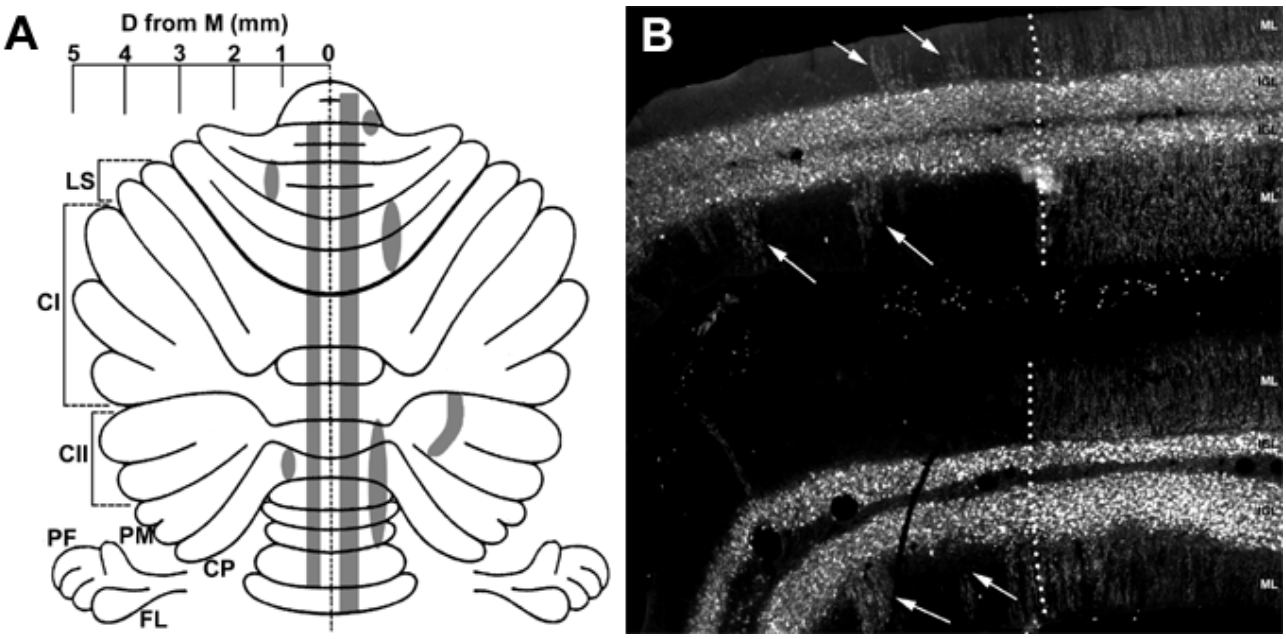


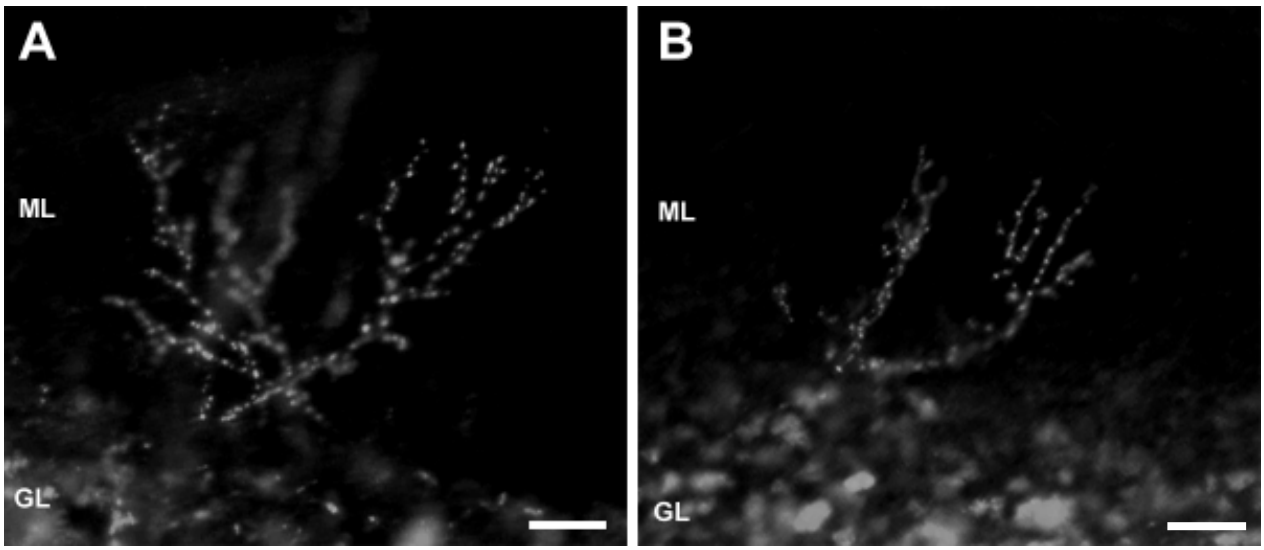
Figure 6.16 Parasagittal banding of reinnervating terminals after extensive exercise

Parasagittal distribution of climbing fibre terminals within the control and reinnervated hemicerebella

(A) A diagram of the unfolded cerebellar cortex (modified from Buisseret-Delmas and Angaut, 1993) showing the distribution of Fluororuby filled climbing fibre terminals in the cerebellar cortex of BDNF injected animals. In the right hemisphere Fluororuby labelling is organised in parasagittal stripes (grey shaded area). Labelled transcommissural reinnervating axons in the left hemivermis are less numerous than on the control right side, but are organised almost symmetrically about the midline.

(B) A photomicrograph of the cerebellar cortex of a BDNF-treated animal showing VGLUT2-labelled climbing fibre and mossy fibre terminals in the left and right hemicerebellum. In the reinnervated left hemicerebellum VGLUT2-labelling is not as dense as the right hemicerebellum and is organised in parasagittal stripes (indicated by arrows). Dotted vertical line indicates the cerebellar midline. Bar = 100 μm.

Key: CI = Crus I; CII = Crus II; CP = copula pyramidis; D = distance; FL = flocculus; GL – granular layer; L = left; LS = lobulus simplex; M = midline; PF = paraflocculus; PM = paramedian lobule; R = right.



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Treatment Group	Left hemisphere (µm)	Right hemisphere (µm)
Px15	170.9 (± 12.4) (n = 9)	186.3 (± 8.2) (n = 4)
Px30	182.3 (± 15.2) (n = 10)	166.7 (± 25.4) (n = 5)

Figure 6.17 Morphology of VGLUT2-labelled reinnervating terminal arbors after extensive exercise
Photomicrographs showing VGLUT2-labelled terminal arbors of transcommissural reinnervating climbing fibres in a Px30 BDNF-treated animal.
(A) Normal climbing fibre arbor ascending through the molecular layer.
(B) An abnormal climbing fibre arbor in the same animal is less branched in the molecular layer.
(C) Table showing arbor size (measured diagonally: mean ± SEM) in the left and right hemisphere of Px15 and Px30 BDNF-treated animals.

