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The Function of Spontaneous and BDNF-induced Repair of the Rat Olivocerebellar System

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Melina Willson 1st September 2007
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Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Melina Willson
1st September 2007
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# Table of Contents

**Abstract** .................................................................................................................. 1

**Resume** ................................................................................................................... 3

**List of Figures** ......................................................................................................... 5

**List of Tables** .......................................................................................................... 8

**Abbreviations** ......................................................................................................... 10

**Introduction** ............................................................................................................ 13

1.0 Regulators of Neural Development and Function:

- the Neurotrophin Family .................................................................................. 17

1.1 Neurotrophins and their receptors ............................................................... 17

1.2 Receptor activation and signalling pathways

   - 1.2.1 Trk receptor activation ........................................................................... 19
   - 1.2.2 Truncated receptor activation ................................................................ 20
   - 1.2.3 p75 receptor activation .......................................................................... 20

1.3 Neuronal properties regulated by neurotrophins

   - 1.3.1 Neuronal survival .................................................................................. 22
   - 1.3.2 Neuronal differentiation and target innervation ..................................... 25
   - 1.3.3 Circuit formation and maturation ........................................................... 28

1.4 The role of BDNF/TrkB in the structural and functional properties of neuronal circuits

   - 1.4.1 BDNF takes an anterograde route ......................................................... 29
   - 1.4.2 BDNF modifies neuronal excitability and synaptic transmission and plasticity .............................................................. 31
   - 1.4.3 BDNF/TrkB mediates axonal guidance and growth .............................. 33
   - 1.4.4 BDNF/TrkB regulates dendritic growth ................................................. 34
   - 1.4.5 BDNF and synaptic stabilisation ............................................................. 35

2.0 The Rat Cerebellum and its Olivary Afferents ................................................. 37

2.1 Cerebellar cortex ............................................................................................... 38

   - 2.1.1 Purkinje cells ......................................................................................... 39
   - 2.1.2 Granule cells ......................................................................................... 40
   - 2.1.3 Interneurons ........................................................................................... 41

2.2 Cerebellar afferents ............................................................................................ 41

   - 2.2.1 Olivocerebellar system .......................................................................... 41
   - 2.2.2 Mossy fibre relay system ........................................................................ 48

2.3 Cerebellar efferents ............................................................................................ 49

2.4 Functions of the cerebellum ............................................................................ 51

   - 2.4.1 Motor function ....................................................................................... 52
   - 2.4.2 Cognitive function .................................................................................. 55
   - 2.4.3 Spatial function ..................................................................................... 56

2.5 Development of the cerebellar neurons and afferents .................................. 61

   - 2.5.1 Purkinje cells ....................................................................................... 61
   - 2.5.2 Granule cells ......................................................................................... 63
2.5.3 Climbing fibres.................................................................64
2.5.4 Mossy fibres..................................................................67
2.6 Neurotrophins and cerebellar development.........................68
  2.6.1 Purkinje cell development..............................................68
  2.6.2 Granule cell development...............................................70
  2.6.3 Climbing fibre development..........................................71
3.0 Post-lesion Models in the Central Nervous System...............75
  3.1 Olivocerebellar reinnervation..........................................81
  3.2 Neurotrophins and olivocerebellar reinnervation...............84
  3.3 Relevance to neural circuit repair and project aims...............85

RESULTS................................................................................87

1.0 The Functional Compensation provided by Transcommissural
  Olivocerebellar Reinnervation in a Spatial Learning Task........88
  Article 1.Developmental neural plasticity and its cognitive benefits:
  olivocerebellar reinnervation compensates for spatial function
  in the cerebellum.................................................................89

2.0 The Role of BDNF to Increase Transcommissural Olivocerebellar
  Reinnervation and its Associated Behavioural Outcomes...........98
  Article 2.BDNF repairs neural circuits: increasing olivocerebellar
  reinnervation and associated complex skills................................99

3.0 The Synaptic Function of Spontaneous and BDNF-induced
  Transcommissural Olivocerebellar Reinnervation in the
  Adult Cerebellum..................................................................139
  Article 3.Effect of BDNF in olivocerebellar system repair: moderate
  modifications of synaptic function at adult climbing fibre-Purkinje cell
  synapses after lesion.........................................................141

DISCUSSION.........................................................................173

1.0 The Functional Accuracy of Olivocerebellar Repair...............175
2.0 The Role of BDNF as a Stimulator of Growth and Repair in the
  Olivocerebellar System..........................................................181
3.0 Behavioural and Synaptic Function Correlate with BDNF-associated
  Olivocerebellar Reinnervation..............................................184
4.0 Future Experiments..........................................................187

REFERENCES.......................................................................189
ABSTRACT
Research on improving recovery of neural function after adult brain injury has focused on axonal regeneration, i.e. the concept of recapitulating developmental axonal growth in the central nervous system (CNS). Although studies that increase axonal regeneration give rise to some functional improvement, it rarely ameliorates complex neural actions. Some attention has been directed to the type of reinnervation that takes place in the neonatal CNS, in which neurons surviving after injury spontaneously develop new axons to replace the damaged path and re-form its specific pattern with correct afferent-target connections. Such reinnervation is partly recreated in the maturing CNS by injection of growth factors e.g. brain-derived neurotrophic factor (BDNF). The aim of this project was to examine in vivo the structure and function of spontaneous and BDNF-associated alternate reinnervation.

We used the rat olivocerebellar projection to characterise the anatomy, physiology and complex (cognitive) neural function of spontaneous and BDNF-associated post-lesion olivocerebellar reinnervation. In the adult, the olivocerebellar path has a well-defined topography where axons enter the cerebellum via the contralateral inferior cerebellar peduncle and terminate as climbing fibres (CFs) onto Purkinje cells (PCs) to regulate motor and spatial functions. Our model involves unilateral axonal transection of this path (pedunculotomy; Px), either at postnatal day (P) 3 to induce spontaneous reinnervation, or P11 when reinnervation only occurs after injection of BDNF.

First, we examined whether spontaneous olivocerebellar reinnervation compensated complex functions such as spatial learning. As reinnervation is partial in the region which mediates spatial cognition, its capacity to mediate navigation was unknown. We tested rats with (Px3) and without (Px11) reinnervation in simple locomotion and spatial (water maze) tasks. Px3 animals performed the spatial task as well as controls despite learning more erratically while Px11 animals did not learn the task. The amount of reinnervation directly correlated with spatial ability, suggesting that even partial reinnervation was associated with functional benefit in a complex task.

Next, we assessed the effect of increasing olivocerebellar reinnervation by BDNF on its associated functions. BDNF/vehicle-treated animals were tested on simple/complex motor and spatial tasks and the amount and distribution of reinnervation were analysed. BDNF did not affect basic motor skills, however on the rotarod BDNF-treated Px11 animals were similar to normal and Px3 groups. They also exhibited better spatial abilities than vehicle-treated Px11 animals. BDNF treatment increased the amount and distribution of reinnervation in both Px3 and Px11 animals. This suggests that neurotrophin-induced reinnervation facilitated appropriate complex (i.e. cognitive) actions.

Finally, as reinnervating synapses do not always induce appropriate target responses, we examined the synaptic function of BDNF-associated olivocerebellar reinnervation to assess any correlation between CF reinnervation and improved behaviour. CF currents (amplitude, paired-pulse depression [PPD], fatigue) were recorded from adult PCs and the structural CF-PC interactions measured. In synapses forming ~6days post Px3, BDNF was associated with impaired CF-PC interaction (smaller synaptic amplitude, greater PPD smaller CFs). Whereas BDNF-induced reinnervation (i.e.Px11) had increased PPD without affecting anatomical attributes. As PPD aids CF-PC transmission at low in vivo frequencies, these synaptic changes are unlikely to affect cerebellar function. Therefore, spontaneous and BDNF-associated reinnervation form functional synapses with associated functional recovery.
La recherche sur la réparation fonctionnelle après un traumatisme cérébral chez l’adulte s’est construite autour du concept de régénération i.e. la capacité de (re)croissance des axones dans le système nerveux central (SNC). Bien que la régénération entraîne une amélioration fonctionnelle, les fonctions complexes sont rarement améliorées. Peu de travaux sont consacrés aux processus de réinnervation chez le nouveau-né, qui permettent aux neurones immatures de survivre après une lésion et de reformer spontanément de nouvelles connexions. Cette forme de réinnervation est reproduite partiellement chez l’adulte dans le SNC par injection de facteurs trophiques comme le brain-derived neurotrophic factor (BDNF). L’objectif de ma thèse a été d’examiner la structure et la fonction des réinnervations spontanée et induite par le BDNF in vivo.

J’ai utilisé la voie olivocérébelleuse chez le rat comme modèle expérimental et étudié l’anatomie, la physiologie du circuit néo-formé et le comportement des animaux après réinnervation post-lésionnelle spontanée et associée au BDNF. Pour cela, les cellules de Purkinje (CPs) ont été désafférentées en sectionnant l’un des pédoncules cérébelleux (Px) soit à 3 jours postnatal (P3) afin d’induire la réinnervation spontanée des CPs par des fibres grimpantes (FGs), soit à P11, la réinnervation étant induite par le BDNF.

J’ai tout d’abord déterminé si la réinnervation spontanée post-lésionnelle chez l’animal jeune (Px3) pouvait compenser une fonction complexe comme l’apprentissage spatial. Dans ces conditions, la réinnervation est partielle dans les régions latérales du cervelet qui sont impliquées dans cette fonction, et donc potentiellement insuffisante pour un comportement normal. J’ai comparé les performances d’animaux Px3 et Px11 non traités par le BDNF dans des tests de comportement moteur et spatial à l’âge adulte. Les animaux Px3 ont acquis la tâche spatiale aussi bien que les contrôles malgré des patrons d’apprentissage plus erratiques, contrairement aux Px11. L’ampleur de la réinnervation chez les Px3 était corrélée au comportement spatial, suggérant qu’une réinnervation même partielle peut améliorer la fonction.

J’ai ensuite évalué les conséquences fonctionnelles de la réinnervation par le BDNF. Les comportements moteur et spatial ont été comparés à la distribution et à l’importance de la réinnervation. Le BDNF n’a eu aucun effet sur le comportement moteur, mais a amélioré considérablement le comportement moteur complexe et spatial des animaux Px11 traités par le BDNF par rapport aux Px11 non traités. L’administration du BDNF chez les Px11 comme chez les Px3 a augmenté la l’importance et l’étendue de la réinnervation. Ces résultats suggèrent que la réinnervation associée au BDNF a permis la récupération des fonctions complexes.

J’ai enfin réalisé une étude électrophysiologique des synapses néoformées en présence de BDNF par la technique du patch-clamp. Le BDNF a entraîné des modifications de la réponse synaptique (amplitude et PPD) et des arborisations des FGs chez les Px3 alors que chez les Px11 seule la PPD était modifiée. La PPD favorise la transmission synaptique à basse fréquence qui est caractéristique du réseau olivocérébelleux in vivo; il est donc peu probable que les modifications observées altèrent le fonctionnement des réseaux cérébelleux. En conclusion, dans ce modèle de réparation post-lésionnelle, la réinnervation qu’elle soit spontanée ou associée au BDNF permet une récupération des fonctions comportementales.
LIST OF FIGURES
Figure 1. Neurotrophin binding selectively to their specific Trk receptor

Figure 2. Signalling pathways initiated after neurotrophin binding to Trk receptor at the cell membrane

Figure 3. Retrograde neurotrophin-Trk receptor induced signalling involved in neuronal survival

Figure 4. Extracellular factors that mediate local signalling cascades to regulate axon growth

Figure 5. Rapid post-synaptic effects induced by exogenous BDNF

Figure 6. Proposed model of the truncated TrkB-p75 complex involved in dendritic filopodial motility

Figure 7. A. Lateral view of the cerebellum and brain stem B. Phylogenetic division of the mammalian cerebellum

Figure 8. The anatomical organisation of the cerebellar cortex

Figure 9. Schematic diagram of the cerebellar circuit

Figure 10. Diagram of the zonal organisation within the cerebellar system

Figure 11. Intracellular recordings of a Purkinje cell after inferior olive stimulation

Figure 12. Purkinje cell loaded with a fluorescent dye to visualise the location of Ca^{2+} transients after climbing fibre stimulation

Figure 13. Coronal section displaying the CF terminal organisation in the cerebellar cortex and deep cerebellar nuclei

Figure 14. The major efferent pathways of the deep cerebellar nuclei

Figure 15. Diagram of the Morris Water Maze primarily used to assess spatial learning and memory

Figure 16. Major developmental events of the Purkinje cell and molecular layer maturation from P3 to P21

Figure 17. Phases of climbing fibre morphology throughout postnatal development

Figure 18. Dendritic differentiation of Purkinje cells in control and target-deleted BDNF (Wnt1-Cre: fBz/fBz) mice

Figure 19. Neurotrophin and receptor synthesis sites during climbing fibre development
Figure 20. Schematic diagram of the cortico-rubral sprouting which occurs after unilateral pyramidotomy

Figure 21. ECM-integrin and neurotrophin-Trk receptor signalling to mediate axon growth

Figure 22. The three myelin associated proteins which interact with Nogo receptor and p75

Figure 23. Diagram of the normal olivocerebellar pathway and collateral reinnervation which occurs prior to postnatal day 10

Figure 24. Camera lucida drawing and photomicrograph of a climbing fibre arbor of a transcommissural olivocerebellar axon

Figure 25. A micrograph showing the collateral of an adult CF branching from an arbor after a subtotal lesion of the inferior olive with 3-AP
LIST OF TABLES
Table 1. Phenotypes of neurotrophin and Trk deficient mice
Table 2. Summary of neuronal processes influenced by neurotrophins
Table 3. Summary of the major efferents of the deep cerebellar nuclei
Table 4. Lesions used to differentiate the relative role of Purkinje cell afferents in navigation tasks
Table 5. Summary of mRNA expression of the neurotrophins and their receptors in the cerebellar cortex, deep cerebellar nuclei and brainstem
ABBREVIATIONS
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABP</td>
<td>Actin-binding protein</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor</td>
</tr>
<tr>
<td>3-AP</td>
<td>3-acetylpyridine</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaM kinase</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CF</td>
<td>Climbing fibre</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element binding</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAO</td>
<td>Dorsal accessory olive</td>
</tr>
<tr>
<td>DCN</td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic ages</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix molecules</td>
</tr>
<tr>
<td>EGL</td>
<td>External germinal layer</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory post-synaptic current</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth associated protein-43</td>
</tr>
<tr>
<td>GC</td>
<td>Granule cell</td>
</tr>
<tr>
<td>Grb-2</td>
<td>Growth factor receptor bound protein-2</td>
</tr>
<tr>
<td>Hcb</td>
<td>Hemicerebellectomy</td>
</tr>
<tr>
<td>IGL</td>
<td>Internal granule layer</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
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<tr>
<td>MAO</td>
<td>Medial accessory olive</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule-associated proteins</td>
</tr>
<tr>
<td>mEPSC</td>
<td>mini excitatory post-synaptic currents</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotrophic glutamate receptor</td>
</tr>
<tr>
<td>MF</td>
<td>Mossy fibre</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NGR</td>
<td>Nogo receptor</td>
</tr>
<tr>
<td>NMMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>nr</td>
<td>nervous</td>
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<tr>
<td>NT-3→7</td>
<td>Neurotrophin – 3 to 7</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocyte myelin glycoprotein</td>
</tr>
<tr>
<td>p75</td>
<td>Pan-low affinity receptor</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal ages</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cell</td>
</tr>
<tr>
<td>pcd</td>
<td>Purkinje cell degeneration</td>
</tr>
<tr>
<td>PF</td>
<td>Parallel fibre</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidylinositol 3- kinase</td>
</tr>
<tr>
<td>PLC$\gamma$-1</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>----------------------------------</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>PO</td>
<td>Principal olive</td>
</tr>
<tr>
<td>PPD</td>
<td>Paired-pulse depression</td>
</tr>
<tr>
<td>Px</td>
<td>Pedunculotomy</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cell</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VOCC</td>
<td>Voltage-gated calcium channel</td>
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INTRODUCTION
Throughout the course of this project, I have examined the function of a neural projection pathway which has the capacity to reinnervate correct target neurons following a lesion to the central nervous system. This form of reinnervation, which involves the ability of one group of neurons to take over the role of those neurons that have been injured or died, utilises collaterals from surviving axons and occurs widely in the developing central nervous system. This phenomenon promoted in the adult central nervous system may be viewed as a potential starting point for repair strategies after a lesion.

Thousands of brain injury cases requiring hospitalisation are diagnosed per year, and thirty percent of these cases require some form of long-term care. The behavioural consequences of brain injury are impairments of both motor and cognitive functions. In order for brain repair to be possible, there must be re-formation of axon-target connections that are functionally beneficial. After injury adult axons give rise to some short-lived regenerative sprouting or longer-lasting sprouting from terminal branches of remaining axons (Caroni and Schwab 1988; Rossi et al., 1995a; Schwab and Bartholdi, 1996; Rossignol et al., 1999; Weidner et al., 2001; Schwab, 2002; Kerschensteiner et al., 2005). Although these recuperation responses may facilitate basic motor functions (Bareyre et al., 2004), they do not compensate for lost skilled movements (Fernandez et al., 1998; Smith et al., 2007) or cognitive function. In contrast, developing axons have a relatively high capacity for re-growth and collateral sprouting (in some cases, through the white matter) which is associated with functional recovery (Leong, 1977; Angaut et al., 1982; Bernstein-Goral and Bregman, 1993; Naus et al., 1984; Spear et al., 1995; Dixon et al., 2005). However, the functional outcome of increased regrowth/sprouting either in the neonate or adult central nervous system has not examined skilled movements or cognitive function.

Developmental studies have shown that the switch from axonal growth and sprouting in the developing central nervous system to the subsequent inhibition in the mature system depends on the local environment encountered by the axons and intrinsic neuronal properties. Important environmental factors include the maturation of oligodendrocytes and myelin, the inhibitory chondroitin sulphate proteoglycans, the diminishing synthesis of growth promoting factors such as the neurotrophins, and the unavailability of molecules that act as guide posts for growth cones. Blocking myelin-associated inhibitory proteins has resulted in increased axonal sprouting following spinal cord
lesions. However, in some cases, these axons synapse onto incorrect target neurons (Z’Graggen et al., 1998; Blochlinger et al., 2001). Application of neurotrophic factors also enhance neurite outgrowth and sometimes long-distance regeneration of lesioned fibres (Horner and Gage, 2000; Lacroix and Tuszynski, 2000). Significantly, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) modulate myelin proteins and increase growth in mature axons (Cai et al., 1999; Cai et al., 2001; Lu et al., 2004).

We have integrated the use of exogenous neurotrophins and the innate capacity of collateral reinnervation in the immature central nervous system and undertaken a detailed characterisation of its anatomical, electrophysiological and behavioural function. This has allowed us to understand how well the developing and later maturing systems can remodel their connectivity to replace lost neuronal circuitry and effectively compensate skilled and/or cognitive neural functions.