Key: Bars = 25 µm; IGL = internal granular layer; ML = molecular layer.

This is in contrast to chapter 5, in which the reinnervating arbors in Px15, but not Px30, BDNF-treated animals were larger than the control arbors.

6.4 DISCUSSION

In this study the anatomical and behavioural sequale of post-lesion reinnervation of the denervated hemicerebellum by transcommissural olivocerebellar axons was examined. While reinnervation during the neonatal period provides some functional recovery (Dixon et al., 2005), it was unknown whether the recapitulation of this plasticity in the adolescent and mature cerebellum, when the Purkinje cell has a large and physiologically mature dendritic tree (McKay and Turner, 2005) would also provide any return of lost function. Therefore, the functional performance and cerebellar anatomy of each group will be discussed.

6.4.1 BDNF-induced reinnervation is distributed in parasagittal bands and develops normal terminal arbors on mature Purkinje cells

This study confirms that BDNF induces climbing fibre reinnervation with appropriate structure and organisation beyond the end of the critical period by recreating the immature cellular milieu (Dixon and Sherrard, 2006). This is supported by 3 lines of evidence:

Firstly, the area of BDNF-induced climbing fibre reinnervation observed in this study is not contained within the injection site and is similar to that observed in chapter 5. Since single olivocerebellar axons terminate in multiple climbing fibre arbors organised in parasagittal bands within multiple cerebellar lobules (Sugihara et al., 2001), then administration of BDNF to only one arbor may increase cellular cAMP, as occurs elsewhere in the CNS (Song et al., 1997), and induce growth of axons into the denervated hemisphere that are not directly acted upon by the BDNF.

Secondly, the results of this study also show that BDNF-induced reinnervating climbing fibres are organised into parasagittal zones. This distribution is similar to that seen in the normal adult olivocerebellar pathway (Buisseret-Delmas and Angaut, 1993;

Sugihara and Shinoda, 2004) and in reinnervating climbing fibres that develop following unilateral olivocerebellar transection in the neonatal or mature cerebellum (Dixon and Sherrard, 2006; Sugihara et al., 2003; Zagrebelsky et al., 1997), or partial olivary destruction by 3-acetylpyridine (Rossi et al., 1991b). The band width of Px15 BDNF-induced climbing fibres in this study (92 – 130 μm, mean = 114.5 μm) is narrower than the normal climbing fibre microzone and that following pedunculotomy at P3 (500 μm and 337 ± 66 μm, respectively: Fukuda et al., 2001; Sugihara et al., 2003), but wider than the BDNF-induced band width of Px30 animals in chapter 5 (48 – 101 μm, mean = 69.6 μm: $p<0.05$: Dixon and Sherrard, 2006). This is in line with increased levels of inhibitory factors as the cerebellum matures (Rhodes et al., 2003), making it more difficult for the axonal branches to traverse the white matter, resulting in a narrower band width. Nevertheless, as in chapter 5, the arbors are still contained within a normal microzone indicating that the large number of denervated Purkinje cells in adjacent areas with their associated growth-attractant signals did not over-ride the cues that guide normal olivocerebellar parasagittal organisation.

Thirdly, in addition to demonstrating a parasagittal organisation, the majority of reinnervating climbing fibres also develop normal terminal arbors, similar to those that reinnervate target cells following either neonatal (Sugihara et al., 2003) or adult (Rossi et al., 1991b) climbing fibre lesion. A small percentage of these arbors were less branched – an abnormality that has also been observed following pedunculotomy on P3 (Sugihara et al., 2003). Importantly however, reinnervating climbing fibre terminals are the same size as those in the control hemisphere, indicating normal growth despite a more mature target. While this is in contrast to the larger arbor size of Px15 reinnervating terminals in chapter 5, the rats in the previous chapter were culled only 8 days post-lesion (P23), compared to the rats subjected to exercise in the current chapter that were culled as adults (~ P90). Therefore, even if the reinnervating terminal arbors in the Px15 animals of the current study (i.e. animals subjected to exercise) were larger than control arbors immediately following the lesion, the terminal arbors in the control hemisphere would have grown in parallel with growth of the Purkinje cell dendritic tree

(until P90), such that when the animal is culled both reinnervating and control terminal arbors are of the same size.

This normality of reinnervating climbing fibre terminals is crucial since climbing fibre circuit reformation does not always lead to functional improvement (Fernandez et al., 1998).

6.4.2 Extensive exercise induces transcommissural climbing fibre reinnervation

In contrast to previous reports (Dixon and Sherrard, 2006; Sherrard and Bower, 2001), climbing fibre terminals were observed extensively throughout the lesioned hemicerebellum of vehicle-treated animals. Since the entire left inferior cerebellar peduncle was transected in these animals and there were no residual climbing fibre terminals remaining in the left hemisphere, the reinnervation could have been induced by the extensive exercise. This is supported by 2 lines of evidence: Firstly, exercise up-regulates BDNF mRNA and its receptor trkB within the central nervous system (Oliff et al., 1998; Widenfalk et al., 1999), including the cerebellum (Klintsova et al., 2004; Neeper et al., 1996) and exogenous BDNF induces climbing fibre reinnervation in the mature cerebellum (see chapter 5: Dixon and Sherrard, 2006). Therefore, widespread cerebellar BDNF upregulation following extensive exercise may induce climbing fibre growth across the cerebellar midline to the denervated Purkinje cells. Secondly, the area of climbing fibre reinnervation in the vehicle treated animals varied depending on the age at which the lesion occurred, i.e. lesions at a younger age had a greater area of reinnervation than lesions at an older age. While BDNF is still upregulated following exercise in the aged rat (19 months old: van Praag et al., 2005) olivary trkB levels decrease with age (Riva-Depaty et al., 1998). This decreased trkB expression, in addition to increased inhibitory molecules within the CNS (Rhodes et al., 2003), is possibly preventing extensive climbing fibre growth at older ages, limiting the distance of reinnervation, thus suggesting that exercise is inducing the reinnervation and is not caused by the presence of a molecule or the injection itself. Additionally, during development, receptor expression still occurs in the olive until P15 (Riva-Depaty et al.,

1998). Therefore olivary neurons in P15-lesioned animals may be primed prior to the injury (i.e. receptor expression until P15 allows a neurotrophin response and thus possibly elevated cAMP levels) enabling a greater distance of reinnervation, by blocking inhibition of MAG (myelin associated glycoprotein) and myelin as happens elsewhere in the CNS (Cai et al., 1999; Ming et al., 1997; Song et al., 1997). This is in contrast to the Px20 group, as trkB and p75 expression is reduced in the olive for more than 5 days prior to the lesion (Nitz et al., 2001; Riva-Depaty et al., 1998), thus olivary neurons may have minimal cAMP levels at P20. As a result the neurons may not be in a 'growth ready' state and this could limit the distance of axonal growth.