We used the rat olivocerebellar pathway to examine the structure and function of reinnervation following a lesion: both spontaneous neonatal collateral reinnervation and that which is pharmacologically-induced later during development. To observe its function, an in vivo model, which can be studied from the synapse to the whole system level, is necessary. The olivocerebellar pathway is ideal because: (i) its normal development, anatomy and synaptic function are well-established (Azizi and Woodward, 1987; Chedotal and Sotelo, 1993; Lohof et al., 1996); (ii) its importance to cerebellar function is illustrated by deficiencies in equilibrium and motor coordination after ablation of the olivocerebellar pathway (Rondi-Reig et al., 1997); (iii) its ablation also impairs spatial learning abilities (Dahhaoui et al., 1992b; Rondi-Reig et al., 2002), implicating the olivocerebellar pathway in this cognitive function; (iv) after a lesion during early development (i.e. prior to postnatal day 10) remaining axons sprout compensatory fibres which develop synapses onto the correct target neurons, the Purkinje cells (PCs), in the deafferented hemicerebellum and they provide motor improvement (Angaut et al., 1985; Sherrard et al., 1986; Dixon et al., 2005); and (v) BDNF appears to be involved in olivocerebellar development (Das et al., 2001) and can induce axonal ingrowth into the deafferented hemicerebellum at ages after the critical period of neonatal reinnervation (Sherrard and Bower, 2001; Dixon and Sherrard, 2006).
The aims of this project were 3 fold:

(1) **to evaluate the extent to which olivocerebellar reinnervation confers normal spatial function.** Previous work has shown that dense olivocerebellar reinnervation is associated with improved motor behaviour while no reinnervation results in cerebellar ataxia. As the distribution of olivocerebellar reinnervation in the lateral region which regulates spatial function is quite sparse, we wanted to further discriminate the extent to which this reinnervation would confer functional compensation in a complex task, i.e. spatial learning.

(2) **to examine whether BDNF treatment increased the amount and distribution of olivocerebellar reinnervation and improved functionally-related behaviours.** We increased the availability of BDNF by a single intracerebellar injection to either increase olivocerebellar reinnervation in partially-reinnervated regions of the neonatal cerebellum or to induce olivocerebellar reinnervation following a lesion after the period of spontaneous reinnervation. We then assessed whether an increase in anatomical reinnervation was associated with olivocerebellar-related behavioural improvements, both motor and spatial function.

(3) **to assess whether the synapses formed during olivocerebellar reinnervation, with or without BDNF, were functionally normal.** As reinnervating fibres synapse onto Purkinje cells later during development than normal, these reinnervating fibres were required to synapse onto a relatively mature and complex dendritic tree and thus normal physiological responses could not be presumed. Furthermore, abnormal synaptic function of reinnervating afferents may modify the target Purkinje cell activity and therefore synaptic integration and outflow of the cerebellar neuronal circuits.

The literature review of my thesis is divided into three parts which includes an overview of one set of factors which regulate neuronal development and function – the **neurotrophin family** – and in particular BDNF; the model system used to analyse collateral reinnervation – the **olivocerebellar system** – its anatomy, physiology and function, and finally a description of transcommissural olivocerebellar reinnervation after lesion. The results obtained are presented in the format of publications. Afterwards, I will integrate the results obtained from the multidisciplinary approach taken during this project, discuss the results in light of other regions of the central nervous system, critique my work and highlight the functional significance of olivocerebellar reinnervation.
1.0 Regulators of Neural Development and Function: the Neurotrophin Family

Nerve growth factor (NGF) was the first neurotrophin discovered and described as a survival factor for sensory and motor neurons (Levi-Montalcini, 1987). The neurotrophin family is now known to regulate almost all aspects of neuronal development and function. The neurotrophins have essential physiological roles in precursor proliferation, neuronal survival, regulation of axon and dendritic growth, membrane trafficking, synapse formation, plasticity and function, as well as glial differentiation and neuronal interactions (McAllister, 1999; Huang and Reichardt, 2001; Chao, 2003; Reichardt 2006). In addition to NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) have also been identified in the mammalian system, whilst neurotrophin-5 (NT-5) is the frog *Xenopus laevis* equivalent of NT-4 and neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) have been identified only in fish (Ip *et al*., 1992; Lai *et al*., 1998).

The neurotrophins are synthesised as pre-pro-neurotrophin precursors (Lessman *et al*., 2003) and then cleaved to yield the mature homodimeric protein (Figure 1). Proteolysis is a key post-transcriptional control point as it has been shown to limit and add specificity to each mature neurotrophin protein (Lee *et al*., 2001). The mature forms of these proteins share a high degree of amino acid sequence homology, except that they differ in the structure of the loop regions (Gotz *et al*., 1992). It has been proposed that NT-3 represents the ancestral-chordate highly conserved sequence from which NGF and BDNF may be derived (Ibanez *et al*., 1991; Ibanez *et al*., 1992; Lewin and Barde, 1996; Hallbook, 1999).

1.1 Neurotrophins and their receptors

The biological function of a neurotrophin is mediated by its binding to cell surface receptors. There are two distinct classes of neurotrophin receptors: the low-affinity pan-neurotrophin receptor (p75) and the high-affinity tropomyosin related kinase (Trk) receptors. Each neurotrophin binds to the p75 receptor with equal affinity but with differing rate constants (Ebadi *et al*., 1997). In contrast, the Trk family of receptors
(TrkA, TrkB and TrkC) are each preferentially activated by one neurotrophin – TrkA by NGF, TrkB by BDNF and NT-4, and TrkC by NT-3 (Figure 1: Lewin and Barde, 1996; Ebadi et al., 1997; Poo, 2001; Reichardt, 2006), although at higher concentrations NT-3 is also able to activate less efficiently TrkA and TrkB (Glass et al., 1991; McAllister, 1999).

Although the interaction between neurotrophin and Trk receptor is thought to be of high affinity, the binding rate of NGF to Trk A and BDNF to Trk B is actually slow (Lindholm et al., 1993b; Mahadeo et al., 1994; Schropel et al., 1995). Regulation of neurotrophin binding to their selected Trk receptor can be modified to higher affinity binding via several processes including: (i) induced dimerisation of Trk receptors and/or (ii) Trk receptors forming a complex with the p75 receptor (Esposito et al., 2001; Hempstead, 2002). Hence, p75 is able to mediate the affinity and selectivity of neurotrophins for the Trk receptors. The p75 receptor does this by either inhibiting the activation of Trk receptors by non-preferred neurotrophins (Beneditti et al., 1993), potentiating the activation of TrkA by suboptimal levels of NGF (Davies et al., 1993) or interacting with TrkA to create high-affinity binding sites for NGF (Esposito et al., 2001).
1.2 Receptor activation and signalling pathways

How do neurotrophins affect processes such as axonal guidance, axon and dendritic growth and synapse formation? Neurotrophic effects are modulated by the location of the neurotrophin-receptor complex (Chao, 2003). Both the Trk and p75 receptors are transported within the cell, either anterogradely to mediate retrograde signalling from the target neuron or to the dendrites to receive afferent-derived neurotrophins (Altar and DiStefano, 1998). It is now clear that after neurotrophin binding, the neurotrophins and activated Trk complex are endocytosed and transported together in vesicles to the cell soma wherein signalling continues (Heerssen and Segal, 2002). The transport of the neurotrophin-receptor complex (upon its arrival at the soma) is responsible for the activation of different intracellular signalling pathways and thus transcription factors, which in turn mediate specific neuronal changes e.g. growth cone behaviour and synaptic function (Patapoutain and Reichardt, 2001; Hempstead, 2002). In addition, local neurotrophin signalling may also activate transcription factors directly at the soma to induce gene expression (Campenot, 1982).

1.2.1 Trk receptor activation

It has been shown that neurotrophin binding to Trk causes the receptors to assemble into dimers, resulting in cross-phosphorylation of each receptor’s multiple tyrosine residues in the cytoplasmic (loop) domain (Jing et al., 1992). The constitution of the receptor’s cytoplasmic domain determines whether phosphorylation will further activate the receptor and produce an autoregulatory loop in order to elevate Trk tyrosine kinase activity, or recruit docking sites for second messengers (Huang and Reichardt, 2001). In the latter case, the src homologues (SHC) adaptor proteins bind to the docking sites, potentially activating three different intracellular signalling pathways: (1) extracellular signal-related kinase (ERKs)/Ras; (2) phosphatidylinositol 3-kinase (PI-3K)/Akt kinases; and (3) phospholipase C (PLCγ-1) (Figure 2). In addition, crossover-activity between these signalling cascades has been shown in NGF-treated PC12 (pheochromocytoma) cells (Ohmichi et al., 1994). In this case, activated PLCγ-1 initiates pathways which produce diacylglycerol (DAG), transiently activates protein kinase C (PKC) and inositol trisphosphate (IP3) which increases Ca^{2+} levels (Kaplan and Stephens, 1994) and subsequently, the phosphorylation of PI-3K increases activity of growth factor receptor-bound protein-2 (Grb-2) and SHC increases the activity of PI-3K, Ras and ERK. The
combined effect of these signals results in the transcription of specific genes through the phosphorylated cyclic AMP (cAMP) response element binding (CREB) transcription factor (Lonze and Ginty, 2002). When CREB is activated by Ras, ERK and PI-3K pathways, it affects neuronal survival and differentiation (Kaplan and Stephens, 1994), whilst cytoskeleton arrangements, growth cone guidance and neurite outgrowth are affected via the PLCγ-1 mediated pathways (Ming et al., 1999; Kaplan and Stephens, 2004). In addition to PLCγ-1, the PI-3K pathway also initiates neurite outgrowth, axon and dendritic growth and synaptic activity (Figure 2; Lonze and Ginty, 2002; Atwal et al., 2000; Jaworski et al., 2005; Zhou and Snider, 2006).

1.2.2 Truncated receptor activation

The truncated Trk receptors do not contain a catalytic tyrosine kinase domain and had been considered to concentrate neurotrophins to specific sites or curtail binding of excess neurotrophins to full-length Trk receptors (Reichardt, 2006). However recent work show that neurotrophins activate truncated receptors to regulate various neuronal and glial processes such as controlling the release of Ca^{2+} from intracellular stores (Rose et al., 2003), activating changes in postsynaptic density (Esteban et al., 2006) and interfering with neuronal differentiation (Guiton et al., 2005).

1.2.3 p75 receptor activation

In contrast to the Trk signalling cascade, p75 not only facilitates neurotrophin-Trk activation but also initiates intracellular signalling cascades independent of Trk. p75 uses a different array of adaptor proteins which in turn activate Jun N-terminal kinases (JNK), NF-κB and RhoA (Chao, 2003). These proteins mediate neuronal death, survival and cytoskeletal changes/neurite outgrowth, respectively (Figure 2; Chao, 2003). Neurotrophins that bind solely to p75 (in conjunction with its co-receptor sortilin) initiate pro-apoptotic actions, whilst neurotrophins that bind both Trk and p75 receptors promote neuronal survival (DeFreitas et al., 2001).
Figure 2: Neurotrophic receptor signaling. The Trk receptors regulate diverse survival pathways through transduction and receptor guidance and growth. The p75 receptor predominantly signals to activate TRK, NT-3, and Ret.

- Prosurvival genes
- Progrowth factors
- Prodifferentiation genes
To further complicate the correlations between neurotrophin-receptor signalling and their specific biological response, other growth factors (excluding neurotrophins) and cytokines also use the same signal transduction pathways. Moreover, factors such as (i) the amount of neurotrophins synthesised and secreted, (ii) the affinity of binding to the receptors, (iii) the duration of activating the downstream molecules (for example, Ras), and (iv) the unique effects produced by each neurotrophin, highlight the complexities between neurotrophins, receptors and their physiological effects.

1.3 Neuronal properties regulated by neurotrophins

As previously mentioned, the neurotrophin family regulates a broad range of neuronal functions. This functional diversity has been suggested to derive from the unique anatomical distributions of each neurotrophin/Trk receptor pair (Conner et al., 1997; Yan et al., 1997a). With the development of the two-site ELISA and in situ hybridization to measure mRNA levels, the sites of most neurotrophin and receptor expression in the peripheral (PNS) and central nervous system (CNS) have been elucidated. However, discrepancies in the distribution of neurotrophins and Trk receptors still exist due to the unpredictability in measured mRNA and corresponding protein levels (i.e independent regulation of mRNA and its protein) and the numerous Trk transcripts encoding both full-length and truncated forms.

In this section, overviews of the role of each neurotrophin and its respective receptor in neuronal development will first be discussed followed by a focussed review on the actions of BDNF and TrkB in the CNS.

1.3.1 Neuronal survival

Deafferentation, genetic ablation of neurotrophins and their receptors, intraperitoneal injections and transgenic overexpression of neurotrophins have all been used to demonstrate that neurotrophic signals are necessary for the survival and development of certain developing PNS and CNS populations. In vitro studies show that target-derived neurotrophins regulate neuronal survival via retrograde signalling (Figure 3). These studies show that neurotrophic factor removal causes the neuron to initiate apoptotic signalling cascades and caspase-mediated cell death (Zweifel et al., 2005). As previously mentioned, it is the PI-3K and ERKs/Ras pathways that mediate retrograde neurotrophin-independent survival.
NGF promotes the survival of neurons predominately in the PNS. NGF is found in neurons of the developing sympathetic and sensory system including the dorsal root ganglion (DRG) and trigeminal ganglion, as well as striatal and cholinergic basal forebrain (Barde, 1989; Chao, 1992; McAllister, 1999). Both NGF and TrkA knock-out mice have greatly reduced populations of DRG and trigeminal neurons by early stages of development, followed by reduced numbers of sympathetic ganglia in the postnatal period (Table 1; Crowley et al., 1994; Smeyne et al., 1994; Snider, 1994).

NT-3 appears to be one of the first neurotrophins expressed embryonically (Maisonpierre et al., 1990; Farinas et al., 1996) and promotes proliferation and survival. The DRG population that will eventually differentiate into proprioceptive neurons express TrkC during the initial stages of neurogenesis. In turn, both NT-3 and TrkC knock-out mice show a complete loss of muscle spindles due to a lack of proprioceptive innervation (Farinas et al., 1996). In addition, a partial loss of trigeminal neurons occurs in the absence of NT-3 or TrkC, while NT-3/BDNF engineered knock-out mutants have major trigeminal neuronal loss and in NT-3/BDNF/NT-4 knock-out mutant mice, these neurons do not survive (Table 1; Fan et al., 2000).
The neuronal losses depicted in Table 1 are expressed as the percentage of neurons lost in the mutant mice compared to wild-type control mice (From Huang and Reichardt, 2001). ND = not done; NS = not significant.

In contrast to NGF and NT-3, BDNF is widely located throughout the nervous system including in neural crest-derived (Sieber-Blum, 1999) and placode-derived sensory neurons (Barde et al., 1982; Davis et al., 1986; Robinson et al., 1996), retinal ganglion cells (Lom and Cohen-Cory, 1999), basal forebrain cholinergic neurons (Alderson et al., 1990), neurons of the hippocampal formation (Yan et al., 1997b), dopaminergic neurons (Knusel et al., 1991), cerebellar Purkinje and granule cells (Segal et al., 1992; Rocamora et al., 1993; Gao et al., 1995; Segal et al., 1995; Lindholm et al., 1997; Das et al., 2001; Dieni and Rees, 2002) and the inferior olive (Yan et al., 1997b). A proportion of these neuronal populations are also responsive to the other preferential ligand of TrkB, NT-4 (Minichiello et al., 1998).

TrkB receptors have been localised in the cerebellum (specifically in Purkinje and granule cells; Ohira and Hayashi, 2003), inferior olive, thalamus, striatum, hippocampus and cerebral cortex (Masana et al., 1993; Chen et al., 1996; Yan et al., 1997a; Dieni and Rees, 2002). As anticipated from their wide distribution, mice lacking BDNF or TrkB receptors display diverse biological and behavioural phenotypes. Both knockouts exhibit death of DRG and trigeminal neurons and some nodose ganglion and cranial motor neurons, and usually die within forty-eight hours (Table 1; Snider, 1994). The few BDNF-/- animals

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Table 1. Phenotypes of neurotrophin and Trk deficient mice

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<th>Determinant</th>
<th>TrkA</th>
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<th>NT-4/5</th>
<th>TrkC</th>
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<td>neurons present</td>
<td>foliation</td>
<td>defect</td>
<td>Increased apoptosis in hippocampal and cerebellar granule cells</td>
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Viability Poor Poor Very Poor Poor Good Moderate Very Poor Very Poor Very Poor Very Poor Very Poor
that survive show behavioural effects including spinning, bobbing heads and ataxic hindlimbs (Jones et al., 1994).

Despite this, most BDNF-responsive central neurons survive in the absence of BDNF (Jones et al., 1994) with the exception of cerebellar granule cells (Schwartz et al., 1997). In the cerebellum with targeted deletions of BDNF, granule cells die, the dendritic growth of Purkinje cells is stunted and defects in foliation patterns are evident (Schwartz et al., 1997; Quartu et al., 2003). Also, TrkB activation is reduced in both of these neuronal types (i.e. granule and Purkinje cells). This implies that in addition to anterograde and retrograde signalling mechanisms induced by BDNF, autocrine and paracrine (Lindholm et al., 1996; Schwartz et al., 1997) activation regulates the survival and morphological phenotypes of developing neurons.

Furthermore, the use of other neurotrophins (such as NGF) or a depolarising agent alone, cannot evoke cell survival or neurite eloration in certain CNS neuronal populations. The promotion of neuronal survival and differentiation in developing Purkinje cells only occurred if the neuron was exposed simultaneously to both NGF and the depolarising agent (Cohen-Cory et al., 1991). This result highlights that in certain populations, neurotrophic factor availability alone is not sufficient for neuronal survival and neural activity is also required.

1.3.2 Neuronal differentiation and target innervation

In parallel with neuronal survival, retrograde signalling by neurotrophins influences steps such as neuronal differentiation and control of correct target innervation (Figure 3). Furthermore, depending on the neuronal type, location and developmental age of each neuronal population, their responsiveness to each neurotrophin is variable. For neuronal differentiation, in vitro and in vivo work demonstrates that NGF induces differentiation of sympathoadrenal precursors into sympathetic neurons rather than adrenal chromaffin cells (Levi-Montalcini, 1987) and also regulates the phenotype of nociceptive neurons (Ritter et al., 1991). In contrast, NT-3 and TrkC expression is required in mouse DRG populations for their initial generation (Ma et al., 1999) and in D-hair afferents in early postnatal development (i.e. fibres responsive to light touch; Huang and Reichardt, 2001). These D-hair afferents switch to NT-4 dependence during later stages of postnatal development while DRG neurons shift to NGF dependence for neuronal outgrowth and
metabolism (Anand et al., 2006). Similar to NT-3/TrkC action, TrkB receptors are also required for the initial generation and differentiation of mouse DRG populations (Ma et al., 1999).

All the neurotrophins promote neurite outgrowth in vitro. NGF regulates neurite outgrowth of sympathetic neuron growth cones as demonstrated in vitro in Campenot’s chamber study (Campenot, 1982). Essentially it was observed that localised NGF signalling is necessary to mediate axon growth and that intracellular events activated at the soma alone are not sufficient. NT-3 has been shown to primarily promote neurite outgrowth in spinal sensory (i.e proprioceptive neurons) and hippocampal neurons (Ip et al., 1993; Farinas et al., 1996). It also appears necessary for the formation of certain sets of sensory endings that detect mechanosensation, with NT-3/TrkC action suppressing Ruffini innervation (slowly adapting mechanoreceptors), to control for appropriate sensory fibre targeting.