If trkB and p75 protein expression was measured prior to and after the lesions, most likely there would be a transient post-lesion increase lasting for 24 – 48 hours, as occurs during development (Nitz et al., 2001) and following climbing fibre modification in adulthood (Li et al., 2001). If cAMP expression was examined in animals of the current study, most likely higher levels would be found prior to the lesion in olivary neurons of the younger P15-lesioned animals, but not P20-lesioned animals. However, it is expected that elevated cAMP levels would occur in all groups post-lesion, due to transient receptor expression increase, as mentioned above in other CNS systems (Ming et al., 1997).

6.4.3 Extensive exercise maintains the ipsilateral climbing fibre pathway and induces transcommissural reinnervation into the denervated hemisphere

In this study retrogradely-labelled neurons were observed within the left and right olivary complex of BDNF-treated animals following a complete unilateral olivocerebellar transection and extensive exercise. Since this is in contrast to previous studies (Dixon and Sherrard, 2006; Sherrard and Bower, 2001), the lesion and retrograde dye sites were carefully examined to ensure a complete transection of the entire inferior peduncle and to exclude animals where the Fast Blue retrograde dye could have spilled across the cerebellar midline, potentially giving false results. The presence of Fast Blue labelled neurons on both sides of the midline indicates

reinnervation of the denervated hemicerebellum by the contralateral (Fig 6.13A) and ipsilateral olivocerebellar pathways (Fig 6.13B); thus extensive exercise has most likely prevented the degeneration of the ipsilateral pathway and induced it to grow across the cerebellar midline into the denervated hemisphere (Fig 6.18). Previously, studies have identified the presence of the ipsilateral olivocerebellar pathway until P40 (Lopez-Roman and Armengol, 1994), although axonal transport has been found to reduce with age (Bower and Payne, 1987). While this study did not measure the functionality of neurons within the ipsilateral pathway and therefore cannot ensure axonal transport is occurring, previous studies have shown that neuronal growth requires axonal transport (Hirokawa and Takemura, 2004). Thus, if exercise has prevented degeneration of the ipsilateral pathway and induced growth into the denervated hemisphere, then it must also be undergoing active transport.

Therefore, animals in this study have cells of the right inferior olivary complex projecting to the left cerebellar hemisphere, similar to the normal path, albeit by an abnormal route.

6.4.4 Unilateral olivocerebellar pathway transection does not affect vestibulo-spinal reflexes

The function of the reinnervation was determined by analysing the behavioural sequale of BDNF- and vehicle-treated lesioned animals. This study confirms that dynamic postural adjustments are relatively independent of cerebellar control (Prendergast and Shusterman, 1982) and that unilateral transection of the inferior cerebellar peduncle does not appear to disrupt vestibulo-spinal reflexes. Evidence for this comes from the normal postural adjustments without directional bias in the performance of all groups, despite different cerebellar connectivity compared with the sham-operated animals. Therefore, the results of other motor tests in this study are indicative of modified cerebellar connectivity, and not altered postural motor reflexes.

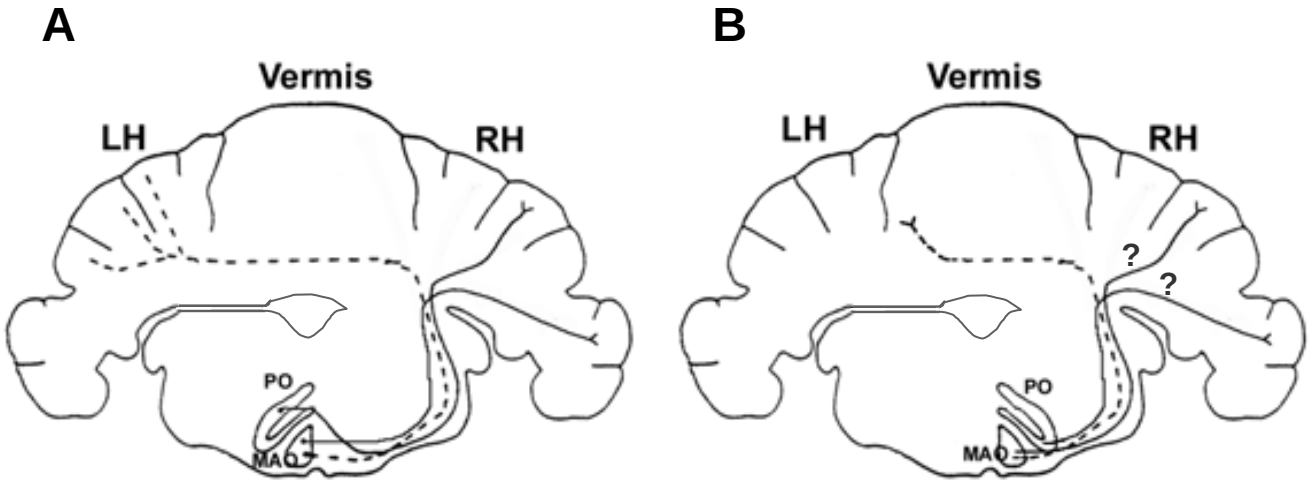

Figure 6.18 Olivocerebellar projection pathways in lesioned cerebellum after extensive exercise

Schemas illustrating the possible olivocerebellar pathways in adult animals subjected to extensive exercise following transection of the left inferior cerebellar peduncle. Only the left **(A)** and right **(B)** inferior olives have been shown for clarity and are out of proportion to the brainstem (modified from Sherrard et al., 1986). Note: for ease of the diagram the majority of the right inferior olivary complex has been drawn, however, the majority of this complex degenerates following transection of the left inferior cerebellar peduncle (Lopez-Roman and Armengol, 1994). Additionally, the solid and dotted lines are representative of single axonal pathways and do not account for the vast topographical arrangement of the olivocerebellar projection.

(A) Illustrates the normal contralateral olivocerebellar pathway (solid lines) and shows that some of these axons have grown across the midline to the reinnervate the previously denervated hemicerebellum (dotted line).

(B) Illustrates the normal supernumerary ipsilateral olivocerebellar pathway (solid lines) that usually regresses during development (it is unclear whether this pathway exists in the adult rats of the current study), although the dotted line indicates that the axons of some of these ipsilaterally projecting neurons have grown across the cerebellar midline into the previously denervated region and are maintained in the adult rat.

Key: LH = left hemisphere; MAO = medial accessory olive; PO = principal olive; RH = right hemisphere

6.4.5 Reinnervation in the mature cerebellum improves functional recovery

The cerebellar cortical circuit controls complex motor coordination (Marr, 1969) and alterations in the cortical circuit impair motor coordination and lead to general ataxia (Fernandez et al., 1998). However, data from the current study revealed that climbing fibre reinnervation improves complex motor skills. On the last day of motor testing (P39), when BDNF- and vehicle-treated animals are likely to have climbing fibre reinnervation (reinnervation is suspected at P39 in Px30V animals based on improvement in rotarod performance from P38), both groups outperform lesioned animals without reinnervation from previous studies (Px30B and Px30V complete the wire task in ~ 11 s and ladder task in ~ 5 s, compared with Px11 animals that take ~ 40 s and ~ 10 s to complete the same tasks at the same age: Dixon, 1998; Dixon et al., 2005).

Additionally, a key function of the olivocerebellar path is synchronisation of gait and learning thereof (Rondi-Reig et al., 1997). This can be tested on the rotarod, as animals synchronise their walking to the rotational speed of the rod to prevent from falling (Auvray et al., 1989; Jones et al., 1995; Ribar et al., 2000; Rondi-Reig et al., 1997). Untrained rats are first able to walk on the rotarod for 180 s at ~ P30 (Caston et al., 1995; Zion et al., 1990), which corresponds to the age at which the cerebellum matures anatomically (Altman, 1982; Armengol and Sotelo, 1991; McKay and Turner, 2005). Therefore, animals lesioned at P30 have already acquired the motor skills necessary to synchronise their gait prior to the lesion and reinnervating terminals are only required to control previously learnt skills. Indeed, data from the current study reveal that reinnervation in the mature cerebellum (Px30), independent of induction method (BDNF or exercise), allows gait synchronisation, similar to sham-operated control animals, on the rotarod at low and moderate speeds, consistent with dense reinnervation to the vermis in previous studies, mediating trunk and limb control (Thach et al., 1992). Furthermore, the inability of Px30 vehicle-treated animals to walk on the rotarod for the

first 3 days of testing (when the vehicle animals do not have any reinnervation) is similar to that observed following complete climbing fibre removal with 3-acetylpyridine in adult rats (dotted horizontal lines on Fig 6.12: Rondi-Reig et al., 1997), and is significantly worse than the Px30 BDNF group (reinnervation is suspected in the Px30B group at P35 since the previous chapter indicates that BDNF can induce reinnervation within 5 days). Therefore, at the start of testing the relative motor deficit between these Px30 groups (BDNF and vehicle) illustrates the functional benefits of climbing fibre reinnervation in the mature system.

Therefore, reinnervation onto mature Purkinje cells improves functional recovery, almost similar to sham-operated control animals and is despite these animals having both contralateral and ipsilateral transcommissural pathways. Although, the following caveat should be noted. Rats develop a smooth regular gait pattern by P15 (Westerga and Gramsbergen, 1990) and master complex motor skills and gait synchronisation by approximately P30 (Zion et al., 1990), which correspond to the ages at which the climbing fibre-Purkinje cell synapses and the cerebellum mature, respectively (Altman, 1982; Crepel et al., 1976; Mariani and Changeux, 1981b). Therefore, despite no pre-lesion training, animals lesioned at P30 have already acquired the skills necessary to synchronise their gait and perform motor skills. Thus, reinnervation of the mature cerebellum is not involved in learning new motor skills; it is only involved in controlling previously learnt skills. Despite this, transcommissural reinnervation in the mature cerebellum appears to be associated with improving motor performance, since both groups (BDNF and vehicle) not only improved their total walking time on the rotarod, but also reduced their relative impairment when compared to the sham-operated control animals (Px30B: 59 % to 10 %; Px30V: 97 % to 16 %). However, when performing difficult tasks (i.e. the highest speed on the rotarod) the functional performance of these animals is not perfect (fall off the rotarod sooner than the sham-operated controls), because although they have a normal gait pattern, these animals develop a side preference ipsilateral to the lesion (as observed in the cliff avoidance task), which is similar following hemicerebellectomy (Molinari et al., 1990), and prevents complete functional recovery .

6.4.6 Exercise-induced reinnervation in the mature-lesioned, but not adolescent-lesioned cerebellum, has delayed functional improvement

The behavioural sequaele of the Px30 vehicle-treated animals, but not Px15 or Px20 vehicle-treated animals shows a delay in improvement over the first 7 days of testing on the rotarod at 10 rpm. While this eventual improvement may result from generalised adaptations involving other motor areas of the CNS, it is more likely that this improvement is due to transcommissural climbing fibre reinnervation. Following extensive exercise, BDNF upregulation within the cerebellum, occurs within 2 to 4 days of the commencement of exercise (Neeper et al., 1996; Widenfalk et al., 1999). Additionally, in neonatal animals' transcommissural climbing fibre terminals take 4 days to grow into the dernervated paravermis and another 2 days to form typical perisomatic plexuses on Purkinje cell somata (Zagrebelsky et al., 1997). Likewise, data from chapter 5 showed that BDNF-induced growth in the mature cerebellum is present by eight days in the lateral hemicerebellum. Thus, the time course of exercise-induced BDNF upregulation and the known time course of transcommissural axonal growth during development and in adulthood correspond with the improvement in functional recovery.

However, the Px15 and Px20 vehicle-treated animals were able to synchronise their gait reasonably well (~ 90 to 139 s) on the first day of rotarod testing and there was no delayed improvement similar to the Px30 Vehicle group. Therefore the question arises, does this suggest that exercise-induced reinnervation in the adolescent-lesioned cerebellum is not involved in gait synchronisation? Perhaps it does, especially since complete climbing fibre removal by 3-acetylpyridine treatment at P15 does not severely impair gait synchronisation at 20 rpm (dotted horizontal line on Fig 6.10: Jones et al., 1995). However, this is unlikely since these animals can walk on the rotarod for longer than Px11 animals without reinnervation (Dixon et al., 2005) (see below). It is currently unclear, however, as to why these animals perform the same as, if not better than, the BDNF-treated animals and future experiments should analyse the cerebellar anatomy of these animals at the end of each day of behavioural testing.