The effect of neurotrophins on axon pathfinding (i.e. influencing the direction of the growth cone response) in the PNS and CNS has been recently explored (Gallo and Letourneau, 2004; Tamura et al., 2006; Yao et al., 2006). This has led to the hypothesis that neurotrophins may control target innervation. Interestingly, neurotrophin binding to TrkB and p75 receptors triggers changes in the actin filament dynamics of the growth cone which in turn regulates growth cone behaviour (Figure 4). Several studies have shown that both βactin messenger RNA and its zipcode-binding protein ZBP1 (i.e. βactin mRNA binds to the RNA binding protein ZBP1) aggregate asymmetrically at the growth cone after neurotrophin-Trk signalling (Gallo and Letourneau, 2004; Yao et al., 2006) and also guidance cues (e.g. netrin-1; Leung et al., 2006).

In addition, in vitro experiments show that neurotrophins (i.e BDNF and NGF) act either as chemoattractants or chemorepellants, dictated by the levels of cyclic nucleotide (cAMP) in neurons (Song et al., 1997; Song and Poo, 1999). The addition of cAMP signalling cascade inhibitors (e.g PI-3 kinase inhibitor) in conjunction with a BDNF/NGF gradient results in the normally chemoattractive neurotrophin cues becoming a chemorepellent cues for the growth cone (Song et al., 1997; Ming et al., 1999) and even inducing growth cone collapse (Wang and Zheng, 1998). This effect not only highlights that cAMP levels regulate growth cone responses but also that activation of the PI-3 kinase signalling cascade is necessary to induce chemoattractive responses. In contrast,
NT-3 gradients remained chemoattractant in the presence of cAMP signalling inhibitors, supporting the view that each receptor activates different signalling cascades and hence different biological responses.

Figure 4. Extracellular factors that mediate local signalling cascades in order to regulate axon growth. The extracellular signals such as the neurotrophins and ECMs activate intracellular signalling pathways (1) locally at the axon that regulate the actions of actin binding proteins (ABPs) and microtubule-associated proteins (MAPs). These two molecules then mediate axon assembly via actin and microtubule polymerisation. In parallel, (2) activated signalling substrates are retrogradely transported from axon to cell body and upon arriving at the soma, (3) induce activation of certain transcription factors that regulate axon growth. The subsequent proteins translated are then used as raw materials for new axons that are (4) transported anterogradely and incorporated into the growing axon (modified from Zhou and Snider, 2006).

The importance of neurotrophin and receptor expression levels for appropriate target reinnervation has also been shown via transgenic mouse lines. In mice lacking or ectopically expressing a neurotrophin, the changes in the normal expression pattern of the neurotrophin results in aberrant axonal paths or disturbed innervation of a specific target (Huang and Reichardt, 2001). For instance, overexpression of BDNF in transgenic mice stalls gustatory sensory fibre growth, as these fibres stall at ectopic BDNF sites and fail to reach the gustatory papillae (Ringstedt et al., 1999). Moreover, dense sympathetic innervation occurs in cells of pancreatic islets, which normally are not innervated, when NGF levels are elevated (Huang and Reichardt, 2001). Furthermore, mice lacking the TrkA receptor have a deficit in cholinergic projections from the basal forebrain which project into the neocortex and hippocampus (Smeyne et al., 1994), indicating that TrkA is essential for normal target innervation.
1.3.3 Circuit formation and maturation

Neurotrophins also regulate the formation of synaptic circuits. Infusion of BDNF into the visual cortex inhibits the development of ocular dominance column formation within the zone of infusion (Cabelli et al., 1995). Ocular dominance column formation occurs when axons from the lateral geniculate nucleus become segregated in the cortex into eye-specific patches (Vicario-Abejon et al., 2002). However, blockade of ocular dominance columns was not observed with NGF or NT-3 infusion, which emphasises the specific involvement of TrkB receptors in axonal segregation in this system. In contrast to BDNF infusion studies (Cabelli et al., 1995; Cabelli et al., 1997), transgenic mice that overexpress BDNF in the visual cortex still developed ocular dominance column segregation (Hanover et al., 1999), but with accelerated maturation of the cortical circuit (Hanover et al., 1999). In addition, Huang et al., (1999) found that in such transgenic mice, maturation of GABAergic innervation and inhibition is also accelerated. Both of these studies suggest that overexpressing BDNF in vivo advances the maturation of synaptic circuits.

The maturation and stabilisation of axonal branches is dependent, amongst other factors, on both neurotrophins and neuronal activity (Cohen-Cory, 1999). A neurotrophic effect on the maturation of functional synapses was first demonstrated in the superior cervical ganglia whereupon modification of NGF levels regulated the strength and number of preganglionic inputs (Nja and Purves, 1978). Since then, overexpression of BDNF in transgenic mice has been shown to increase synaptic number in the sympathetic ganglia while BDNF knock-out mice have decreased synaptic innervation (Causing et al., 1997). In addition, BDNF and NT-3 (but not NGF) advance axon morphology toward their mature phenotypes (Wang et al., 1995). In terms of stabilisation, BDNF has been found to prevent growth cone collapse and retraction from repulsive substances, which usually eliminate mistargeted axons (Ernst et al., 2000). In this study, only BDNF (and neither NT-3 nor NGF) could prevent growth cone collapse (Ernst et al., 2000).

In addition, neurotrophin mRNA and Trk receptor expression in the adult animal is tightly regulated by both sensory input and neuronal activity. Neuronal activity, such as epileptiform activity in the hippocampus and cortex, induces increased NGF and BDNF expression levels (Gall and Isackson, 1989; Ernfors et al., 1991). In particular, the increased BDNF mRNA in turn increases expression of TrkB and regulates surface
expression of TrkB receptors (Castren et al., 1992; Meyer-Franke et al., 1998). Other forms of neuronal activity (i.e. non-epileptic activity) also increase neurotrophin expression. These include depolarisation (via high potassium concentrations), glutamate agonists and acetylcholine. These factors increase the secretion of NGF and BDNF from hippocampal slices (Blochl and Thoenen, 1995; McAllister, 1999).

In summary, it has been described that neurotrophin-Trk signalling takes an important position in the survival of certain neuronal populations, matching the right innervating axons to their correct target neuronal type and inducing neurite outgrowth. In the case of axonal injury, the increased availability of neurotrophins has been shown to promote neuronal survival and stimulate neurite outgrowth. In the model system used in the present study, the olivocerebellar system, BDNF has been implicated in both its development and reinnervation following a lesion. As our neurotrophin candidate to increase reinnervation is BDNF, the next section includes a detailed review of BDNF-TrkB signalling effects.

1.4 The role of BDNF/Trk B in the structural and functional properties of neuronal circuits

Considerable interest has been drawn to BDNF due to its effects on: synaptic transmission at developing synapses (Lohof et al., 1993; Wang et al., 1995), synapse formation (Martinez et al., 1998; Luikart et al., 2005) and stabilisation of appropriate synapses (Luikart et al., 2005). The following section will discuss the unique role of BDNF and also the potential actions of exogenously introduced BDNF into developing and mature systems (refer to Table 2).

1.4.1 BDNF takes an anterograde route

The distribution and concentration of BDNF protein is greater than that of the other neurotrophins in the CNS. Neurons rich in BDNF mRNA at their somata project to target regions with correspondingly high BDNF protein (Conner et al., 1997; Yan et al., 1997b). This suggests that, unlike NGF (Curtis and DiStefano, 1994), BDNF is anterogradely transported and potentially influences postsynaptic targets (via the amount of BDNF released) in the CNS. The anterograde transport of BDNF has been confirmed in several
experiments: (i) BDNF mRNA is localised in cortical and nigral neurons which innervate the neostratium (which contains BDNF protein; Altar et al., 1997), (ii) BDNF mRNA increases in dentate granule neurons, in conjunction with increased BDNF protein in mossy fibres, during induced seizures (Smith et al., 1997) and (iii) BDNF protein is localised in axon terminals throughout the brain.

Table 2. Summary of neuronal processes influenced by neurotrophins.

<table>
<thead>
<tr>
<th>Neuronal Property</th>
<th>Neurotrophin(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation of precursors</td>
<td>NT-3</td>
<td>Kalchiem et al., 1992</td>
</tr>
<tr>
<td>Survival of precursors</td>
<td>NGF, NT-3</td>
<td>Birren et al., 1993; DiCicco-Bloom et al., 1993; Huang and Reichardt, 2003 (review)</td>
</tr>
<tr>
<td>Differentiation along specific lineages</td>
<td>NGF, BDNF</td>
<td>Sieber-Blum, 1991; Anderson, 1993</td>
</tr>
<tr>
<td>Programmed cell death</td>
<td>All</td>
<td>Davies, 1994 (review)</td>
</tr>
<tr>
<td>Axon growth (cytoskeletal elements)</td>
<td>NGF, BDNF, NT-3</td>
<td>Snider and Johnson, 1989 (review); Patel et al., 2000; Zhou and Snider, 2006 (review)</td>
</tr>
<tr>
<td>Axon collateral branching into target fields</td>
<td>NGF, BDNF, NT-3</td>
<td>Schnell et al., 1994; Huang and Reichardt, 2003</td>
</tr>
<tr>
<td>Neurotransmitter enzymes</td>
<td>NGF, BDNF, NT-4</td>
<td>Henderson et al., 1993</td>
</tr>
<tr>
<td>Calcium binding and buffering protein</td>
<td>BDNF, NT-3</td>
<td>Ip et al., 1993; Jones et al., 1994</td>
</tr>
<tr>
<td>Synaptic efficacy</td>
<td>BDNF, NT-3</td>
<td>Lohof et al., 1993</td>
</tr>
<tr>
<td>Synaptic rearrangement</td>
<td>NGF, BDNF</td>
<td>Maffei et al., 1992; Bamji et al., 2006</td>
</tr>
<tr>
<td>Dendritic arborisation</td>
<td>NGF, BDNF</td>
<td>Snider, 1988; Schwartz et al., 1997</td>
</tr>
</tbody>
</table>

Modified from Snider (1994).
The pivotal study by von Bartheld and colleagues (1996) showed that exogenous BDNF is also transported in an anterograde direction from cell bodies to axon terminals as opposed to well-established retrograde transport of NGF. Importantly, the neurotrophin is released after anterograde transport and endocytosed by the post-synaptic target neuron in the developing chick brain (von Bartheld et al., 1996). These authors observed labelled neurotrophin (either $^{125}$I-labelled NT-3 or BDNF) associated with presynaptic vesicles and localised in the dendrites and soma of post-synaptic neurons.

These findings further support the hypothesis that endogenous and exogenous BDNF may act as anterograde trophic messengers, as seen in the developing noradrenergic and dopaminergic neurons and visual system and also in the adult CNS post-axotomy (Fawcett et al., 1998; Alonso-Vanegas et al., 1999; Spalding et al., 2002; Caleo et al., 2003).

1.4.2 BDNF modifies neuronal excitability and synaptic transmission and plasticity

It had been previously thought that only neurotransmitters could have a rapid effect on the membrane potential of neurons. However, BDNF application results in membrane depolarisation within milliseconds (Kafitz et al., 1999) which means there is a change in the membrane potential which can possibly initiate an action potential. In this experiment, BDNF applied as puffs onto neurons expressing TrkB triggered trains of action potentials, whilst the other neurotrophins failed to induce the same response when applied. Because BDNF is a large molecule and neither of its receptors are ion channels, the mechanism underlying BDNF-induced depolarisation was explored. Firstly, this depolarisation triggers an increase in the levels Ca$^{2+}$ in the spines of postsynaptic neurons (Kovalchuk et al., 2002). Secondly, BDNF causes an inward flow of Na$^+$ ions (via the channel Na$_v$1.9) thus explaining the rapid depolarisation (Figure 5; Blum et al., 2002). Blum and colleagues (2002) proposed that BDNF produces a conformation change in the TrkB receptor that is transferred without delay to the Na$_v$1.9 channel which then becomes permeable to Na$^+$. This view was substantiated when targeted elimination of Na$_v$1.9 blocked BDNF-evoked depolarisation (Blum et al., 2002).
Figure 5. Rapid post-synaptic effects induced by BDNF. BDNF binding to TrkB activates Na\(^+\) channel (Na\(_{\text{v}1.9}\)) opening. The depolarisation induces Ca\(^{2+}\) influx through voltage-gated calcium channels (VOCCs) thereby enhancing NMDA-receptor channel activation (Rose et al., 2004).

In addition to BDNF-induced neuronal excitability, BDNF evokes rapid presynaptic effects. Lohof et al. (1993) showed that the application of BDNF (or NT-3) resulted in an elevation of the frequency but not the amplitude of spontaneous synaptic currents (mEPSCs) at the developing Xenopus neuromuscular junction (Lohof et al., 1993). Similarly, at CNS neurons, BDNF and NT-4 rapidly enhance spontaneous synaptic activity in cultured hippocampal neurons (Lessman et al., 1994).

BDNF also modulates the postsynaptic ligand-gated N-Methyl-D-Aspartic acid (NMDA) receptor channel. BDNF increases the phosphorylation of the NMDA receptor channels and the ‘open probability’ of the receptor (Jarvis et al., 1997). In the presence of K252, an inhibitor of Trk activation, the enhancement of NMDA currents by BDNF did not change, suggesting that the mechanism was independent of Trk phosphorylation. In contrast, the effect of BDNF on AMPA receptor-mediated transmission remains unclear. In one instance, BDNF was found to enhance synaptic transmission through NMDA receptors yet suppressed AMPA receptor-mediated currents in cultured hippocampal neurons (Song et al., 1998). In contrast, it was revealed that BDNF upregulates the surface expression of AMPA receptors suggesting that BDNF increases AMPA-mediated transmission (Narisawa-Saito et al., 2002). Supporting the latter case, the application of BDNF activates AMPA receptor trafficking into synaptic sites and shifts the expression levels from NMDA receptor only synapses to AMPA containing ones (without affecting NMDA receptor transmission; Itami et al., 2003). This result suggests that BDNF is a key player for the maturation of AMPA-mediated transmission.

The involvement of BDNF-induced rapid depolarisation of neurons and the consequent intracellular mechanisms (Figure 5) has led to intense research on the role of BDNF in activity-dependent long-term plasticity (Poo, 2001; Kovalchuk et al., 2002). Hippocampal long-term potentiation (LTP) entails enhanced neurotransmitter (i.e.
glutamate) release during high frequency synaptic stimulation. Hippocampal LTP is substantially reduced in BDNF deficient mice, both in homozygotes and in heterozygotes (Korte et al., 1995; Patterson et al., 1996) and can be rescued by viral re-expression of the BDNF gene (Korte et al., 1995). Substantiating the involvement of BDNF in LTP, blockade of endogenous BDNF function by TrkB fusion proteins also reduces LTP (Figurov et al., 1996). Recently, the site of BDNF action during the induction of LTP has been explored, and BDNF appears to have a presynaptic role during LTP. Most studies imply that BDNF to TrkB signalling does not directly induce the changes underlying LTP in the postsynaptic neuron. Instead BDNF modulates the efficiency of the presynaptic neuron, which then modifies the response of postsynaptic cell (Pozzo-Miller et al., 1999; Minichiello et al., 1999; Xu et al., 2000a).

It is well established that BDNF signalling via TrkB activates intracellular events that then modulate ligand-gated, voltage-gated and cation channel (e.g. TRPC3) currents (Figure 5). The interaction of TrkB with the Na\textsubscript{v}1.9 channel throws additional light on the role of BDNF-TrkB in LTP. One study revealed that BDNF-evoked Na\textsuperscript{+} influx is not responsible for a different form of LTP (i.e pairing weak afferent stimuli with brief BDNF pulses instead of conventional tetanic stimulation), but induces rapid amplification of the LTP induction process (Kovalchuk et al., 2002). It has been suggested that the fast BDNF-induced excitation of postsynaptic neurons by TrkB-Na\textsubscript{v}1.9 channels, which triggers Ca\textsuperscript{2+} influx through VOCCs, may lead to the activation of transduction pathways (such as cell adhesion molecule (CaM) kinase and CREB). In turn, their activation could induce activity-dependent gene expression and neuronal plasticity.

1.4.3 BDNF/TrkB mediates axonal guidance and growth

Neurotrophins play an important role in axonal guidance: growth cone turning can be induced through a Trk-dependent mechanism (Gallo et al., 1997; Song and Poo, 2001) and filopodial dynamics through a neurotrophin-p75-dependent mechanism (Gehler et al., 2004). Axonal filopodia contribute to axon extension, guidance and arborisation. BDNF has been shown to directly regulate axonal filopodia length and motility. Application of BDNF-coated beads causes filopodial sprouting of DRG axons (Gallo and Letourneau, 1998) whilst no filopodial recruitment is seen, and decreased synapese numbers are found, in conditional knock-out mice with deleted TrkB in pre and postsynaptic neurons (Luikart
et al., 2005). Furthermore, endogenous BDNF mediates the formation of optic axon arborisation in the tectum (Cohen-Cory and Fraser, 1995).

The majority of studies clearly demonstrate that in addition to axonal guidance, short-term elevations of BDNF and TrkB activation enhances outgrowth of peripheral and central axons both in vitro (McAllister et al., 1995; Segal et al., 1995; Lentz et al., 1999; Ernst et al., 2000) and in vivo (Cohen-Cory and Fraser, 1995; Causing et al., 1997; Cohen-Cory, 1999; Lom and Cohen-Cory, 1999; Alsina et al., 2001; Gillespie et al., 2003). Only one report, in which BDNF inhibited axonal growth of neurons in the PNS (Griffin and Letourneau, 1980), contradicts these findings. BDNF application is sufficient to increase axonal elaboration (i.e. branching or lengthening), for example in the tectum (Cohen-Cory, 1999), in parallel with increased in synapse formation (Alsina et al., 2001). The observed increased number of synapses per axon terminal after exogenous BDNF application (Alsina et al., 2001) appears to be due to the neurotrophin gradient providing chemo-attractive cues to axons (Ming et al., 1997). BDNF’s chemo-attractive role has been shown by inducing extensive formation of lamellipodia at multiple sites along the neurite shaft and its growth cone (Ming et al., 1997; Tucker et al., 2001). These experiments suggest that BDNF not only acts as a neurotrophic but also a chemo-attractive agent (i.e. neurotropic).