6.4.7 Exercise-induced reinnervation outperforms BDNF-induced reinnervation in the adolescent cerebellum

In the adolescent cerebellum, animals with exercise-induced reinnervation have greater post-lesion return of function than animals with the combination of exogenous BDNF and exercise. Although not statistically significant, on the rotarod the trend for the Px15V group is to synchronise their gait more effectively than the Px15B group, such that their mean total walking time is almost the same as that of the sham-operated control group (Px15V: 179.2 ± 0.7 s; sham-operated: 178.7 ± 0.8 s: mean ± SEM: $p>0.05$). This is in contrast to the total walking time of the Px15B animals (136.7 ± 27 s), which cannot walk on the rotarod for longer than animals' with unilateral or bilateral climbing fibre removal during adolescence (climbing fibre removal at P15 ≈ 128 s; unilateral climbing fibre removal at P11 = 135.9 ± 20 s: Dixon et al., 2005; Jones et al., 1995). The functional benefit of exercise-induced reinnervation is also evident with coordination tasks, for example, the Px15V animals are able to climb the ladder faster than their age-matched BDNF counterparts, and the Px20V group is as fast as the sham controls, while the Px20B group is not as fast as the sham controls. Thus exercise-induced reinnervation in the adolescent cerebellum provides improved gait synchronisation and co-ordination, which are climbing fibre and cerebellar functions.

Exercise-induced reinnervation in the adolescent cerebellum is therefore associated with improved learning of gait synchronisation and complex motor coordination, over the BDNF-treated animals. This is consistent with the anatomical organisation of the reinnervation. While, the area of reinnervation in vehicle-treated animals is not greater than in BDNF-treated animals, the vehicle-treated animals appear to have a more even distribution of reinnervating terminals, compared with the BDNF-treated animals. Therefore an even distribution of terminals, as opposed to a greater area of reinnervation, is clearly important when recreating olivocerebellar circuit re-formation. Indeed, this is consistent with previous studies showing that groups of Purkinje cells fire synchronously in response to olivary input and these Purkinje cells converge on specific motor zones within the DCN, thereby contributing to the firing of motor-neuronal pools responsible for executing a movement (McCormik, 1995; Welsh et al., 1995). It is

possible that the uneven distribution of reinnervation within the BDNF-treated groups may disrupt this rhythmical grouping of Purkinje cells, altering the firing of specific motor-neuronal pools, thus ultimately impairing motor coordination and gait synchronisation.

6.4.8 Earlier reinnervation in the adolescent cerebellum improves gait

As well as exercised-induced reinnervation providing greater functional improvement than BDNF-induced reinnervation, there is also an age effect, particularly evident in the vehicle treated animals.

The trend from this study reveal that climbing fibre reinnervation in the adolescent cerebellum induced after lesions at P15 enable animals to synchronise their gait more effectively than lesions at P20. At the lowest speed on the rotarod (10 rpm) the Px15B animals did not walk for significantly less then the sham-operated controls, while the Px20B animals fall off the rotarod sooner (this is also the trend for the vehicle groups, although since the Px15V group reach the UTL the actual comparison between Px15V and Px20V may not detect a real difference). Additionally, the Px20B and Px20V groups walk on the rotarod for less time than animals with unilateral climbing fibre removal at P11 or animals with complete climbing fibre removal at P15 (Dixon et al., 2005; Jones et al., 1995). Similarities are seen in other studies on the cerebellum, where climbing fibre removal with 3-acetylpyridine, hemicerebellectomy or cerebellectomy at earlier ages leads to a greater ability to synchronise gait (Auvray et al., 1989; Caston et al., 1995; Jones et al., 1995; Molinari et al., 1990; Petrosini et al., 1990; Rondi-Reig et al., 1997; Zion et al., 1990).

This age effect is also evident in the bridge test as the Px20B animals appear slower to complete the task than the Px15B animals. Although all groups used the same strategy - walking - to get across the bridge, the larger stride width of the Px20B group (Px20B: 4.3 ± 0.3 cm), which was larger than the 3 cm wide bridge, adversely affected their performance i.e. they were slower. However, since the majority of Px20B animals succeeded in walking across the bridge this contrasts with the severe locomotory deficits

following vermal ablation (Joyal et al., 1996) and following unilateral climbing fibre removal at P11 (only about 20 % can walk across the bridge: Dixon et al., 2005), indicating the importance of vermal circuits for this task. This reinforces the efficacy of the vermal/paravermal reinnervation by transcommissural olivocerebellar axons at P15 and P20.

Therefore, prior to cerebellar maturity the age at which reinnervation occurs is crucial for improved performance and these motor patterns can be explained by the maturity of target cells that the transcommissural fibres are reinnervating. Purkinje cells become physiologically mature at P18 (McKay and Turner, 2005), thus deafferentation prior to P18 (Px15) leads to reinnervation of physiologically immature Purkinje cells, while deafferentation at P20 leads to reinnervation of mature Purkinje cells. Thus, the reduced physiological maturity of the target cells is associated with a decreased ability to learn gait synchronisation. While deafferentation at P30 also leads to reinnervation of physiologically mature Purkinje cells, animals lesioned at P30 have learnt gait synchronisation prior to the injury (Zion et al., 1990), thus reinnervating climbing fibres are only required to control temporal movements, not learn them. Therefore, animals lesioned at P20, which have not yet learnt gait synchronisation (Zion et al., 1990), are developmentally disadvantaged, because the reinnervating climbing fibres on mature physiologically Purkinje cells are required to both learn and control temporal tasks.

6.5 CONCLUSION

The data from the current study show that climbing fibre reinnervation of mature Purkinje cells, independent of method of induction, results in improved functional recovery of gait synchronisation and complex motor tasks, functions of the climbing fibre pathway and cerebellum, respectively. The delay in improved gait synchronisation skills in the Px30 vehicle-treated animals corresponds with the known time course of BDNF upregulation in the cerebellum and climbing fibre reinnervation during the neonatal period, thus the function can be associated with the anatomy. Additionally, in the adolescent cerebellum, the physiological maturity of the target cell and the density of reinnervation appear to be critical factors in improving functional recovery. While

reinnervation can occur with normal morphology on physiologically mature target cells, if the lesion takes place before the skill has been learnt, then the functional recovery is impaired. As these anatomically appropriate paths can be reproduced in other regions of the central nervous system (Ballermann and Fouad, 2006; Naus et al., 1984; Spear, 1995), this study has significance for the development of potential therapeutic strategies after traumatic injury involving axonal damage.

CHAPTER 7

GENERAL DISCUSSION

7.1 TRAUMATIC BRAIN INJURY: WHERE DO WE CURRENTLY STAND?

The adult mammalian brain has limited recovery from injury due to a combination of intrinsic neuronal properties (Spencer and Filbin, 2004) and inhibitory molecules in the extracellular microenvironment (Rhodes et al., 2003).