1.4.4 BDNF/TrkB regulates dendritic growth

Likewise, neurotropic action occurs at dendritic filopodia, which in turn influences not only dendritic arborisation but also dendritic spine morphology. The application of BDNF in dissociated hippocampal cultures increases dendritic filopodial density (Eom et al., 2003). Intriguingly, an experiment by Hartmann et al., (2004) revealed that truncated TrkB–p75 complex signalling underlies the enhanced filopodial motility of dendrites, whereas catalytic TrkB in association with truncated TrkB or p75 opposes this action (Figure 6; Hartmann et al., 2004). Furthermore, the overexpression of p75 induces filopodial growth of proximal dendrites (Hartmann et al., 2004). All of these responses are independent of BDNF binding to the receptors. Therefore it appears that dendritic filopodia respond differently depending on the receptor composition on the dendrite.
The role of BDNF and TrkB in dendritic growth is unclear. Cortical pyramidal neurons that overexpress BDNF display increased sprouting of their basal dendrites in the ferret visual cortex (McAllister et al., 1995). Moreover, deletion of TrkB decreases the complexity of dendrites in pyramidal neurons (Xu et al., 2000b). In contrast, application of BDNF to retinal ganglion cells decreases the complexity of dendritic arbors, and neutralising endogenous BDNF (with Trk ‘receptor bodies’ i.e. fusion bodies) increases dendritic complexity (Lom and Cohen-Cory, 1999). These divergent results suggest that BDNF can act either to stimulate or to inhibit growth of dendritic arbors depending on the concentrations of BDNF, the presence of catalytic versus truncated TrkB receptors, and the neuronal population. Highlighting the subtleties in BDNF concentrations, dose-response experiments by Ji and colleagues (2005) show that low doses of BDNF (5ng/mL) increase only spine densities while higher doses (25ng/mL) increase filopodial density, spine density and dendritic growth (Ji et al., 2005).

In addition, different neurotrophins have been found to oppose one another in regulating dendritic growth. For instance, in cortical layer 6 (a site of endogenous NT-3 activity for growth and maintenance of dendritic arbors), exogenous BDNF application inhibits dendritic growth (McAllister et al., 1997). This emphasises that BDNF and other neurotrophins tightly regulate the balance between growth and inhibition of dendritic arbors.

1.4.5 BDNF and synaptic stabilisation

The presence of BDNF can lead to dendritic and axonal arbor modifications, hence producing changes in synapse formation and stabilisation. Application of BDNF into the optic tectum increases the synapse number in dendritic arbors of tectal neurons (without affecting arbor branching), in parallel with the increase in presynaptic sites on RGC axons (Sanchez et al., 2006). Furthermore, the overexpression of BDNF decreases the stability of dendritic spines (Horsh et al., 1999), possibly due to modifications in dendritic arbors.
and the turnover of synapses (Horsh et al., 1999). Therefore, BDNF overexpression may initiate ‘remodelling’ of dendritic arbors to compensate for the new patterns of BDNF activity. Whilst downregulation of TrkB receptors does occur with short-term elevates of BDNF (Frank et al., 1996), overexpression of BDNF in the hippocampal dentate gyrus of mature brains does not increase or change TrkB expression or downstream TrkB signalling (Tolwani et al., 2002). This difference in TrkB expression found between short-term and long-term applications of BDNF could be explained by the fact that in experiments with short-term exposure, BDNF application is extracellular and thus is not regulated like endogenous neurotrophin release (Blochl and Thoenen, 1995).

Blocking neuronal activity is known to limit axonal branch stabilisation by increasing addition and elimination of these branches (Cohen-Cory, 1999). However, in the presence of BDNF, blockade of neural activity does not inhibit the branching or lengthening of an axon (Cohen-Cory, 1999). Furthermore, in the hippocampus, mice overexpressing BDNF do not show alterations in the neurite growth promoter protein (GAP-43) or induce mossy fibre sprouting (Qiao et al., 2001). In the latter case, mossy fibre sprouting has been induced when these fibres are exposed to hyper-excitability during seizures (Scharfman, 2000). This result suggests that in the hippocampus, BDNF may not directly induce axonal sprouting without neuronal activity.

To conclude, we have seen that neurotrophin-induced signalling, and in particular BDNF-TrkB signalling, mediates neuronal growth, synaptic stabilisation and activity. These processes contribute to the precise synaptogenesis which occurs during development. As the extracellular factors which create precise connections during development are also those that limit the regenerative potential of the CNS following a lesion, one approach is to induce repair by recapitulating development. A related approach is to further characterise and maximise spontaneous reinnervation that takes place after an injury in the developing system. To maximise this system, neurotrophin treatment can be administered as one method to recreate similar reinnervation in the later developing CNS. In order to assess the benefits and limitations of developmental reinnervation, we have used the developing olivocerebellar system as a model. The following section describes fundamental anatomy, physiology and function of this system.
2.0 The Rat Cerebellum and its Olivary Afferents

The cerebellum of the adult rat is a highly folded hindbrain structure that lies dorsal to the pons and medulla (Figure 7A). Three pairs of cerebellar peduncles, the inferior and middle (predominately afferent pathways) and superior (main efferent pathway) connect the cerebellum to the brainstem. The cerebellum is bilaterally symmetrical: two lateral hemispheres are separated medially by the central longitudinal vermis (Larsell, 1952). It is also divided phylogenetically into anterior and posterior lobes by a transverse primary fissure, and a ventrally located flocculonodular lobe. Shallow horizontal fissures further subdivide the anterior and posterior lobes into transversely oriented lobules, or folia that are categorised by roman numerology I to X (Figure 7B; Larsell, 1952).

Figure 7. (A) A lateral view of cerebellum. The brainstem is dissected to view the cerebellar peduncles. The superior peduncle (green) is located medially while the middle peduncle (blue) lies most lateral and the inferior peduncle (red) sits between the two. (B) The anatomical nomenclature of the unfolded mammalian cerebellum, displaying the divisions of the anterior (shaded grey) and posterior lobes (unshaded) and flocculonodular lobe (shaded black). This view also shows the lobules which make up the vermis, paravermis and hemisphere (modified from Voogd and Glickstein, 1998).
2.1 Cerebellar cortex

Each single folium comprises a cortex or superficial grey matter that overlies a central white matter core, the medullary centre. The cortex has a highly uniform microstructure across the entire cerebellum and is organised into three layers comprising: (i) the outermost molecular layer, which consists mainly of granule cell axons called parallel fibres, Purkinje cell dendrites plus stellate and basket interneurons; (ii) the middle Purkinje cell soma layer; and (iii) the innermost granular cell layer, which contains mostly compacted granule cell somata and also Golgi cells (Figure 8; Ramon y Cajal, 1911). The cortex receives two major types of extracerebellar afferents, the mossy fibres and the climbing fibres, and a third afferent that contains various amines which course as fine varicose fibres in the granule and molecular layers and contact Purkinje cells (e.g. serotonin, noradrenaline, acetylcholine derived from the raphe nuclei, reticular formation and locus coeruleus; Ramon y Cajal, 1911; Palay and Chan-Palay, 1974; Ito, 2006). Underlying the cortex, the medullary centre contains four sets of deep cerebellar nuclei (DCN; i.e. medial, interpositus anterior and posterior, and lateral) that provide the only output from the cerebellum (Palay and Chan-Palay, 1974).

Figure 8. Anatomical architecture of the cerebellum: (a) parasagittal section of the cerebellum and the corresponding organisation of the cerebellar cortex (b) consisting of three layers: granular layer, Purkinje cell layer and molecular layer (Ramnani, 2006).
2.1.1 Purkinje cells

Purkinje cells (PCs) are the principal neurons of the cerebellar cortex and serve as the sole output from the cortex to the DCN (Palay and Chan-Palay, 1974; Ito, 1984). PC somata form a dense monolayer between the granular and molecular layers (Figure 8; Armstrong and Schild, 1970; Palay and Chan-Palay, 1974). Within this layer, PCs express biochemical heterogeneity of certain proteins (such as zebrin I and II) that segregate PCs into sagittally organised bands (Hawkes and Leclerc, 1987; Sotelo and Wassef, 1991). The PC soma is approximately 21µm in diameter and 25µm in length and the dendritic tree spans 300 to 400µm in width but only 15µm deep (Palay and Chan-Palay, 1974). This planar dendritic arborisation is oriented in the sagittal plane (perpendicular to the long axis of the folium and to parallel fibres) and is formed from one or two primary branches that further subdivide into secondary and tertiary branches (Palay and Chan-Palay, 1974; Berry and Bradley, 1976; Ito, 1984). Two types of spines are located along the dendritic tree: (i) spines that are restricted to major dendritic trunks and interact primarily with climbing fibres and (ii) spines that stem from distal tertiary branches which synapse with parallel fibres (PF) (Ramon y Cajal, 1911; Ito, 1984). This type of distribution means that the afferent populations are strictly segregated and interact with specific domains on the PC dendritic tree.

The axon of the PC emerges from the basal pole of the soma, descends through the PC layer and the internal granular layer and synapses mainly onto the deep cerebellar nuclei (DCN) neurons, providing GABAergic innervation (Figure 9; Palay and Chan-Palay, 1974). Some PC axons from specific lobules (lobule X and the flocculus) synapse onto the vestibular nuclei to mediate balance (Ito, 1984). Collaterals are also emitted along the PC axon’s descent: these collaterals re-enter the PC layer in the same sagittal plane as the PC dendrites and contribute to supra- and infraganglionic plexuses, which form above and below the PC layer, inhibiting adjacent PCs, Golgi, basket and stellate cells (Palay and Chan-Palay, 1974).

The PC electrophysiology involves tonic discharge in the absence of any stimuli (Cerinmara and Rawson, 2004). It was previously thought that the discharge of PCs was due to sustained post-synaptic depolarisation by the mossy fibre-parallel fibre pathway, however, neither deafferentation nor an agranular cerebellum (Eccles et al., 1967; Siggins et al., 1976) abolishes the spontaneous simple spike activity in PCs. The PC also has the
capacity to generate dendritic spikes which are Ca\(^{2+}\) instead of Na\(^{+}\) dependent (Llinas and Sugimori, 1980), and which are decrementally propagated towards the PC soma.

### 2.1.2 Granule cells

Granule cells (GCs) are found in the internal GC layer and relay input from mossy fibres to the PCs. GC somata are 5 to 8\(\mu\)m in diameter and their dendrites, which are generally shorter than 30\(\mu\)m, receive mossy fibre terminals (rosettes) to form complex synapses (glomeruli; Palay and Chan-Palay, 1974; Ito, 1984; Voogd and Glickstein, 1998). Their GC axons ascend through the molecular layer, bifurcate and run parallel to the longitudinal axis of the folium as parallel fibres (PFs; Figure 8; Palay and Chan-Palay, 1974; Voogd and Glickstein, 1998), and form en passant synapses on PC dendrites and inhibitory interneurons (Figure 9; Palay and Chan-Palay, 1974). The physiological action of the parallel fibres is a simple spike discharge in PCs and repetitive discharges in stellate and basket cells which induces an overall inhibition that lasts for more than 100 milliseconds.

Figure 9. Schematic diagram of the cerebellar circuit with marked excitatory (+) and inhibitory (−) innervation (modified from Medina et al., 2002).
2.1.3 Interneurons

There are at least five types of interneurons in the cerebellar cortex: basket, stellate, Golgi, Lugaro and unipolar brush cells (Palay and Chan-Palay, 1974). All of these interneurons, with the exception of the unipolar brush cells, have been shown to be inhibitory (Eccles et al., 1966c; Aoki et al., 1986). The basket and stellate axons provide lateral inhibition in the parasagittal plane to adjacent PC soma and dendrites, respectively (Palay and Chan-Palay, 1974). The Golgi cell dendrites receive input from GCs as well as mossy fibres, and provide feed-back inhibition to GCs (Figure 9; Palay and Chan-Palay, 1974) thereby forming a closed inhibitory circuit in the cerebellar cortex, in preparation for the next mossy fibre input.

2.2 Cerebellar afferents

The fibres from the olivocerebellar axons (i.e. climbing fibres) and the mossy fibre system constitute the two major cerebellar afferent systems. Both systems carry sensorimotor information directly to the DCN and also to the cerebellar cortex to regulate PC firing. In turn, the inhibitory projection of PCs onto the DCN regulates the extent to which the climbing and mossy fibres activate the DCN (Figure 9). The differences that emerge in the synaptic strength within the cerebellar cortex are thought to form the basis of cerebellar plasticity during learning (De Zeeuw and Yeo, 2005).

2.2.1 Olivocerebellar system

Anatomy. Climbing fibres (CFs) are one of the main operational inputs into the cerebellar cortex. Electrophysiological (Eccles et al., 1966a), autoradiographic (Courville and Cantin-Faraco, 1978) and degeneration (Desclin, 1974) techniques show that CFs originate exclusively from neurons in the inferior olive of the medulla. The inferior olive sends olivocerebellar axons to the cerebellum, where the final segment of the axon takes the form of a CF in the cerebellar cortex. The olivocerebellar projection predominately crosses the medullary midline, terminating in the contralateral hemicerebellum with the addition of a very small uncrossed component innervating the ipsilateral hemicerebellum (Chan-Palay et al., 1977; Sugihara et al., 1999). From the inferior cerebellar peduncle, axons extend into the white matter and branch into the cortex in the parasagittal plane.
In the rat, the olivocerebellar projection has an organised topography that is arranged into a pattern of parallel longitudinal zones in the cerebellar cortex (Azizi and Woodward, 1987; Buisseret-Delmas and Angaut, 1993; Sugihara et al., 2001). In general, each subnucleus of the inferior olive projects contralateral CFs that closely adhere to one or more parasagittal PC zones (for example, defined by PC expression of the marker zebrin), which then projects to a specific part of the DCN (Armstrong et al., 1974; Campbell and Armstrong, 1983; Wassef et al., 1992; Buisseret-Delmas and Angaut, 1993; Sugihara et al., 2001). Furthermore, each olivary subnucleus gives off collaterals to the DCN (Figure 9), which receives PC input from the same parasagittal zone(s) (Andersson and Armstrong, 1987) and in turn, the DCN neurons project to the same olivary subnuclei from which they receive collaterals (De Zeeuw et al., 1998; Ruigrok and Voogd, 2000). In summary, the PCs in each longitudinal zone receive CFs from a discrete zone within the inferior olive and these PCs sends axons to a specific region in the DCN thereby creating an olivo-cortico-nuclear circuit (Figure 9).

The inferior olive is a homogeneous population with few interneurons (<0.1%). It is subdivided into three major subnuclei, the medial accessory olive (MAO), the dorsal accessory olive (DAO) and the principal olive (PO); and four minor subnuclei, the nucleus beta, the dorsal cap (dc), the ventrolateral outgrowth (vlo) and the dorso medial cell column (dmcc) (Azizi and Woodward, 1987). Each of these subnuclei are further divided according to their axon terminations (Buisseret-Delmas and Angaut, 1993). In general, the caudal half of MAO projects CFs to the flocculus and the vermis whilst the hemispheres are innervated by CFs from the PO and rostral half of MAO. The DAO provides fibres to the paravermal cortex (Figure 10; Azizi and Woodward, 1987; Buisseret-Delmas and Angaut, 1993).
Figure 10. A simplified diagram of the zonal organisation within the olivocerebellar system. The lamellae (folds) of DAO (1 & 2) and the horizontal lamella of MAO (3) send fibres predominately to the anterior vermis and paravermis. The medial MAO (vertical lamella, 4) projects to the posterior vermis as well as the flocculus. The rostral lamella of MAO (5) and the dorsal and ventral PO (6 & 7) projects fibres to the lateral hemisphere. The inferior olive receives afferents from the spinal cord, vestibular nuclei, tectum, red nucleus, diencephalons and cortex. The zonal distribution of the olivary projections into the cerebellar cortex is thought to be associated with different aspects of motor control. Modified from Azizi and Woodward (1987).

In addition, the olivary neurons contain two morphological types that are differentially located. Olivary cell bodies with smaller diameter dendritic arbors are typically situated in the rostral MAO, DAO and PO, while neurons with long diffuse, sparsely branching spiny dendritic arbors occupying a large dendritic tree are found predominately in the caudal MAO (Scheibel and Scheibel, 1955; Ruigrok and Voogd, 1990). Furthermore, the dendritic spines of different olivary neurons are electrotonically coupled by gap junctions wherein the spine necks cluster together and one olivary neuron can have 500 to 1000 gap junctions (De Zeeuw et al., 1997). A recent study has shown that these gap junctions are required for coordinated membrane oscillations (Leznik and Llinas, 2005). It has been proposed that this allows the generation of synchronous olivary activation which may correlate to the initiation and performance of movements.
In the cerebellar cortex, PC activation is usually simultaneous (within 1 millisecond) within their parasagitally organised bands (Sugihara et al., 1993) which corresponds to the electrotonic coupling in inferior olive neurons (Llinas and Yarom, 1981). It had been proposed that the conduction velocity is tuned so that longer CFs propagate action potentials with a greater conduction velocity than shorter CFs (Sugihara et al., 1993), to give a uniform conduction time. However, using the climbing fibre reflex (Eccles et al., 1966a) rather than electrical stimulation in the medulla (Sugihara et al., 1993), a recent study has demonstrated that conduction time does vary so that deep PCs will be activated prior to superficial PCs (Baker and Edgley, 2006). The difference in conduction time was in the order of several milliseconds (Baker and Edgley, 2006). Although this result does not contest the importance of CFs in synchronised PC firing, it implies that this synchronicity does not have submillisecond precision.

In the white matter, the olivocerebellar axons are thick (2 to 3µm in diameter) and myelinated (Palay and Chan-Palay, 1974; Sugihara et al., 1999). However, as they ascend towards the granular cell layer, the axons become unmyelinated as they extend into the PC layer and synapse onto the soma and the thick dendritic trunks of PCs (Palay and Chan-Palay, 1974; Sugihara et al., 1999). At the PC dendritic tree, CF arborisations emit fine beaded tendrils (including varicosities approximately 2µm thick) that run along the PC dendrites and synapse onto spines and the soma (Palay and Chan-Palay, 1974; Sugihara et al., 1999). These CF-PC synapses are entirely covered by sheets of Bergmann glia membranes (Palay and Chan-Palay, 1974; Xu-Friedman et al., 2001) which decreases glutamate spill-over at this synapse (Wadiche and Jahr, 2001). In addition to CFs projecting onto PCs, CFs also send collateral branches to the deep cerebellar nuclei (Palay and Chan-Palay, 1974; van der Want, 1989; Sugihara et al., 1999; Ruigrok and Voogd, 2000; Sugihara et al., 2001). Therefore, the anatomy of the olivocerebellar system has been described in order to provide us with a template for analysing certain anatomical features of olivocerebellar reinnervation.

**Physiology.** The morphological and physiological characteristics of the CF-PC synapses have been extensively studied (Ramon y Cajal, 1911; Eccles et al., 1966a; Palay and Chan-Paly, 1974; Konnerth et al., 1990; Mariani et al., 1990; Callaway et al., 1995; Hashimoto and Kano, 1998; Hashimoto and Kano, 2003). A salient feature of the adult rat cerebellum is that each PC receives input from only one CF, but every CF can
innervate 5 to 7 PCs (Eccles et al., 1966a; Schild, 1970; Sugihara et al., 1999). CFs elicit a powerful and low frequency all-or-none complex spike (Eccles et al., 1966a; Konnerth et al., 1992; Callaway et al., 1995; Hashimoto and Kano, 1998; Scelfo et al., 2003). Eccles and colleagues (1966a) were the first to observe the complex spike response after CF stimulation in vivo. The complex spike consists of a brief burst of sodium action potentials and Ca$^{2+}$-dependent dendritic spikes (Figure 11; Eccles et al., 1966a; Weber et al., 2003). In contrast to the simple spikes generated by the mossy fibre-GC-parallel fibre system in PCs, the complex spikes discharge occurs at low rates (~1.5 Hz) in awake animals (Armstrong and Rawson, 1979).

![Intracellular recordings of a PC after inferior olive stimulation. A to F shows the CF-induced PC response. Note the initial Na$^+$ spike followed by the slower spikelets on the declining phase of the depolarisation and the all-or-none response in C and F (Eccles et al., 1966a).](image)

The generation of a complex spike remains unclear as the pharmacological blockers of voltage conductances also affect synaptic release. However, it is known that the burst of fast spikes is associated with somatically-activated Na$^+$ conductances while the depolarising spike bursts are mediated by dendritically activated Ca$^{2+}$ conductances (Figure 12; Schmolesky et al., 2002). Patch-clamp recordings have shown that the Na$^+$ spike propagation is completely passive in PC dendrites and initiated in the PC soma or axon (Callaway and Ross, 1997) by fast, inactivating Na$^+$ conductances (Schmolesky et al., 2002). Instead, at the proximal dendrites, the spike activity upon CF activation clearly highlights the involvement of the voltage-dependent Ca$^{2+}$ conductances through P/Q as well as T-type channels (Llinas et al., 1989; Watanabe et al., 1998; Talley et al., 1999; Pouille et al., 2000; Isope and Murphy, 2005). The activation of Ca$^{2+}$ conductances involved in the generation of this depolarisation is the dominant cellular event associated with the complex spike, which can stimulate intracellular cascades having potentially-important functional implications (discussed below).
In addition to CF-excitatory postsynaptic currents (EPSCs) inducing an all-or-none complex spike in PCs, they also exhibit paired-pulse depression (PPD) upon repeated stimulation. PPD involves a reduction in neurotransmitter release after the second stimulation, and is thought to have functional significance for controlling the size of CF-EPSCs (i.e. acting as a low-pass filter; Silver et al., 1998). In this way, the high-probability of neurotransmitter released after the 1st stimulation allows for reliable olivocerebellar transmission while the presynaptic depression following the 2nd stimulation ensures limited transmission during high-frequency stimulation. Upon CF stimulation, glutamate is released into the synaptic cleft and binds to post-synaptic AMPA and metabotropic glutamate receptors (mGluR). Because of the high number of CF synaptic contacts and the high release probability at each CF terminal, the resulting EPSC is large (Silver et al., 1998).

It was considered that PPD (i.e. the decrease in neurotransmitter release) might modify the complex spike. Interestingly, at high frequency stimulations of 100 Hz, complex spikes could be activated (Eccles et al., 1966b). However, Hashimoto and Kano (1998) demonstrated that PPD decreased the number of spikelets in the complex spike with interstimulus intervals of 1 Hz indicating that the complex spike form can be affected depending on the firing rate of the CF afferents. PPD is primarily a presynaptic function of the CFs, as it is unaffected by antagonists of mGluRs, GABA receptors, or adenosine receptors, and does not change upon postsynaptic receptor desensitisation or modifications of the number of release sites (Hashimoto and Kano, 1998).
Function. Although olivary neurons discharge at low firing rates, the CFs have an important role in normal cerebellar function. It has been shown that CF loss translates into motor deficits (Llinas et al., 1975; Rondi-Reig et al., 1997). That CFs exert an inhibitory effect on PCs has been shown by experiments involving (i) the inactivation of the inferior olive which increased PC simple spike discharge (Montarolo et al., 1982; Savio and Tempia, 1985) and (ii) sustained CF stimulation, which decreased PC spontaneous simple spike activity (Rawson and Tilokskulchai, 1981); these experiments suggest that CFs control the PC ‘pacemaker’ generator. In addition, the CFs also exert a strong effect on the parallel fibre-PC synapse. CFs appear to regulate synaptic plasticity at the parallel fibre input. It does this by inducing large dendritic Ca\textsuperscript{2+} influx that acts as a trigger for long-term decrease in synaptic strength - i.e. long-term depression (LTD) - rather than long-term potentiation (LTP) at parallel fibre-PC synapses (Coesmans et al., 2004).

In addition to CF induction of LTD at the parallel fibre-PC synapse, CFs also exhibit homosynaptic LTD. This means that tetanic stimulation (>5 Hz) of the CF alone can lead to LTD at the CF-PC synapse (Hansel and Linden 2000; Carta et al., 2006) via a postsynaptic mechanism (Shen et al., 2002) which is not associated with changes in PPD. This CF-LTD entails a reduction by twenty percent of the CF-EPSC, the Ca\textsuperscript{2+} influx and the spikelets in the complex spike (Hansel and Linden 2000; Weber et al., 2003; Schmolesky et al., 2005). The LTD of CF-induced Ca\textsuperscript{2+} dendritic signalling may be functionally significant. First, long-term changes in dendritic Ca\textsuperscript{2+} signalling may activate signals for synaptic modifications at other synaptic inputs at the PC. In this instance, large Ca\textsuperscript{2+} transients evoked by CF stimulation are a prerequisite for PF-LTD (Hansel et al., 2001). Second, CF-LTD may be a neuroprotective mechanism for the PC (Weber et al., 2003). During high periods of elevated complex spike activity, the high amplitude Ca\textsuperscript{2+} signals can be neurotoxic to the PC (O’Hearn and Molliver, 1997). Third, CF-LTD is associated with a decrease in the after-hyperpolarisation following tetanic CF stimulation. The after-hyperpolarisation of the complex spike is due in part to specific Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents (Schmolesky et al., 2005). A change in after-hyperpolarisation could potentially change the length of the complex spike-induced ‘pause’ and generate changes in PC output. Last, a reduction in the number of spikelets in the complex spike may lead to failure to initiate the respective spike in the axon hillock (Monsivais et al.,
2005). This may change the axon spike pattern and hence overall inhibition levels on the DCN. Therefore, as changes in the synaptic strength at the PC is assumed to underlie certain forms of learning, the long-term changes in Ca\(^{2+}\) dendritic signalling at CF-LTD could also have important consequences at a behavioural level.

Thus, the integration of the synaptic inputs onto the PC is important in mediating the PC’s activity. In certain cases, PC firing that is markedly disrupted can affect sensorimotor processing (Hoebeek et al., 2005). The described CF physiology can be used to understand the synaptic efficacy of reinnervating CFs onto PCs and the potential functional significance of these re-formed synapses.

### 2.2.2 Mossy fibre relay system

Mossy fibres (MFs) are the second main cerebellar afferent and have different morphologic and anatomic characteristics to the CFs. They are derived from multiple sensory sources in the brainstem and the spinal cord (e.g. spinocerebellar, vestibulocerebellar and lateral reticular, pontine reticulotegmental and external cuneate nuclei (Palay and Chan-Palay, 1974; Voogd and Glickstein, 1998). Those MFs that arise in the brainstem or pons enter the cerebellum via the contralateral middle cerebellar peduncle (Mihailoff et al., 1981), while spinal MFs enter the cerebellum through the inferior and superior peduncles (Chan-Palay et al., 1977). Similar to CFs, the MFs run as myelinated axons in the white matter and emit collaterals to the deep cerebellar nuclei (Arsenio-Nunes et al., 1998). From the white matter, MFs ascend into the GC layer to synapse onto GC soma and dendrites (Palay & Chan-Palay, 1974). Their synaptic terminals form ovoid swellings called rosettes that are enveloped by GC dendrites and Golgi cell axons.

Previously, it was thought that MF terminals had a very different spatial distribution in the cerebellar cortex compared to CFs. Yet new anatomical analysis shows the convergence of both afferent systems (Pijpers et al., 2006) such that non adjacent cerebellar zones that receive the same CF input also share the same MF input. Unlike their distribution, the action of MFs on PCs is different from that of CFs. First, excitatory activation of MFs is exerted onto PCs indirectly by the GC axons, PFs. Second, each PF contacts many PCs in the translobular plane and has few synapses with a single PC.
Lastly, PFs generate a single Na\(^+\)-dependent soma action potential (or simple spike) in the PC.

### 2.3 Cerebellar efferents

The major input to the DCN is the PC axons (Voogd and Ruigrok, 2004) and the organisation of the cortico-nuclear projection gives rise to the three functional zones of the cerebellum. PCs whose axons terminate onto the medial nucleus are located predominately in the vermis, ventral paraflocculus and flocculus, while PC axons which terminate in the interpositus and lateral nuclei primarily compose the paravermis and hemisphere, respectively (Figure 13). In turn, these three functional zones differ in the type of afferents received and are assumed to be implicated in different aspects of motor activity. For example the vermis is considered to control balance both in stance and locomotion (Diener et al., 1984; Thach et al., 1992; Thach and Bastian, 2004) whereas goal-directed and visually-guided movements, in addition to the modulation of rhythmic–synchronised movements by the paravermis and hemisphere (Schwartz et al., 1987; Cooper et al., 2000).

![Figure 13](image.png)

**Figure 13.** Frontal cerebellar section displaying the climbing fibre terminal organisation in the cerebellar cortex and DCN after PHA-L injection into the olive. It also provides a description of the different sagittal cerebellar zones and their corresponding cortico-nuclear output targets in the ipsilateral DCN. FN= fastigial/medial nucleus, RB= restiform body, NIA= nucleus interpositus anterior, NIP= nucleus interpositus posterior, LVN= lateral vestibular nucleus, LCN= lateral cerebellar nucleus. Modified from Van der Want et al., (1989).
Furthermore, each DCN provides output from the cerebellum in either descending or ascending pathways (Table 3) through which the cerebellum is involved in motor control and through the lateral nucleus links to the prefrontal cortex to cognitive function (Middleton and Strick, 1994; Kelly and Strick, 2003).

**Table 3. Summary of the major efferents of the DCN.** *Gray shading indicates a minor component of DCN output.*

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Descending</th>
<th>Ascending</th>
</tr>
</thead>
</table>
| **Medial** (mainly via inferior peduncle) | -vestibular nuclei  
                       -inferior olive  
                       -pontine reticulotegmental nuclei  
                       -lateral reticular nuclei  
                       -reticular formation | -superior colliculus  
                       -visual structures in midbrain  
                       -ventrolateral complex in motor thalamus |
| **Interpositus** (via superior peduncle) | -pontine reticulotegmental nuclei  
                       -MAO and PO | -red nucleus  
                       -ventrolateral complex in motor thalamus |
| **Lateral** (via superior peduncle) | -pontine reticulotegmental nuclei  
                       -reticular nucleus  
                       -inferior olive | -red nucleus  
                       -ventrolateral complex in motor thalamus  
                       -frontal eye field  
                       -dorsolateral prefrontal cortex |

In summary, the cerebellar circuit is formed by the aforementioned neurons and afferents (Figure 9). The key neuron of the circuit is the PC, as it integrates a variety of inputs and is the sole efferent of the cortex. PCs receive 2 main excitatory inputs: CFs synapse direct onto PCs in a 1:1 (CF: PC) ratio and MFs indirectly via GC axons in approximately 100,000:1 (MF: PC) ratio (Eccles *et al*., 1966a; Ito, 1984). CFs stimulate sagittally oriented bands of PCs, whilst PFs activate a transverse beam, and it is presumed that at the intersection of these two afferents that cerebellar function takes place. Simultaneously, PC activity is modulated by the basket and stellate axons (located in a plane perpendicular to PFs) which sharpen the PC output by inhibiting sagittally adjacent PCs. The Golgi cells provide feedback inhibition to the MF-GC synapse and inhibit the transversely activated PC beam (Palay and Chan-Palay, 1974). The PC axons exit the cortex and inhibit the DCN and in turn the DCN axons exit via the superior and inferior peduncles and transmit information primarily to other motor centers (for instance, the red nucleus and thalamus).
2.4 Functions of the cerebellum

The anatomical organisation of the cerebellum and its afferents and efferents are particularly important when considering the role of the cerebellum in different forms of processing information. However, the relative contribution of each structure as well as the functional changes that occur between these structures and other motor and non-motor centres of the brain during learning and memory is unclear. Overall, in terms of cerebellar efferent flow, there appears to be two closed efferent ‘loops’: one with the red nucleus and the other with the premotor/motor cortex (Figure 14). In the first case, the cerebellar DCN projects axons to the red nucleus which in turn projects to the precerebellar nuclei of the spinal cord, medulla (e.g. external cuneate nucleus and inferior olive) and pons (e.g. pontine gray and vestibular nuclei) that also receive peripheral input. The precerebellar nuclei innervate the cerebellar cortex and a subset of DCN neurons to form one closed circuit (Qvist, 1989; Altman and Bayer, 1997). As the precerebellar nuclei receive peripheral input (by way of the spinal cord) and cerebellar input (by way of the DCN and red nucleus), they could be part of a regulatory or readjustment system between both external and internal input (Altman and Bayer, 1997).

In the second case, the cerebellar DCN project directly to the thalamus that receives input from the motor cortex, and the premotor, parietal and prefrontal cortices. Also, the thalamus and the motor cortex project to the pontine nuclei, which in turn project to the DCN forming another closed circuit. This massive descending path from the cerebral cortex to the pontine nuclei is thought to carry information about an intended action (Altman and Bayer, 1997). The structural link between the cerebellar cortex-DCN and cerebellar function provides a site where the process of motor learning and non-motor activities may occur, although the mechanisms involved are not fully elucidated.
Figure 14. Schematic diagram outlining the major efferent pathways of the cerebellar DCN. Within the lower closed loop (in blue), there is also projections from the red nucleus to the DCN (direct) or via the precerebellar nuclei to the DCN (indirect). The other closed loop (in red) predominately involves the thalamus, cerebral cortex and pontine gray nuclei (Modified from Altman and Bayer, 1997).

2.4.1 Motor function

Cerebellar motor learning

The structure and function of the cerebellar cortical network has inspired several theoretical models of its function. Theorists have focused on the role of the cerebellar cortex and its connections in the control of action and acquisition of motor memory (Brindley, 1969; Marr, 1969; Albus, 1971; Kawato and Wolpert, 1998), emphasising the differences between the two main cerebellar afferents. Briefly, in the Marr-Albus theory, learning to link new actions and the contexts in which they should be executed was modelled on experience-dependent acquisition (Marr, 1969). In these contexts, the strength of synapses between PFs and PC dendrites could encode these links to eventually make an action automatic (Marr, 1969; Albus, 1971). Therefore, the PFs convey information to the PC concerning the context in which a movement is made and this context is represented as patterns of high firing rates of PCs (Marr, 1969; Albus, 1971).
also specifies that when an error in movement has occurred, it is the CF input which conveys error-information to the PCs via low frequency but synaptically powerful firing (Marr, 1969; Albus, 1971). This adjusts the synaptic strength of synapses of simultaneously active PFs on PC dendrites. Thus as motor skill improves, CF-PC synapses are reduced in strength so that when optimal motor function is achieved, the error-induced CF input is eliminated and the inferior olive returns to slow background firing. Therefore, after behaviour is learned, CFs can be experimentally deleted but appropriate motor operations in that context can still occur, via the remaining PF-PC synapses (Marr, 1969; Albus, 1971; Ito, 1984; Raymond et al., 1996). This form of physiological learning relies on the CF pathway, as inferior olive lesions (with an intact cerebellar cortex) prevent subsequent motor learning (Llinas et al., 1975; Welsh et al., 1995). Hence the CF pathway is an important participant within the motor system that generates motor learning.

Effects of cerebellar lesions on motor functions

The importance of the cerebellum and its components in motor functions are demonstrated by studies using cerebellar lesions and mutant mice. The removal of the whole cerebellum causes a lack of sensorimotor coordination and impaired equilibrium in both neonatal and adult rats (Zion et al., 1990; Caston et al., 1995). Comparatively, the removal of half of the cerebellum has a differential effect on adult and neonatal rats (Molinari et al., 1990). In adult rats the effects include an asymmetrical gait, deficits in dynamic postural adjustments and coordination and a side preference contralateral to the lesion (Molinari et al., 1990). However, neonatally hemicerebellectomised rats exhibit a normal gait but a delay in acquiring dynamic postural adjustments and a permanent impairment of motor skills such as crossing a narrow path or suspension from a wire (Petrosini et al., 1990). The transition from normal motor development to defective motor behaviour following neonatal hemicerebellectomy, referred to as ‘growing into a deficit’, is evidenced in the progressive reduction of hindlimb grasping techniques and a direction bias in posture correction (Petrosini et al., 1990). It is suggested that these motor abnormalities are independent of cerebellar control in early development, but as the rat matures, the retention of these motor skills requires a cerebellar circuit (Petrosini et al., 1990). Collectively, these studies indicate that the age of lesion affects the functional recovery of the animal.
Further investigations using mutant mice indicate the relative roles of the DCN and the
cortex in cerebellar function. These mutant mice have included those with cerebellar
cortical and inferior olive degeneration (e.g. Lurcher mice), GC degeneration (e.g. 
staggerer mice) or development of an excess of neurons due to an over-expression of the
human bcl-2 gene (Hu-bcl-2). Lurcher mutant mice are ataxic and limited in equilibrium,
motor coordination and postural sensorimotor skills in complex motor tasks (i.e. rotarod; 
Caston et al., 1998; Le Marec et al., 1997a; Hilber and Caston, 2001). Because DCN
output is the only part of the cerebellar circuit remaining in this mutant, evidence suggests
that DCN activity is adequate for simple sensorimotor tasks but not for more complex
tasks. Similarly, transgenic Hu-bcl-2 mice that possess supernumerary PCs, GCs and
inferior olive neurons, display impairment of complex motor abilities such as
synchronised walking movements but not in basic motor tasks (Rondi-Reig et al., 1999).
This indicates that one function of the DCN is to learn and maintain simple sensorimotor
behaviour while a correct cerebellar cortical circuit is necessary for complex learning.

Since the cerebellar cortical circuit requires an input in order to function, the cerebellar
afferents, CFs and MFs, are also necessary for complex motor tasks (Jones et al., 1995;
Rondi-Reig et al., 1997; Gasbarri et al., 2003). Bilateral lesions to the inferior olive, by
using 3-acetylpyridine (3-AP) which kills the neurons of the inferior olive, initially causes
rats to display deficiencies in motor coordination and static equilibrium (Rondi-Reig et
al., 1997). However, if rats undergo pre-lesional training, some of the motor
incoordination deficits are decreased (Jones et al., 1995), indicating that CFs are needed
to learn the task but not to retain a learned task. One reason for the reduction in motor
deficits is that undamaged adult CFs sprout to reinnervate the deprived PCs (Rossi et al.,
1991a; Rossi et al., 1991b). However, given that adult CF structural modifications do not
lead to motor compensation for highly synchronised movements (e.g. equilibrium ability
on the rotarod at 10 to 20 revolutions per minute; Rondi-Reig et al., 1997), this
explanation seems incomplete. Comparably, juvenile CF lesioned rats are initially
inefficient in equilibrium tasks yet improve with training on the rotarod (Jones et al.,
1995). Furthermore, bilateral pontine nuclear degeneration (i.e. MF loss) causes deficits
in static equilibrium and motor coordination on the rotarod, replicating results of CF
lesion studies (Gasbarri et al., 2003).
Therefore while the the DCN are required for simple motor tasks, loss of the cerebellar cortex and both afferents affect both equilibrium and synchronisation of movements (i.e. rotating rotarod). This concides with the CFs and MFs being afferents to the DCN and cerebellar cortex, and notably the CF pathway appears to be involved in learning complex motor tasks.