Following traumatic injury to the adult rodent there is limited growth of injured axons into the denervated region, even when growth factors are added or inhibitory factors are reduced in the lesion area (Hiebert et al., 2002; Schnell et al., 1994). This is thought to be due to the formation of a prominent glial scar in the lesioned area (reviewed in Stichel and Muller, 1998). Accordingly, no studies show an improvement in behavioural recovery that can be attributed to growth of the injured axons (Ruitenberg et al., 2005). In an attempt to improve regeneration of injured axons some studies have applied growth factors to the cell body, which reverses atrophy and upregulates growth promoting genes such as GAP-43 (Kobayashi et al., 1997). Unfortunately, however, studies using cell body applied growth factors have not shown the growth of injured axons into the denervated region, only into peripheral nerve grafts (Kobayashi et al., 1997; Kwon et al., 2002). Therefore, it is still unclear whether these methods can improve recovery.

Alternatively, in adult rats with partial lesions there is spontaneous collateral sprouting from axon terminals nearby (ipsilateral to) the lesion, which grow into the denervated area (Hagg et al., 2005; Liu et al., 1999; Rossi et al., 1991b; Ruitenberg et al., 2003). However, this sprouting does not involve long distance axonal elongation and in many cases is aberrant and undirected (Ruitenberg et al., 2003). Furthermore, there have been no studies as yet to analyse the physiological and topographical organisation achieved by this sprouting. It is thought that sprouting is due to local upregulation of diffusible factors such as neurotrophins, despite low levels of growth promoting factors in the mature system (reviewed in Hagg, 2006) and occurs in regions where myelination is

only moderate or weak, thus, low amounts of myelin-associated neurite growth inhibitors are present in the target region (Kapfhammer, 1997). An important question is whether such collateral terminal sprouting has any useful functional consequences. Unfortunately, in some areas of the CNS, aberrant sprouting can give rise to maladaptive behaviour (reviewed in Gramsbergen, 1993), and while there is some evidence that spontaneous collateral sprouting can improve functional recovery (Liu et al., 1999; Ruitenberg et al., 2003), this may be attributed to plasticity of other neurons (Commissiong et al., 1991).

In contrast, after complete unilateral lesions in the neonatal rodent, there is growth of uninjured axons from the contralateral uninjured side into the denervated region (Angaut et al., 1985; Ballermann and Fouad, 2006; Barth and Stanfield, 1990; Finlay et al., 1979; Naus et al., 1986; Sherrard et al., 1986; Spear, 1995; Zagrebelsky et al., 1997). These reinnervating fibres grow over long distances, develop similar physiology, topography, branching patterns and arborisations to the normal path (Kuang and Kalil, 1990; Sugihara et al., 2003) and improve the animals' functional recovery (Ballermann and Fouad, 2006; Barth and Stanfield, 1990; Dixon et al., 2005; Weber and Stelzner, 1977). Thus improvement in function can be correlated with the physiology, topography and organisation of the new paths (Dixon et al., 2005; Sugihara et al., 2003; Zagrebelsky et al., 1997).

Until now it was unclear as to why this type of repair occurs in neonatal animals but not in adults, especially since, as mentioned above, axonal growth from salvaged pathways following partial lesions can occur in adult rats. One theory is that growth factors are involved in this reinnervation during development, and the upregulation of diffusible growth factors in adulthood is not sufficient enough to reach the contralateral spared side (Stichel and Muller, 1998). Specifically, data from this thesis (chapter 4) revealed for the first time that this type of repair during development is dependent on BDNF. Neutralisation of this peptide in the neonatal system using blocking antibodies inhibited growth into the treatment area. However, reinnervation during development occurs when there is limited myelination and inhibitory factors within the cerebellum

(Reynolds and Wilkin, 1991), thus it was unclear whether adding BDNF to the mature system would also induce post-lesion axonal growth into a denervated region.

Indeed, data from this thesis (chapter 5) revealed for the first time that the addition of BDNF to the mature system induces axonal growth from the contralateral uninjured side into the denervated side, with appropriate terminal morphology and organisation. Thus, this suggests that growth factor application recreates (to a certain degree) an immature cellular milieu, despite increased levels of inhibitory factors in the mature system. Additionally, reinnervation occurred beyond the area of growth factor injection (even in different lobules) suggesting that growth factor application altered the intrinsic capacity of the neurons into a "growth-ready" state. The BDNF may have increased intracellular cyclical nucleotide levels and protein kinase activation, thereby affecting microtubule assembly and cytoskeletal protein expression, contributing to appropriate axonogenesis and synaptogenesis (Spencer and Filbin, 2004). This in turn may have affected olivocerebellar pathway collaterals that were not directly acted upon by the exogenous BDNF, giving reinnervation into distant uninjected lobules. Importantly, this thesis also revealed (chapter 6) that reinnervation onto previously denervated target cells is associated with improved gait synchronisation and motor skills, specific to the olivocerebellar path and cerebellum (Ito, 1993; Rondi-Reig et al., 1997). Thus, using BDNF to recreate neuronal circuitry with appropriate post-lesion topography and morphology leads to improved functional recovery.

Furthermore, data from this thesis also revealed (chapter 6) that post-lesion exercise induces transcommissural reinnervation of the denervated area. Previous studies have identified that exercise upregulates BDNF and trkB expression within the CNS (Widenfalk et al., 1999), including the cerebellum (Klintsova et al., 2004) and results in a redistribution of BDNF protein within neurons (from the cell body into the dendrites: Macias et al., 2005). It is therefore proposed that this widespread upregulation of BDNF within the cerebellum also recreated an immature cellular milieu (i.e. growth associated gene upregulation), allowing reinnervation onto previously denervated Purkinje cells, especially since exercise is also known to upregulate synaptic-plasticity-related proteins

(Ding et al., 2006). Therefore, these data provide the first evidence (albeit indirect) that the mature system is capable of upregulating sufficient levels of a growth factor to induce growth from uninjured areas, across the CNS midline into the denervated region. Additionally, this reinnervation was organised with appropriate topography and terminal morphology, and resulted in improved functional recovery (albeit delayed). This improvement had limitations though, which was dependent on the developmental state of the cerebellum and whether exogenous BDNF had been applied or not: (1) in adolescent animals, tasks that were not learnt prior to the injury were more difficult to perform post-lesion and (2) all animals with BDNF-treatment performed more poorly than vehicle-treated animals, which may reflect an uneven distribution of reinnervating terminals following BDNF-treatment.