2.4.2 Cognitive function

Support for a role for the cerebellum in cognitive functions is derived from a variety of sources, including neuroanatomical, functional imaging, clinical and experimental studies (Botez et al., 1985; Botez-Marquard and Botez, 1993; Schmahmann, 1998; Lalonde and Botez-Marquard, 2000; Rapoport et al., 2000; Marien et al., 2001). The extensive interconnections between the neocortex and lateral cerebellar hemispheres has led to the interpretation that these two structures are functionally related (Middleton and Strick, 2000) hence implying a cerebellar role in cognition. Although studies demonstrate cerebellar processing in non-motor tasks, the specific regions of the vermis and the lateral zones involved in these functions remain poorly defined. Generally, lesions of the lateral zone of the rat cerebellum induce selective deficits in spatial orientation tasks but not in visuomotor abilities, while lesions to the vermis impair only visuomotor abilities (Joyal et al., 1996). Furthermore, vermal lesions induce autistic-like symptoms, such as limited attention capacity and decreased anxiety (Caston et al., 1998; Bobee et al., 2000).

Associative learning

The earliest studied example of cerebellar cognition was in associative and non-associative forms of learning (McCormick and Thompson, 1984; Berthier and Moore, 1986). Cerebellar involvement in associative learning is shown in an avoidance-conditioning task (Dahhaoui et al., 1990). In this task, cerebellectomised rats learn as fast as intact rats to avoid an electric shock when given an auditory stimulus (Dahhaoui et al., 1990). However, retention of the acquired conditioned response does not occur, indicating that the cerebellum is required for maintaining learned behaviour (Dahhaoui et al., 1990). In contrast, Lashley and McCarthy’s (1926) study did not find this effect, as animals showed no retention deficits. This finding may be due to the differing experimental protocol used, as Lashley and McCarthy (1926) did not quantify the acquisition phase of
learning the task. Although many studies exist on the relative role of the cerebellar cortex and DCN in the acquisition of eyblink conditioning, it will not be reviewed here.

Non-associative learning

Cerebellar lesions also reveal impairments in non-associative forms of learning, such as habituation (Lalonde and Botez, 1985; Lalonde and Botez, 1990; Dahhaoui et al., 1992a). Habituation is defined as the decrease in explorative behaviour in response to the repeated presentation of the same environment (Lalonde and Botez, 1990). In one study, it was found that cerebellectomised rats that undergo post-lesion training show no deficits in retaining habituated behaviour (Dahhaoui et al., 1992a). However, rats experiencing pre-lesion training are retention-impaired. Habituation has also been evaluated under other paradigms such as in complex environments (for example, learning the spatial orientation of holes on a board; Lalonde and Botez, 1985). Nervous (nr) mutant mice which have damaged PCs and DCN, lack the ability to retain the learned behaviour in their complex environment compared to controls (Lalonde and Botez, 1985). This study suggests that an intact cerebellar cortical circuit and afferents are needed for learning and retention of spatially demanding tasks.

2.4.3 Spatial function

Recent reports indicate that one cognitive function affected by cerebellar pathology is the inability to cope with spatial demands (Petrosini et al., 1996; Molinari et al., 1997; Gandhi et al., 2000). Anatomically, the cerebellum is connected to the neural structures responsible for visuospatial abilities, specifically, the frontal and parietal cerebral cortex regions, the limbic system and the superior colliculi (Schmahmann, 1998). Although cerebellar lesions cause deficiencies in visuospatial abilities, illustrating the importance of visual input for spatial processing, an awareness of space implicates mnemonic functions and learning the location of relevant objects in a given environment. Until recently, only two brain regions, the hippocampus and the associative parietal cortex are described as structures in spatial processing (O’Keefe and Nadel, 1978; DiMattia and Kesner, 1988; Whishaw and Tomie, 1997). However, studies also indicate the cerebellum is inclusive, particularly in the procedural components of spatial processing (Petrosini et al., 1996; Mandolesi et al., 2001). The procedural elements are defined as controlling the way in which an object can be reached, an environment is explored and spatial knowledge is
acquired in relation to personal orientation (Petrosini et al., 1996). Subsequently, cerebellar function relates to how to find an object rather than where an object is (Petrosini et al., 1998; Leggio et al., 1999).

*How to find an object* also referred to as spatial learning, primarily involves the integration of both the environment (sensory) and the exploratory (motor) acts of an animal (Poucet and Benhamou, 1997; Petrosini et al., 1998; Mandolesi et al., 2001). In this study, spatial learning is operationally defined as the ability to learn self-controlled movements in space in order to reach a non-visible target (Rondi-Reig et al., 2002). This requires the integration of internal (vestibular, proprioceptive) and external (auditory, olfactory, visual) sensorimotor information, to locate and orient oneself towards a non-visible target (Rondi-Reig et al., 2002). Sensory motor input is derived from the vestibular system, spinal cord and superior colliculi, and from other brain regions and is conveyed to the cerebellar cortical circuit via the two major cerebellar afferents, the CF and the MF-GC-parallel fibre pathway (Berthoz and Viaud-Delmon, 1999). Damage to these afferents inhibits spatial learning but spares the execution of the motor act (Rondi-Reig et al., 2002).

The main experimental apparatus used to analyse spatial learning is the Morris Water Maze (MWM; Morris et al., 1982). In this maze, rats or mice are repeatedly placed into a circular pool filled with water in order to learn how to escape (Figure 15). Their escape is possible if they locate a platform, either a visible one (cued test) or one that is submerged (referred to as the hidden platform; Morris, 1984). Different strategies used by rats to locate the platform are identified as: (i) praxic strategies – using a learned sequence of movement; (ii) taxic strategies – using cues proximal to the platform; or (iii) mapping strategies – using egocentric or allocentric encoding to form an internal map (O’Keefe and Nadel, 1978; Poucet, 1993; Petrosini et al., 1998). Egocentric encoding is referencing spatial information about the environment with respect to the animal’s own body space (i.e. ‘place’ cells in the hippocampus fire at relevant locations) while allocentric encoding is referencing with respect to aspects of the external world (i.e. learning the relation of certain visible objects to the platform; O’Keefe and Nadel, 1978; Poucet, 1993). Animals learn locate and reach a non-visible target through the utilisation of both egocentric and allocentric cues.
Figure 15: Schematic diagram of the Morris Water Maze which can include 4 starting positions (North, South, East and West) for the animal. External cues (e.g. green triangle) are kept in fixed positions to aid navigation.

Effects of cerebellum lesions and genetic mutations on spatial function

Both cerebellectomised and hemicerebellectomised rats are unable to develop an effective searching behaviour in the MWM (Le Marec et al., 1997b; Petrosini et al., 1996). Their repetitive circling around the periphery of the pool (i.e. thigmotaxis) resembles the initial searching behaviour exhibited by intact developing animals, characteristic of rats at postnatal days 17 to 24 (Petrosini et al., 1996), when cerebellar maturation is incomplete (Altman and Bayer, 1985). However, when the platform is visible these lesioned animals are able to reach it (Petrosini et al., 1996; Leggio et al., 1999), indicative of inefficient strategies rather than poor motor ability.

In contrast, Lurcher mutant mice that possess a decorticate cerebellum and intact DCN, show deficits only in controlling their swimming trajectory in the MWM, regardless of whether the platform is visible or hidden (Lalonde et al., 1998). Yet, Lurcher mice can significantly decrease their escape latencies to reach the hidden platform, implying increased efficiency in learning to find the hidden platform (Lalonde et al., 1998).

In addition, animals with a selective loss of GCs (weaver mutant; X-irradiation) have a decreased ability to locate the hidden platform (Lalonde and Botez, 1986; Le Marec et al., 1997b) although without visuomotor deficits (Le Marec et al., 1997b). Therefore the loss of GCs is associated with a failure to learn the task rather than with motor disability.

Comparable to complete GC loss, PC degeneration, e.g. in PC degeneration (pcd) and nervous (nr) mutant mice that are characterised by PC degeneration after development and maturation of the CFs and cortical circuit, interferes with the acquisition of the spatial
task (Goodlett et al., 1992; Lalonde and Strazielle, 2003). Similarly, a specific lesion to PCs (via immunotoxin OX7-saporin) also significant impairs performance in the hidden platform task (Gandhi et al., 2000). In addition, Hu-bcl-2 adult transgenic mice, which possess supernumerary neurons in the cerebellar cortex, display spatial deficits (Rondi-Reig et al., 2001). Hence, the presence of PC output and correct cerebellar circuitry (that is, the correct number of neurons) appear to be necessary for normal spatial learning abilities.

As CFs and MFs are crucial to cerebellar cortical function, it is no surprise that a few studies examining the sequela of CF or MF lesions have implicated these afferents in spatial navigation (Table 4; Dahhaoui et al., 1992b; Meignin et al., 1999; Rondi-Reig et al., 2002; Gasbarri et al., 2003). Animals with bilateral CF lesions are significantly slower to learn the maze but eventually resemble control animals (Dahhaoui et al., 1992b; Rondi-Reig et al., 2002). Interestingly, this experimental group also forget the task more quickly (Dahhaoui et al., 1992b), suggesting that disruption of CF input interferes with learning and retaining spatial information. In contrast, when either both afferents or only the PFs are totally lesioned the animals remain impaired in spatial learning (Le Marec et al., 1997b; Rondi-Reig et al., 2002). This indicates that learning is dependent on both intact CF-PC and PF-PC synapses.

Table 4. Various lesions used to differentiate the relative role of both PC afferents in navigation tasks. Modified from Rondi-Reig and Burguiere (2005).

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Hidden platform</th>
<th>Visible (cued) task</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Complete CF lesion- Intact PF</td>
<td>Delayed</td>
<td>Not done</td>
</tr>
<tr>
<td>Intact CF- Partial PF lesion</td>
<td>Delayed</td>
<td>Not done</td>
</tr>
<tr>
<td>Intact CF- Complete PF lesion</td>
<td>Impaired</td>
<td>Not done</td>
</tr>
<tr>
<td>Complete CF lesion- Partial PF lesion</td>
<td>Impaired</td>
<td>Normal</td>
</tr>
<tr>
<td>Complete CF&amp; PF lesion</td>
<td>Impaired</td>
<td>Normal</td>
</tr>
</tbody>
</table>

In summary, the cerebellum is involved in motor learning and motor control with the cerebellar cortex and its afferents essential for fine motor control (i.e. equilibrium and posture). Notably, the CFs are necessary for learning complex motor functions e.g. the control of highly synchronised movements (on the rotating rotarod), while the DCN is mainly implicated in simpler sensorimotor abilities. In addition, the CFs appear to be
required for normal spatial functions potentially by aiding procedural components. The role of PFs in spatial functions remains elusive as the control test for proper swimming capabilities (i.e. the visible platform test) has not yet been undertaken (Table 4).

In order to assess whether or not olivocerebellar reinnervation conferred normal behavioural function, we analysed the spatial learning abilities of animals with and without reinnervation (as motor development and function had been previously examined; Dixon et al., 2005). By knowing the relative role of CFs in spatial function, this gave us a comparator for the behaviours seen in reinnervated and non-reinnervated animals. Moreover, it also enabled us to examine the effect of BDNF treatment on complex behavioural functions (i.e. the rotarod).

In the following section, the normal developmental sequences of the rat cerebellum are considered. As a number of factors, including the neurotrophins, influence cerebellar development, an outline of normal development appeared essential in order to understand the function of spontaneous and BDNF-associated reinnervation post-lesion.
2.5 Development of the cerebellar neurons and afferents

In the rat, most neurons of the olivocerebellar system are produced prenatally while the cortical circuitry of the cerebellum develops mainly postnatally and maturation is complete by postnatal day 30 (P30) (Altman and Bayer, 1985). The first neurons to form are those of the inferior olive, followed by DCN and then PCs, which leave the ventricular neuroepithelium and settle in the developing cerebellar cortical plate before birth (Altman and Bayer, 1985). The inhibitory interneurons also originate from the neuroepithelium of the 4th ventricle, migrate like the PCs but continue to proliferate during their transit through the deep cerebellar mass (Zhang and Goldman, 1996, Schilling, 2000). Similarly, a secondary neuroepithelium, the external germinal layer (EGL), is generated prenatally, the precursor cells migrating onto the surface of the cerebellar plate by birth (Altman, 1972a). This latter epithelium produces the GCs (Altman, 1972a; Altman and Bayer, 1997).

2.5.1 Purkinje cells

The PCs originate in the neuroepithelium of the 4th ventricle from embryonic day 13 (E13; Altman, 1975). They ascend through the zone of the DCN to the cortical plate on the surface of the cerebellar primordium anlage by E15 (Altman and Bayer, 1978), leaving their axons behind and establishing synaptic contacts with DCN neurons as early as E20 (Eisenman et al., 1991). From E20, CF axons are in the PC plate (Chedotal and Sotelo, 1992) and make transient contacts with the PCs (Morara et al., 2001), suggesting very early influence of CFs on PCs. At birth, the morphological features of PCs are clearly apparent at the inner boundary of the EGL and are arranged in a plate 6 - 12 cells deep (Addison, 1911; Altman and Bayer, 1985). Their somata contain a large clear oval nucleus and minimal cytoplasm that emits short fine processes across the EGL (Addison, 1911; Altman, 1972b).

From birth to postnatal day 2 (P2), transient synapses exist between CF axon terminals and emerging transient dendrites of the PCs (Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993). From P3 to P4, PCs begin to align in a monolayer (Addison, 1911; Altman, 1972b), which is thought to be influenced by parallel fibre growth from above and GC migration to below the PCs (Goldowitz & Hamre, 1998). They display fine somatic
processes that receive immature CF arbors (Altman, 1972b; Mason et al., 1990; Armengol & Sotelo, 1991). From P5 to P7, a large apical cone develops increasing the PC somatic cytoplasm and extending into the molecular layer (Figure 16; Addison, 1911; Altman, 1972b). At P7, PCs begin to develop primary dendrites which receive PF synapses (Tanaka et al., 1994; Scelfo and Strata, 2005) while their perisomatic processes receive CF synapses (Altman, 1972b).

![Figure 16](image)

**Figure 16.** Representation of the major developmental events of the PC and molecular layer maturation from P3 to P21. S= stellate cell, B= basket cell. Modified from Altman 1972b.

The morphology of the PC has transformed by P10, with somatic processes reabsorbed into the soma and basket cell axons contacting PC somata (Berry and Bradley, 1976; Altman, 1972b). By P12, the apical dendrites enlarge forming numerous secondary and tertiary branches (Altman, 1972b). From P12 to P15, the PC dendritic arbor increases in complexity, first in the lateral domain and then growing in height in parallel with the molecular layer (Berry and Bradley, 1976) and generation of PF synapses (Altman, 1972b). By P14, the distal branches of the PC dendrites and spines located in the lower
half of the molecular layer receive PFs (Altman, 1972b). At P15, the PC dendritic tree has formed its full width and the PC soma is synaptically mature (Berry and Bradley, 1976). By P21, PFs synapse with the distal spines on the dendritic tree in the upper half the molecular layer (Altman, 1972b). The growth of the PC dendritic tree continues through the upper molecular layer until P30 as PFs continue to lengthen (Berry and Bradley, 1976).

A number of factors influence the development of the PC dendritic tree, notably PFs, CFs, endogenous electrical activity and neurotrophins. In the absence of PFs (i.e. weaver, reeler and postnatally X-irradiated rats; Sotelo, 1978; Crepel et al., 1980; Doughty et al., 1999; Sugihara et al., 2000), the PC layer is poorly aligned and the dendrites stunted, less branched and studded with extraneous ectopic spines. Notably, glutamate-mGluR1 signalling at the PF-PC synapse affects PC dendritic growth and differentiation (Catania et al., 2001; Kapfhammer, 2004). In contrast, CF-deprived PCs exhibit grossly normal dendritic morphology with primary, secondary and tertiary branchlets (Sotelo and Arsenio-Nunes, 1976). However, these PC trees are reduced in length and number of branch points (Bradley and Berry, 1976), with the proximal PC dendrite studded with spines contacted by PFs, stellate and basket cells (Sotelo and Arsenio-Nunes, 1976). Therefore, CFs do not seem to be essential for the initial phases of PC dendritic development but become important for complete growth of the PC tree and separation of different afferent input (Sotelo, 2004). Electrical activity also appears to be necessary for the refinement of PC dendritic morphology, as spontaneous activity blockade by tetrodotoxin (TTX) allows dendrites to continue branching – reproducing their earlier dendritic growth phase (Schilling et al., 1991) and ectopic spinogenesis (Bravin et al., 1999). The effect of neurotrophins on PC dendritic differentiation will be discussed in the following section.

2.5.2 Granule cells

At birth, the EGL has formed 6 to 8 rows of differentiating GC precursors (Addison, 1911). These cells undergo rapid proliferation until P9 and the EGL increases in depth with 8 to 12 rows of cells (Altman, 1972a). The EGL displays two distinct zones of cells: an outer proliferative zone and an inner premigratory zone of bipolar cells (Altman, 1972a). The bipolar cells of the premigratory zone grow processes that elongate laterally and run parallel to the developing pial surface to become PFs whilst the bipolar cell
somata remain on this plane (Altman, 1972a). Once the length of the PFs has reached their optimal value, the soma begins to migrate vertically to its position in the internal granular layer (IGL) while the PF remains in the upper region of the molecular layer (Altman, 1972a). By P6, functional synapses exist between PFs and PCs (Tanaka et al., 1994; Scelfo and Strata, 2005). From P7 to P12, the GC numbers increase reaching peak generation during P10 to P11 and maximum migration between P9 to P17 (Altman, 1972a). From P15, the GCs in the IGL develop short claw-like dendrites that envelope the immature MF rosettes (Altman, 1972c).

2.5.3 Climbing fibres

CF development is concurrent with the development of the cerebellar cortex. The inferior olive neurons are generated in the dorsal neuroepithelium of the caudal hindbrain between E12 and E13 (Altman and Bayer, 1978; Bourrat and Sotelo, 1991) and their axons extend and invade the cerebellar cortical plate by E17 where they are arranged in broad sagittal zones resembling the adult CF distribution (Chedotal and Sotelo, 1992; Chedotal and Sotelo, 1993). During this period, immature CFs appear as bundles distributed amongst PC clusters (Chedotal and Sotelo, 1992) and their targeting appears to be influenced by chemoattractant molecules from the embryonic cerebellum (Chedotal et al., 1997; Sotelo and Chedotal, 1997; Zhu et al., 2003). The onset of transient (non-synaptic) contacts between CF axon terminals and transient PC somatic processes first occur embryonically (E20; Morara et al., 2001; Chedotal et al., 1993) and not postnatally (P0 to P2; Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993). But it is postnatally that CFs undergo four major developmental stages.

1. The creeper stage: At birth, immature CFs are unbranched terminals with small tapered growing tips or growth cones (Chedotal and Sotelo, 1993) which are distributed in broad sagittal zones resembling the adult olivocerebellar distribution (Sotelo et al., 1984). By P2, they begin to ‘creep’ as mini arbors to associate with immature PC somata and their transient somatic processes via attachment plates within the PC plate (Figure 17; Chedotal and Sotelo, 1993). Although this contact between CFs and transient PC processes regresses by P3, a large proportion of CFs retain contact onto several adjacent PCs (Mason et al., 1990; Chedotal and Sotelo, 1993). These early postnatal contacts are functional (Crepel et al., 1976), despite their perisomatic location. At P3, the EPSPs
induced by CFs are similar to the ones initiated by adult CFs, except they are of longer
duration and smaller synaptic amplitude (Crepel, 1971; Hashimoto and Kano, 2003).

2. **The pericellular nest stage**: By P5, CFs make ‘nests’ onto the PC somata aligned in a monolayer (Figure 17; Mason et al., 1990; Chedotal and Sotelo, 1993). Each PC receives input from several CFs, with a maximum mean number of 3.5 CFs synapsing onto perisomatic processes of each PC (Crepel et al., 1976; Mariani and Changeux, 1981).

3. **The capuchon stage**: Between P8 and P9, CFs progressively ‘climb’ upwards and terminate onto the main trunk of the developing PC dendritic tree (Mason et al., 1990). With CF translocation, there is a regression of multi-innervation that reduces the percentage of PC innervation by more than 50% (Crepel et al., 1976).

4. **The young CF stage**: From P10 to P15, reorganisation of CF terminals occurs so that by 2\(^{\text{nd}}\) (rat) to 3\(^{\text{rd}}\) (mouse) week, the adult 1:1 relation between CF:PC is achieved (Crepel et al., 1976; Crepel et al., 1981; Mariani and Changeux, 1981a & b; Kano et al., 1995; Kano et al., 1997; Kano et al., 1998) i.e. CF-EPSCs change from having multiple

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**Figure 17.** Phases of CF morphology throughout postnatal development. The PC layer is highlighted as grey zones. Modified from Sugihara (2006).
CF-EPSP induced steps during early development (P3 to P6) but produce only one CF-EPSP step in adulthood (Mariani and Changeux, 1981a & b; Hashimoto and Kano, 2003). The developmental elimination of surplus CFs to each PC is concurrent with CF-PC synapse maturation.

One salient feature of the developing CF-PC synapse is the elimination of transient CF multi-innervation. Many studies have been examined the correlation between neuronal death in the inferior olive and CF regression. Some studies concluded that neuronal death occurred from P1 to P5 but was followed by an apparent increase in neurons from P5 to P15 (Bourrat and Sotelo, 1984; Delhaye-Bouchaud et al., 1985; Armengol and Lopez-Roman, 1992), suggesting that CF elimination was independent of inferior olive cell death and actually a process of CF collateral retraction (Bourrat and Sotelo, 1984; Delhaye-Bouchaud et al., 1985; Shojaeian et al., 1985; Armengol and Lopez-Roman, 1992), which is also associated with the narrowing of the sagittal bands to adult width (Dupont et al., 1981). However, another study has shown that the survival of inferior olive neurons decreases from P10 onwards, indicating that regression is temporally congruent with inferior olive death (Cunningham et al., 1999). Differences in results stem from the fact that astrocytes and neurons were morphologically differentiated. Despite this decrease in inferior olive neuronal numbers coinciding with synapse elimination, the small proportion of neuronal death in the inferior olive cannot completely account for CF regression (Sugihara, 2006).

This CF elimination is highly dependent on the presence of GCs and the formation of normal PF-PC synapses (Crepel et al., 1976; Mariani & Changeux, 1980; Sugihara et al., 2000). Multiple innervation of CFs persists when GC numbers are decreased in mutant mice strains, weaver and reeler (Crepel et al., 1976; Puro and Woodward, 1977; Mariani et al., 1977; Mariani, 1982) and irradiated rats (Delhaye-Bouchaud et al., 1978; Mariani et al., 1990). Multiple-innervation is also retained when PFs are unable to form normal synapses with PCs in the staggerer and Lurcher mutant mice (Mariani and Changeux, 1980; Mariani, 1982; Sotelo, 1990). In addition, mutant mice deficient in proteins that initiate postsynaptic signalling pathways (via PF-PC synapses) display partially impaired transition from multiple to mono CF innervation. This is illustrated in mutant mice deficient in the glutamate receptor subtype mGluR1 (Kano et al., 1997), PKC (Kano et al., 1995), CaMK4/7 (Ribar et al., 2000) and PLCβ4 (Kano et al., 1998). In turn, the
PLCβ4 and CaMK4−/− mice display locomotor ataxia specifically of the hindlimbs (Kano et al., 1998; Ribar et al., 2000), consistent with CF dysfunction.

The restructuring of CF axonal branches during development also appears to depend on the electrical activity of the post-synaptic neuron. CFs and PFs release glutamate and the NMDA receptor has been implicated, at least partially, in CF synapse elimination (Rabacchi et al., 1992b; Kakizawa et al., 2000). From P7 to P15, PC somata and primary dendrites express NMDA receptor immunoreactivity whilst GCs are weakly reactive (Hafidi and Hillman, 1997). This expression coincides with GC generation, PF-PC synaptogenesis, CF translocation from PC soma to dendrite and CF elimination. Therefore, CF redundancy may be driven by cues from PCs (Rabacchi et al., 1992a; Bravin et al., 1995). This proposition is likely, given that PCs regulate structural modifications in the mature cerebellum (Rossi and Strata, 1995). Because synapse elimination involves the rearrangement of overproduced collaterals during development, it is likely that spontaneous reinnervation which involves the production of axonal collaterals to correct target neurons following target denervation may also be dependent on similar neuronal regulators of synapse formation and subsequent retraction.

2.5.4 Mossy fibres

MFs derived from the brainstem nuclei also develop embryonically and emerge into the white matter of the cerebellum by P3 (Arsenio-Nunes and Sotelo, 1985). From P3 to P5, they invade the internal granular cell layer to synapse onto GC dendrites and by P7 MFs are organised in the topographical arrangement matching that of the adult MF input (Arsenio-Nunes and Sotelo, 1985). By P15, the MFs are biochemically mature with rosettes and GC dendritic branches formed (Altman, 1972c). However, the presence of only minimal numbers of glomeruli prevents MF-GC maturity until P21 (Altman, 1972c) even though PF-PC synapses are active at P7 (Scelfo and Strata, 2005).
2.6 Neurotrophins and cerebellar development

Both the mRNA and protein levels of the neurotrophins and their receptors synthesised in the cerebellum have highlighted their importance in cerebellar development. Despite an incomplete view of some sites of neurotrophin synthesis, an overview of the interactions of neurotrophins during cerebellar neuronal and afferent development is considered in the next section (Table 5). This has been attempted in order to shed light on the rationale for studying BDNF in spontaneous and induced olivocerebellar reinnervation following injury.

2.6.1 Purkinje cell development

The first neurotrophin identified to promote PC survival was NGF (Cohen-Cory et al., 1991). Interestingly, the action of NGF on PCs does not involve its preferential receptor, TrkA since PCs do not express TrkA (Aloe and Vigneti, 1992; Pioro and Cuello, 1990). PC survival and neurite elaboration requires the co-activation of mGluR or p75 which makes PCs responsive to NGF (Cohen-Cory et al., 1991; Mount et al., 1993; Mount et al., 1994; Mount et al., 1998; Florez-McClure et al., 2004). In contrast, if metabotropic stimulation or other excitatory input does not occur, PCs die (Mount et al., 1994). Hence, it appears that NGF promotes the maintenance of PCs only with the addition of other stimulation.

NT-3 is expressed in the embryonic cerebellum (Maisonpierre et al., 1990). Although PCs do not synthesise NT-3 mRNA, they do express TrkC and p75 at the same developmental stage (Ernfors et al., 1992; Lindholm et al., 1993a; Rocamora et al., 1993; Velier et al., 1997). This occurs at a time when EGL cells synthesise NT-3 (Taniuchi and Johnson, 1985). It has been proposed that GCs influence PC survival via a paracrine action or anterograde axonal delivery of NT-3 (Lindholm et al., 1993a; Mount et al., 1994). In parallel, TrkC is localised on PCs at P7 (Velier et al., 1997), when GC axons (PFs) begin to synapse onto PCs (Scelfo and Strata, 2005). Therefore, it has been hypothesised that NT-3 released by PFs coincides with the outgrowth of PC dendrites as observed in vitro (Lindholm et al., 1993a).
The role of BDNF in PC survival remains unclear: exerting either no effect (Mount et al., 1994) or promoting PC survival (Larkfors et al., 1996; Morrison and Mason, 1998; Shimada et al., 1998). Also, BDNF increases in the postnatal cerebellar cortex (Neveu and Arenas, 1996; Das et al., 2001) with peak synthesis occurring at P7 (Das et al., 2001). TrkB mRNA is found in immature PC somata and their apical dendrites, potentially linking a direct effect of BDNF-TrkB signalling to PC differentiation (i.e. primary, secondary and tertiary dendrites) particularly from P8 until P15 (Carter et al., 2002). Until recently, the effect of BDNF on PC dendritic differentiation gave conflicting results. It was previously shown that BDNF did not change PC dendritic formation although spines were more densely packed at BDNF-exposed dendrites (Shimada et al., 1998). However, BDNF manipulation does not produce consistent effects. Less BDNF signalling, via TrkB antibodies or BDNF +/- mice, decreased PC dendritic development (Schwartz et al., 1997; Hirai and Launey, 2000). In contrast, no abnormality in PC dendritic differentiation was found when: (i) the TrkB gene was conditionally deleted in mice (Wnt1: Cre:fBz/fBz; Rico et al., 2002; Figure 18) or (ii) BDNF was applied to cultured PCs (Adcock et al., 2004). Furthermore, PC dendritic development appeared normal by P24 using the same model as Schwartz and colleagues (above; Carter et al., 2002). It was also noted that these early PC dendritic abnormalities were a result of altered p75 signalling (Carter et al., 2003). Therefore, BDNF does not appear to affect PC dendritic differentiation but only spine formation, which has led to the hypothesis that spine development is probably not dependent on dendritic differentiation (Sotelo, 2004).

Figure 18. The dendritic differentiation of PCs in control and mutant mice (TrkB gene deleted). The white arrows outline normal primary dendrite formation in both groups. PCs are labelled by calbindin staining (Rico et al., 2002).
Thus, while the role of NGF remains unclear, BDNF does not appear to promote dendritic outgrowth but potentially spine formation on PCs.

2.6.2 Granule cell development

Both NT-3 and BDNF regulate GC survival, differentiation and migration. These two neurotrophins have complementary temporal expression patterns in developing GCs (Rocomora et al., 1993; Katoh-Semba et al., 2000; Das et al., 2001). NT-3 mRNA is synthesised in the prenatal cerebellum as the EGL is formed (Ernfors et al., 1992). During GC genesis and differentiation, there is a transient increase in cerebellar NT-3 mRNA (Neveu and Arenas, 1996), while differentiating GCs in the IGL and EGL express NT-3 and its receptor TrkC at P5 and P10, respectively (Lindholm et al., 1993a; Rocamora et al., 1993; Segal et al., 1995; Neveu and Arenas, 1996). Although a deletion of a single target gene for TrkC in mice does not cause major cerebellar abnormalities (Klein, 1994), the addition of NT-3 accelerates the exit of post-mitotic GCs from the EGL (Doughty et al., 1998). Moreover, mice lacking NT-3 have abnormal cerebellar development, particularly in the posterior lobe, and increased GC death, which suggests that NT-3 acts as a survival factor for some GCs (Bates et al., 1999).

By contrast, BDNF has a role in GC development later during development (i.e. from P10 to P20 when BDNF is upregulated in contrast to NT-3 (Rocomora et al., 1993; Das et al., 2001). GCs upregulate expression of both BDNF and TrkB (Wetmore et al., 1991; Rocamora et al., 1993; Segal et al., 1995), the latter being synthesised when postmitotic GCs have migrated from the EGL (Gao et al., 1995). BDNF also protects cultured postnatal GCs from apoptosis (Lindholm et al., 1993b; Kubo et al., 1995; Courtney et al., 1997). Thus, BDNF -/- mice not only have increased GC death in the IGL, but also increased thickness of the EGL (Schwartz et al., 1997), which is consistent with impaired GC migration in the absence of BDNF (Borghesani et al., 2002). Furthermore, BDNF and NT-4 influence the speed of GC growth cone migration and increase neurite elongation (Segal et al., 1995; Tanaka et al., 2000). Thus, NT-3 seems to contribute mainly to GC differentiation and migration, while BDNF/NT-4 – TrkB signalling promotes survival and axonal growth.
2.6.3 Climbing fibre development

Neurotrophic function has also been recently implicated in the development of cerebellar afferents. MFs derived from pontine neurons express TrkB which coincides with increasing BDNF synthesis in the cerebellum and synaptogenesis of MFs and GCs (Rabacchi et al., 1999). In CFs, as the inferior olive begins axonogenesis into the cerebellar cortical plate, NT-3 and NT-4 are synthesised in the embryonic cerebellum (Taniuchi and Johnson, 1985; Timmusk et al., 1993). This expression correlates with p75 transcripts in the inferior olive and TrkB and TrkC in the brainstem (Yan et al., 1988), so that by birth both neurotrophin receptors are expressed in the inferior olive (Ernfors et al., 1992; Ringstedt et al., 1993; Riva-Depaty et al., 1998). Although neither NT-3 nor NT-4 mRNA have been detected in the inferior olive, their proteins are present (Friedman et al., 1998). As neurotrophin receptors are anterogradely transported along their axons to their terminals (Bhattacharyya et al., 2002) where neurotrophin-receptor binding can occur, cerebellar NT-3 or NT-4 could bind to the CF receptor and be retrogradely transported to the inferior olive. This is one possible explanation for the presence of NT-3 and NT-4 protein in the inferior olive (Friedman et al., 1998). In addition, this suggests that cerebellar neurotrophins may act in a target-derived manner on CF development.

From birth until P5, immature CF terminals synapse on PC perisomatic processes and by P5 each PC is innervated by several CFs (Mariani and Changeux, 1981). BDNF expression increases both in the cerebellum and inferior olive at P5 (Figure 19; Sherrard and Bower, 2002).
Rocamora et al., 1993). Likewise, TrkB is abundantly expressed in the inferior olive and PCs (Riva-Depaty et al., 1998; Shimada et al., 1998). These findings raise the possibility that inferior olive neurite growth may be maintained by one of several mechanisms including autocrine and paracrine actions (Sherrard and Bower, 2002). First, this may involve autocrine stimulation of olivary TrkB receptors by olivary BDNF. Second, because the expression of BDNF and TrkB temporally overlaps between PCs and the inferior olive, it has been suggested that CFs may anterogradely transport TrkB receptors to their terminals in the cerebellum (Sherrard and Bower, 2002). The binding of cerebellar BDNF to TrkB receptors may generate mechanisms responsible for the growth of olivocerebellar axons and their collaterals (Sherrard and Bower, 2002).

A role for BDNF in CF development is further supported by the olivocerebellar expression patterns of other neurotrophins. The expression of TrkA in the inferior olive is low (Riva-Depaty et al., 1998) and NGF mRNA expression decreases (Neveu and Arenas, 1996), suggesting that NGF is not a major participant of CF growth and synaptogenesis (Sherrard and Bower, 2002). Moreover, NT-3 mRNA is absent from the inferior olive (Rocamora et al., 1993) but it is present in cerebellar GCs when TrkC is synthesised in the inferior olive (Ringstedt et al., 1993; Riva-Depaty et al., 1998). Although, GC NT-3 paracrine activity may be involved in CF-PC interactions, at this developmental stage there are only a few mature GCs to have this function (Sherrard and Bower, 2002). Therefore, it is unlikely that NT-3 – TrkC signalling gives rise to CF growth throughout the cerebellum (Sherrard & Bower, 2002).

As CFs ascend the PC primary dendrites from P7 to P10, neurotrophin expression in the cerebellum increases, notably BDNF, NT-3 and NT-4 (Figure 18; Neveu and Arenas; 1996; Das et al., 2001). In contrast, PC expression of BDNF and NGF mRNA begin to fall in PCs (Friedman et al., 1998). Similarly, decreased synthesis of BDNF, TrkB, TrkC and p75 also occurs in the inferior olive (Maisonpierre et al., 1990; Rocamora et al., 1993; Neveu and Arenas, 1996; Riva-Depaty, 1998; Das et al., 2001; Nitz et al., 2001). This diminishing expression of BDNF and TrkB in the inferior olive could begin to limit signalling at the CF terminal (Sherrard and Bower, 2002). In addition, from P10 to P15, the production of BDNF, TrkB, TrkC and p75 in the inferior olive continues to decrease (Ringstedt et al., 1993; Rocamora et al., 1993; Riva-Depaty et al., 1998) until CF mono-innervation is complete. Notably, a role for TrkB signalling in CF regression has been
recently suggested by Johnson et al., (2007). As both BDNF and receptor expression falls during CF maturation, it is possible that BDNF is implicated in both CF-PC interactions and CF retraction (Sherrard and Bower, 2002). This reduction in neurotrophin expression could be attributed to the temporally congruent cell death of inferior olive neurons and/or the change in axonal dependency/responsiveness to neurotrophins. Moreover, although the relationship between BDNF and CF-PC interactions are not clear, there is a coherency between diminishing BDNF expression and the cessation of spontaneous olivocerebellar reinnervation

In summary, it appears that at the stage of CF retraction and maturation, BDNF is most likely to be the major neurotrophin participant, although the role of NT-4 cannot be discounted. Therefore, increasing the availability of neurotrophic support during a period when neurotrophin levels are diminishing, may lead to increased axonal outgrowth and therefore post-lesional reinnervation. The use of neurotrophins in post-lesion CNS models and particularly in olivocerebellar reinnervation will now be reviewed.
Table 5. Summary of mRNA expression of the neurotrophins and their receptors.

<table>
<thead>
<tr>
<th>Region</th>
<th>p75R</th>
<th>TrkB</th>
<th>TrkC</th>
<th>BDNF</th>
<th>NT3</th>
<th>NMDA R(NR1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGL ML</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
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<td>PCL</td>
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<td>IGL</td>
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<td>DCN</td>
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<tr>
<td>IO</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

- = absent  
+ = present or low level  
++ = intermediate level  
+++ = high expression
3.0 Post-lesion Models in the Central Nervous System

Similar to the situation during early development, regenerative axonal growth post-injury requires the advance of the growth cone from injured neurons (i.e. ‘true’ axonal regeneration) or uninjured neurons nearby to grow to the denervated target (i.e. collateral sprouting). In the case of regeneration, the growth cone must transverse an inhospitable terrain (i.e. growth-inhibitory molecules associated with myelin) and an altered signalling environment (i.e. limited availability of target-derived growth factors and rearrangement of the distribution of guidance molecules). These factors make it less likely that regenerating axons re-establish the precise path taken during development and connect to the appropriate target neuron.