A possible reason why the functional recovery is not perfect following this type of brain repair may be because one side of the brain is now controlling both sides of the body. Improved performance may occur if the supernumerary ipsilateral pathways, which degenerate during development, can be maintained into adulthood and induced to grow across the midline into the denervated region. That way the side of the brain contralateral to the lesion would still be controlling the correct side of the body. Although neurotrophin application to the cell body reverses lesion-induced atrophy in the red nucleus (Ruitenberg et al., 2004), BDNF applied to the cell body of olivary neurons is unable to prevent the natural cell death of ipsilaterally projecting olivary neurons (chapter 2), despite an increased level of available target neurons. While trkB expression after BDNF treatment was not examined in the current study, at the age when this experiment was performed trkB expression within the inferior olivary complex is almost at its peak (Riva-Depaty et al., 1998). Thus, olivary BDNF does not salvage the ipsilateral olivocerebellar pathway from natural regression. Future work could confirm trkB expression profiles for all treatment regimes.

In contrast however, exercise, which upregulates BDNF and trkB expression within the cerebellum (Klintsova et al., 2004) was able to maintain the persistence of the supernumerary ipsilateral pathway and induce it to grow across the midline into the

denervated side (chapter 6). Growth of such an ipsilateral pathway into the denervated region has never before been shown. While axonal growth indicates axonal transport is occurring (Hirokawa and Takemura, 2004), when this pathway is most likely growing into the denervated region (given that the normal contralateral pathway takes 4 days to grow into the denervated paravermis: Zagrebelsky et al., 1997), there is no improvement in the animals' gait synchronisation skills at the time. Unfortunately, it is currently unknown how long the ipsilateral pathway takes to grow into the denervated hemisphere, therefore it cannot be concluded that this pathway does not improve the animals' functional recovery. Additionally, since the animals in the current study have both an ipsilateral and contralateral projection into the denervated hemisphere it is impossible to determine whether development of the ipsilateral pathway on its own could improve the animals' functional recovery, similar to that of sham-operated animals.

7.2 TRAUMATIC BRAIN INJURY: WHERE TO FROM HERE?

While this study has made notable inroads into the involvement of BDNF in the development of an alternate olivocerebellar pathway, there are additional areas that deserve attention in order to more clearly understand what has been learnt.

The data from the current study show that climbing fibre reinnervation of mature Purkinje cells, independent of method of induction, results in improved functional recovery of gait synchronisation and complex motor tasks, functions of the climbing fibre pathway and cerebellum, respectively. Unfortunately, the improved function is not perfect and thus there should be 2 new areas to investigate: Firstly, the area of climbing fibre reinnervation found in the current study is not identical to the normal path; there is a gradient of terminal density as the hemivermis is densely reinnervated while the lateral hemisphere is sparsely reinnervated. While extensive exercise can increase the terminal density more laterally, this reinnervation is still less dense than the normal circuitry; therefore there is a need to qualitatively measure and establish what causes this lack of terminal density compared with the normal cerebellum. Since exercise causes widespread BDNF upregulation within the cerebellum (Klintsova et al., 2004), one

future direction could be to investigate the extent of reinnervation in the cerebellum following widespread BDNF upregulation using viral vectors. For example, the BDNF DNA sequence could be inserted into an Adeno-associated viral vector and administered to the mature cerebellum following unilateral olivocerebellar transection. Secondly, after closure of the critical period, the normality of synaptic connections formed between the reinnervating climbing fibre terminals and Purkinje cells has not yet been analysed. Preliminary data indicates that BDNF-induced climbing fibre reinnervation at P11 (just past the closure of the critical period) has more depressed synapses and an increased paired-pulse depression in the vermis compared with normal climbing fibre-Purkinje cell synapses (Willson, personal communication). This abnormality leads to reduced cerebellar outflow and thus animals have less ability to learn and control motor skills. Therefore, reinnervating synaptic connections formed within the mature cerebellum may not be the same as control synapses, thus further investigation is required.

Additionally, the circuitry formed by mature neurons reinnervating the denervated cerebellar hemisphere is not clearly understood: neurons from both the ipsilateral and contralateral olive appear to reinnervate the denervated Purkinje cells. This abnormal circuitry may contribute to an inability of these animals to obtain full motor recovery. Identification of factors that prevent the degeneration of the transient ipsilateral pathway in the normal cerebellum without inducing growth from the contralateral pathway may result in correct post-lesion circuitry with improved return of lost function. Unfortunately, while BDNF is known to prevent neuronal death within many areas of the central nervous system (Friedman et al., 1995; Gillespie et al., 2003; Lu et al., 2001; Tuszynski et al., 1996), data form the current study show that BDNF applied to the cell body of olivary neurons does not salvage neurons of the ipsilateral pathway. Although olivary neurons express trkB and p75 (Merlio et al., 1992; Riva-Depaty et al., 1998; Rocamora et al., 1993), it is unknown if the subset of olivary neurons that project their axons ipsilaterally also express these receptors. Furthermore, olivary neurons also express growth associated proteins such as GAP-43 (Buffo et al., 2003), which are thought to enable their remarkable post-lesion remodelling capabilities. Therefore in the

future, these ipsilaterally projecting neurons need to be retrogradely labelled and characterised, e.g. trkB and GAP-43 protein and mRNA expression measured through immunohistochemistry, Western blots and *in situ* hybridisation.

Lastly, NT-3 has also been shown to induce olivocerebellar reinnervation beyond the end of the critical period at P12 (Sherrard and Bower, 2001). As stated in the introduction, one reason the current study chose to investigate the effects of BDNF is because BDNF-induced reinnervation at P12 is more efficacious than NT-3-induced reinnervation (as indicated by the number of retrogradely-labelled olivary neurons). However, it is possible that NT-3 may induce topographically, morphologically and physiologically correct reinnervation in the mature cerebellum also providing functional recovery. Additionally, NT-4 also activates trkB (Klein et al., 1989), therefore, future work should not be limited to BDNF-treatment, but ideally should investigate the potential effects of NT-3 and NT-4 in the adult CNS.

7.3 CONCLUDING REMARKS

As stated in the introduction, reducing the long-term disabilities associated with traumatic brain injury will ease an individual's suffering and reduce the long-term costs to society. Although clinical trials are currently underway in promoting post-lesion axonal regeneration and sprouting in the mature human CNS (Mackay-Sim, 2005), there is currently little evidence to suggest that this type of repair will improve functional recovery. In contrast, data from this thesis has provided information on an alternative repair mechanism of the CNS that can restore appropriate post-lesion topography and terminal morphology and improves functional recovery. Possibly in the future post-lesion traumatic CNS injury management can include induction of these axonal pathways to reinnervate regions of denervation to improve post-lesion disabilities.

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APPENDIX I

ANAESTHETIC

KETAMINE/XYLAZINE/ACEPROMAZINE

For animals that weigh more than 100g

Per Kg bodyweight:

50 mg Ketamine (dissociative anaesthetic)

10 mg Xylazine (analgesic/sedative)

0.75 mg Acepromazine (analgesic/sedative)

For animals that weigh less than 100g

Per Kg bodyweight:

35 mg Ketamine (dissociative anaesthetic)

7 mg Xylazine (analgesic/sedative)

0.05 mg Acepromazine (analgesic/sedative)

Inject ketamine/xylazine/acepromazine (i.p.) using a 1ml syringe with a 25 gauge needle. Wait until the animal no longer has eye-blink response and/or foot-pinch withdrawal.

LIGNOCAINE 2 % (w/v)

Apply local anaesthetic directly onto the skin and muscle approximately 5 seconds before cutting.

DIETHYL ETHER

Approximately 20 ml diethyl ether in a 150 mm^3 jar (inhaled).

SODIUM PENTOBARBITONE (LETHOBARB 150 mg/ml)

For overdose: inject 30 mg (0.2 ml, i.p.) sodium pentobarbitone per adult rat using a 1ml syringe with a 25 gauge needle. Wait until the animal no longer has eye-blink response and/or foot-pinch withdrawal.

SOLUTIONS FOR CEREBELLAR AND OLIVE INJECTIONS

ANTI-BDNF ANTIBODY (CEREBELLAR INJECTIONS)

500 µg/ml

20 µl Stock anti-BDNF IgY (500 µg/1000 µl)

100 µg/ml

10 µl Stock anti-BDNF IgY (500 µg/1000 µl)

40 µl PBS/BSA (0.1 %)

10 µg/ml

2 µl anti-BDNF IgY (100 µg/ml)

18 µl PBS/BSA (0.1 %)

2 µg/ml

2 µl anti-BDNF IgY (10 µg/ml)

198 µl PBS/BSA (0.1 %)

Mix with vortex

K252a 50 nM (CEREBELLAR INJECTIONS)

First dissolve K252a in DMSO (0.1 %)

50 µg K252a

1000 µl dimethylsulphoxide

Mix with pipettor in 1000 µl eppendorf tube until dissolved

Then dilute to 0.05 µg/ml

10 µl K252a in DMSO

10 000 µl PBS

Mix with pipettor until dissolved

BDNF/CYTOCHROME C 4 µmol/L (CEREBELLAR INJECTIONS)

For Px15 Cerebellum

10 µl BDNF (1 mg/ml) or cytochrome C (1 mg/ml)

10 µl Saline (0.9 %)

For Px20 Cerebellum

15 µl BDNF (1 mg/ml) or cytochrome C (1 mg/ml)

5 µl Saline (0.9 %)

For Px30 Cerebellum

20 µl BDNF (1 mg/ml) or cytochrome C (1 mg/ml)

Mix with pipettor in a centrifuge tube until dissolved. Inject 1 µl per animal.

UNINFECTED CHICKEN SERUM (CEREBELLAR INJECTIONS)

This serum was collected by Dr Cheryl Johansen (UWA). It was collected with a syringe and allowed to clot overnight at 4^{o}C. The serum was collected after centrifugation and stored at -20^{o}C. Bradford assay indicates stock chicken serum is 46.51 mg/ml protein.

500 µg/ml

1.1 µl stock chicken serum

100 µl PBS/BSA (1 %)

2 µg/ml

4 µl 500 µg/ml chicken serum

996 µl PBS/BSA (1 %)

Mix with vortex in a centrifuge tube

FAST BLUE DYE (2 %) (CEREBELLAR INJECTIONS)

1 mg Fast blue dye

50 µl Distilled H_2O

Mix with pipettor in a centrifuge tube until dissolved

BDNF/CYTOCHROME C WITH FLUORORUBY (4 %) (OLIVE INJECTIONS)

1 mg Fluororuby dye

25 µl BDNF (1 mg/ml) or cytochrome C (1 mg/ml)

Mix with pipettor in a centrifuge tube until dissolved

TRANSCARDIAC PERFUSION SOLUTIONS

SALINE (0.9 %)

1000 ml Distilled H_2O

9.0 g NaCl

HEPARINISED SALINE

1000 ml Saline (0.9 %)

5.0 ml Heparin (1000 units/ml)

PHOSPHATE BUFFER (0.4 M)

92.0 g Anhydrous disodium phosphate (Na_2HPO_4)

23.72 g Acid sodium phosphate ($NaH_2PO_2.H_2O$)

2000 ml Distilled H_2O

Stir until dissolved. pH to 7.4.

PARAFORMALDEHYDE (4 %)

80 g Paraformaldehyde

1500 ml Distilled H_2O

1 drop 10 M Sodium hydroxide

500 ml 0.4 M Phosphate buffer (pH 7.4)

Add 800 ml water to paraformaldehyde and heat while stirring (no more than 55 ^oC). When paraformaldehyde has dissolved, add remaining water. Add sodium hydroxide to clear the solution. Store in fridge until cool. pH to 7.4. Add phosphate buffer when cool. Note: perfuse animal with paraformaldehyde at 4 ^oC, not hot.

HISTOLOGICAL SOLUTIONS

SUCROSE SOLUTION FOR CUTTING

30 g Sucrose

70 ml Paraformaldehyde (4 %)

Dissolve sucrose in paraformaldehyde

SUBBING SLIDE SOLUTION

10 g Gelatine

1 g Chromium potassium sulphate

2000 ml Distilled H_2O

Dissolve the chromium in a little of the water using a stirring bar. Add the rest of the water. Stir and heat to approximately 40 ^oC (not above 50 ^oC). Slowly add the gelatine while stirring.

METHYLENE BLUE DYE (0.5 %)

500 ml Distilled H_2O

2.5 g Methylene blue dye

Add water to dye and stir overnight on an agitator

PHOSPHATE BUFFER (0.1 M)

11.5 g Anhydrous disodium phosphate (Na_2HPO_4)

2.96 g Acid sodium phosphate ($NaH_2PO_2.H_2O$)

1000 ml Distilled H_2O

Stir until dissolved. pH to 7.4.

PHOSPHATE BUFFERED SALINE (0.1 M)

1000 ml 0.1 M Phosphate buffer

9 g Sodium chloride

Stir until dissolved

TRITON-X-100 IN PBS (0.25 %)

2.5 ml 0.1 M PBS

1000 ml Triton-X-100

Stir until dissolved

GELATIN IN PBS/TRITON (0.2 %)

0.20 g Gelatin

100 ml PBS/triton

Heat and stir until dissolved.

PBS/BOVINE SERUM ALBUMIN (0.1 %)

100 ml 0.1 M PBS

1 g Bovine serum albumin

Stir until dissolved. Store at -20^{o}C

CITIFLUOR (COVERSLIPPING SOLUTION)

90 ml Glycerol

10 ml PBS

0.1 g Sodium azide

2.5 g DABCO (anti-quenching agent)

Dissolve DABCO in glycerol and azide in PBS, then combine. Mix for 1 hour on shaker. Store at 4 ^{o}C in dark bottle.