Many studies have aimed to increase regeneration and provide functional recovery following adult CNS lesions, but results of these studies are often conflicting. The three basic approaches that have been employed to encourage regeneration in the CNS (i.e. primarily in the spinal cord) are: (i) increasing the availability of neurotrophic factors (ii) blocking the inhibitory factors and their associated receptor, and/or (iii) modifying the internal state (i.e. growth-readiness) of the neuron so that its responsiveness to these signals is no longer inhibitory. These three approaches are now considered, in addition to factors that reduce growth of regenerating axons.

Neurotrophins. There are five major families of growth factors associated with neuronal development and repair (Henderson, 1996). In this review, the neurotrophins are only considered because they have been thoroughly studied, are relevant to the model system used in this thesis, and are the most widely distributed family in the CNS. Regeneration studies that have used neurotrophins have shown a divergent pattern of success. In the PNS, controversy remains concerning the role of neurotrophins in regeneration remains. Some experiments report improved axonal regeneration in the presence of neurotrophin application whilst others show that regeneration is inhibited (Diamond et al., 1987; Linnarsson et al., 2001). In particular, NGF was not required for normal sensory axon regeneration in an in vivo study where endogenous signalling of NGF was neutralised with a specific antibody (Diamond et al., 1987). However, NGF is needed for collateral sprouting of terminals in the target. In contrast, NGF does promote axonal regeneration in in vitro experiments (Lewin and Barde, 1996) although
the effect may not be direct as these regenerating axons may not express the appropriate receptors for NGF-activated signalling (McMahon et al., 1994).

In contrast to the PNS, exogenous application of neurotrophic factors has been shown more consistently to increase regeneration after injury in a number of animal models of CNS injury (Bregman et al., 1997; Grill et al., 1997; Jakeman et al., 1998; Coumans et al., 2001; Lu et al., 2004). In spinal cord transplant studies of embryonic tissue, administration of BDNF, NT-3 or NT-4 increases both the density and extent of axonal ingrowth into the transplants compared to vehicle-treated control-transplants only (Bregman et al., 1997). Furthermore, cerebellar neurons which are first cultured overnight (primed) with BDNF are resistant to axonal growth inhibition by myelin-associated inhibitory proteins (Cai et al., 1999). By priming neurons with BDNF, neuronal cAMP increases and if a protein kinase A inhibitor is also added, myelin-associated inhibition of axonal growth/neurite extension is completely suppressed (Cai et al., 1999). Previously, neurotrophin signalling was not considered to activate the cAMP pathway. However, Gao and colleagues (2003) reported that neurotrophins elevate cAMP by an ERK-dependent inhibition of the phosphodiesterases; a process that occurs in many cells including non-neuronal cells (Hoffmann et al., 1999; Baillie et al., 2000). Thereby, neurotrophin signaling cascades appear to overcome growth inhibition due to myelin-associated proteins and to enable interaction between ERK and cAMP signalling pathways.

A combination treatment including neurotrophins and embryonic transplants has been used to induce remaining undamaged neuronal pathways to sprout (recently shown also to occur spontaneously without post-lesion treatment; Bareyre et al., 2004) or to increase regeneration of damaged axons into the transplant (Bernstein-Goral et al., 1997; Bregman et al., 2002). This approach has led to improvement in behavioural recovery, particularly simple locomotor and plantar foot placement tests after spinal cord lesions (Z’graggen et al., 1998; Coumans et al., 2001). However, as the recovery of rhythmic and alternating stepping movements involved in locomotion relies on the intrinsic system within the spinal cord (i.e. the spinal cord pattern generator), the extent to which these activities relate to the induced regeneration remains unclear. This is further evident in studies which use specific behavioural tests (e.g. skilled fore-limb movements for stroke or unilateral cortico-spinal lesions; Z’graggen et al., 1998;
Figure 20) when axonal sprouting takes place by cortico-rubro-spinal fibres instead of cortico-spinal fibres and does not improve the dysfunction of skilled movements (Priestley, 2007; Smith et al., 2007).

**Figure 20.** Schematic diagram of the normal corticospinal circuit in the adult rat and also post-lesion. The grey box illustrates a unilateral pyramidotomy and the subsequent plasticity of the cortico-rubral fibres. RN=red nucleus; CST=corticospinal tract. A schematic diagram representing the results of Z’graggen et al., (1998) after unilateral pyramidotomy.

As previously mentioned, in addition to the neurotrophins, other factors intrinsic and extrinsic to the axon influence the promotion or suppression of growth post-injury, and in certain cases, the neurotrophins also influence these factors. These factors include the extracellular matrix molecules, Nogo/p75 receptors and the intrinsic capacity of regenerating neurons, and are also considered separately below.
**Extracellular matrix molecules (ECMs)-integrin signalling.** Extracellular matrix molecules and their receptors (integrins) are key players in axon growth during development and thus potentially may regulate axonal regeneration. The ECMs such as laminin and cell adhesion molecules (CAMs; for example L1 and neural adhesion molecules) bind to the integrins and promote a substrate for growth of the growth cone protrusion through interaction with the growth cone actin filaments and/or neurotrophin-induced signalling pathways ([Figure 21](#); Zhou and Snider, 2006). Extracellular guidance molecules bound to integrin induce axonal growth, independent of the PI-3K and ERK signalling cascades (Zhou et al., 2006). Interestingly, NGF induces clustering of integrins at the growth cone leading edge during regeneration (Liu et al., 2002). Although few studies have explored the role of ECM-integrin induced axon growth in regeneration, one study has reported that over-expression of integrin promotes axon growth over inhibitory proteoglycans (Condic, 2001).

![Figure 21. ECM-integrin signalling in conjunction with neurotrophin-Trk receptor signalling (blue dotted outline) to mediate axon growth. Integrin interacts with PI-3K and Raf-MEK-ERK pathways and also directly regulates actin dynamics and growth cone motility by controlling two actin-based motor proteins (myosin X and myosin II; Zhou and Snider, 2006).](image)

**Nogo and p75 interaction.** It has been suggested that it is the myelin-inhibitory factors in the CNS rather than the deficiency in growth-promoting molecules that makes the extracellular milieu a ‘barrier’ for regenerating axons (Berry, 1982; Schwab and Thoenen, 1985). Maturing astrocytes and oligodendrocytes express three main membrane proteins which cause growth cone collapse and are thought to contribute to poor regeneration. These proteins are NogoA, oligodendrocyte myelin glycoprotein...
(OMgp) and myelin-associated glycoprotein (MAG). All three proteins recognise the Nogo receptor (NGR) in neurons (Figure 22; Schwab, 2004).

The NGR does not contain a cytoplasmic domain, which implies that it associates with other receptors in order to function. Recently, it was observed the p75 is a co-receptor of NGR and the transducer for MAG (Yamashita et al., 2002). When MAG binds to NGR, it requires the presence and action of p75 (i.e. NGR and p75 interacting via their extracellular domains) to induce Rho activity and block axonal growth (Dergham et al., 2002; Yamashita and Tohyama, 2003). Specifically, the NGR-p75 complex enables Rho to bind to the cytoplasmic domain of p75 and it has been suggested that p75 then activates signalling cascades in neurons to induce growth cone collapse and inhibit neurite outgrowth. This is consistent with the finding that in the p75 knock-out mouse, MAG does not inhibit neurite outgrowth (Yamashita et al., 2002).

**Figure 22.** Nogo, OMgp and MAG interact with the NGR-p75 receptor, activating Rho activity, which inhibits neurite outgrowth (Filbin et al., 2003).

Targeted gene deletions or antibodies blocking inhibitory myelin proteins or their receptors have given varying degrees of success in terms of regeneration and behavioural recovery. For instance, depending on the isoform of Nogo and its receptor NGR blocked, there can either be no improvement in regeneration, limited regeneration, or robust regeneration after their action is blocked; and in some cases also behavioural recovery (Kim et al., 2003; Kim et al., 2004; Simonen et al., 2003; Zheng et al., 2003;
Zheng et al., 2005). Overall, this approach appears promising given that the lack of spontaneous regeneration in the adult mammalian brain coincides with the onset of myelination (Ferreti et al., 2003).

Intrinsic capacity of neurons. Differences in the intrinsic capacity for axon growth of neurons also determine their regenerative potential post-injury. It is well-established that both embryonic CNS and adult PNS neurons have a high intrinsic capacity for axon growth, such that adult PNS neurons can extend axons in the adult CNS, but adult CNS neurons cannot grow axons. However, a pre-conditioning lesion in the PNS prior to a lesion in the CNS induces a regenerative response in mature CNS neurons (Neumann and Woolf, 1999). The active state of neurons in regeneration has been examined in retinal ganglion cells (RGCs; Fischer et al., 2004). Mice with a dominant-negative form of NGR have RGCs whose axons cannot regenerate; but if the RGCs are primed with macrophage-derived factors (which activate their growth program); axonal regeneration takes place in spite of the presence of myelin-based inhibitor molecules (Fischer et al., 2004). Furthermore, by comparing axon growth of embryonic and postnatal RGCs, Goldberg and colleagues (2002) showed that the intrinsic growth capacity of mature neurons is irreversibly reduced during maturation (due to low endogenous cAMP levels; Cai et al., 2001) so that embryonic RGCs grew longer axons than postnatal RGCs in the same extracellular milieu (Goldberg et al., 2002).

In the PNS, the induced upregulation of cAMP post-axotomy changes the intrinsic properties of adult neurons by altering the responsiveness of the neuron to inhibitory molecules (Neumann et al., 2002). Two factors that change the intrinsic properties of regenerating neurons are: (i) sustained upregulation of cAMP which can convert a repulsive signal into an attractive cue (refer to section 1.3.2 page 26) and makes the surviving neuron have greater regenerative ability (Shen et al., 1999) and (ii) subsequent activation of inducible transcription factors such as CREB (Gao et al., 2004). As one example, the transcription factor c-Jun is upregulated in the regenerating PNS but not in CNS neurons (Broude et al., 1997). c-Jun conditional null mice show delayed axonal regeneration of the facial nerve, however the lack of c-Jun had no effect on axon growth during development (Raivich et al., 2004). This suggests that c-Jun regulates regenerative axon growth and that certain transcription factors are specifically
involved in axonal growth in regeneration but not developmental axon growth in the PNS.

In summary, although neurotrophin-Trk signalling has an important role both during neuronal development and axonal regeneration, the limited availability of growth factors is not the only barrier to regeneration in the mature CNS: the presence of myelin-inhibitory proteins, altered expressions of guidance cues and recruitment of inflammatory cells are a few of the additional factors. Given the difficulty of mimicking the developmental re-expression of guidance cues for regeneration and the aberrant sprouting induced when blocking myelin-associated inhibitory proteins, one aim of this thesis is to examine the efficacy of increasing neurotrophin availability to promote target-specific appropriate reinnervation.

In comparison to these three general approaches to increase post-lesion reinnervation, a related method is to optimise spontaneous neonatal reinnervation post-lesion aiming to recapitulate this process from surviving neurons in the maturing brain. In the immature nervous system, undamaged neurons have the capacity to sprout axon collaterals and reinnervate denervated tissue. This has been observed at the neuromuscular junction (Edds, 1953; Brown et al., 1981) and in the hippocampus (Raisman, 1969). It has also been noted in the olivocerebellar system where developing axons have the ability to grow a whole new projection pathway after axotomy derived from a homologous separate inferior olive population (Angaut et al., 1982; Angaut et al., 1985; Sherrard et al., 1986; Zagrebelsky et al., 1997). The reinnervation provided by the olivocerebellar system both during development and in the adult is discussed below.

3.1 Olivocerebellar reinnervation

Transcomissural reinnervation of the olivocerebellar system is seen after a unilateral transection of the inferior cerebellar peduncle (a pedunculotomy; Px) in newborn rats (Figure 23; Bower and Waddington, 1981). In this condition, new CF collaterals arise from the intact ipsilateral inferior olive extend to reinnervate PCs of the denervated hemicerebellum (Angaut et al., 1982; Angaut et al., 1985; Sherrard et al., 1986; Zagrebelsky et al., 1997; Sugihara et al., 2003) and form functional synapses (Sugihara et al., 2003). The new CFs begin to cross the vermal midline in 18 hours post-injury and within 4 days have made terminal plexuses around PCs in the paravermal cortex
Within 6 days, the CFs complete their reinnervation and the axotomoised inferior olive neurons contralateral to the pedunculotomy die (Armengol and Lopez-Roman, 1996).

This reinnervation is organised in parasagittal stripes replicating the normal projection in the intact hemicerebellum (Angaut et al., 1985; Sherrard et al., 1986; Zagrebelsky et al., 1997), and aligned with the zebrin PC bands (Zagrebelsky et al., 1997). At the ultrastructural level, new CFs show correct distribution of normal synapses along proximal PC dendrites in the molecular layer (Angaut et al., 1982). Although reinnervation is topographically specific, it is also incomplete as shown by the number of PCs which are not reinnervated (Angaut et al., 1982; Sugihara et al., 2003). The reinnervation occurs extensively in the vermal cortex reaching 86% of PCs (Sugihara et al., 2003) but is only sparse in the lateral regions of the deprived hemisphere (Angaut et al., 1985; Zagrebelsky et al., 1997) innervating only 40% of PCs (Sugihara et al., 2003). Furthermore, retrograde studies reveal that olivocerebellar axons arise mainly from the intact caudal MAO and to a lesser extent from DAO and PO (Angaut et al., 1985; Sherrard et al., 1986; Neppi-Modona et al., 1999), potentially contributing to the greater number of reinnervating CFs in the vermis as opposed to lateral zones. The CF arbors formed onto PCs are morphologically normal (Figure 24; Sugihara et al., 2003) with appropriate topography (Zagrebelsky et al., 1997). These CFs undergo similar developmental CF-PC synaptogenesis processes of multi-innervation and regression (Lohof et al., 2005). This form of reinnervation also provides motor compensation of gait (Dixon et al., 2005).
Figure 24. Camera lucida drawing and photomicrograph of a climbing fibre arbor of a transcommissural olivocerebellar axon (Sugihara et al., 2003).

Analysis of adult CF remodelling studies also provides insight into the factors which may control developmental CF reinnervation. In the adult, the reinnervation of adult CFs is limited to axon terminal remodelling. CF terminal sprouting is displayed when PCs are deprived of their normal innervation after CF injury (Beneditti et al., 1983). Following a subtotal lesion of the inferior olive by 3-AP (which causes 90 to 99% degeneration of the inferior olive), the surviving adult CFs are able to sprout into the molecular layer to reinnervate adjacent deafferented PCs (Figure 25; Beneditti et al., 1983; Rossi et al., 1989; Rossi et al., 1991a; Rossi et al., 1991b). An extrinsic factor that allows CF branching in the molecular layer could be that myelin proteins are absent in this layer. The increased CF terminal branches innervate approximately 50 PCs (Beneditti et al., 1983), compared to the normal adult CF: PC innervation of 1: 7 (Schild, 1970; Sugihara et al., 2001) and their synapses are functional (Benedetti et al., 1983). Morphologically, these terminals extend from the intact arbors of surviving CFs and cover the proximal dendrites of denervated PCs (Rossi et al., 1989; Rossi et al., 1991a; Rossi et al., 1991b). CF branches along the PC dendrites are rich in varicosities, their terminal arbours are morphologically similar to normal CFs and they are restricted by the boundaries of the zebrin-specific PC compartments (Zagrebelsky et al., 1996). Despite the presence of CF terminal sprouting, it is not associated with behavioural improvement (Fernandez et al., 1998).
Experimental evidence indicates that such adult CF remodelling is specifically regulated by PCs. In these studies, embryonic cerebellar tissue is inserted into a lesion cavity or grafted onto the surface of the adult cerebellum (Rossi et al., 1992; Rossi et al., 1994; Rossi et al., 1995a; Rossi et al., 2002). The CFs which are either axotomised or uninjured CFs expand their terminal branches onto the grafted embryonic PCs (Rossi et al., 1992; Rossi et al., 1994; Rossi et al., 1995a; Tempia et al., 1996; Rossi et al., 2002) and these new contacts on PCs replicate the morphology and location of normal CF input. Furthermore, CFs respond to PC degeneration by retracting their terminal arbors (Rossi et al., 1993; Rossi et al., 1995b). Therefore, these studies show that adult axonal terminal branching is regulated by PCs. Although adult CFs do not grow a new projection pathway, it is postulated that the CF-PC interactions during development may also be active in adult animals.

3.2 Neurotrophins and olivocerebellar reinnervation

Studies suggest that olivocerebellar reinnervation during development is dependent on age and growth factor availability (Angaut et al., 1985; Sherrard et al., 1986; Sherrard, 1997; Nitz et al., 2001; Li et al., 2001a; Li et al., 2001b; Sherrard and Bower, 2001). In rats pedunculotomised at P3, new CFs terminate in parasagittal strips of the deafferentated hemicerebellum in a distribution that is symmetrical about the midline (Angaut et al., 1985; Sherrard et al., 1986). Reinnervation gradually diminishes with age at lesion, so that by P10 no reinnervation occurs (Sherrard et al., 1986). It has been observed that transcommissural olivocerebellar reinnervation is temporally congruent with an increase in p75 receptor expression in inferior olive neurons (Nitz et al., 2001) and an increase in inferior olive neuronal survival (Armengol and Lopez-Roman, 1992).
One mechanism promoting the upregulation of p75 in the inferior olive may be the increased retrograde neurotrophin signalling by target neurons in the denervated hemicerebellum (Nitz et al., 2001).

Furthermore, when growth factors such as BDNF, NT-3 or insulin growth factor-1 are injected into the cerebellar cortex 24 hours post-pedunculotomy at P11 or later (i.e. P30) transcommissural CFs develop to reinnervate the deafferented hemicerebellum (Sherrard, 1997; Sherrard and Bower, 2001; Dixon and Sherrard, 2006; Letellier et al., 2007). In addition, BDNF produces more CF reinnervation than NT-3, as reflected in the greater number of retrogradely labeled inferior olive neurons in BDNF-treated versus NT-3 –treated animals (Sherrard and Bower, 2001). Since CFs synapse only onto PCs, and BDNF (not NT-3) is synthesised in the PC (Neveu and Arenas, 1996), it is proposed that exogenous BDNF injections could be chemoattractive to TrkB-carrying CF terminals. This is likely given that CF retraction and maturation may be influenced by falling BDNF and TrkB levels in the inferior olive (Sherrard and Bower, 2002).

The potential role of BDNF in CF reinnervation in the maturing cerebellum is also evident following unilateral labyrinthectomy (Li et al., 2001a; Li et al., 2001b). Following labyrinthectomy, adult animals initially have ataxia and vertigo, but these symptoms disappear in several days (Li et al., 2001a; Li et al., 2001b). The observed functional improvement is attributable to CF terminal reorganisation (Li et al., 2001a; Li et al., 2001b). During this reorganisation, BDNF mRNA is upregulated in the plasticising inferior olive (i.e. contralateral to the labyrinthectomy), while no changes occur in olivary expression of TrkB or cerebellar BDNF (Li et al., 2001a; Li et al., 2001b). This suggests that CFs anterogradely transport olivary BDNF to the PCs and potentially modify synaptic function and thus change PC outflow to the vestibular nuclei and provide functional compensation (Li et al., 2001a; Li et al., 2001b). Although it is unknown whether or not BDNF synthesis increases in the inferior olive during post-pedunculotomy transcommissural olivocerebellar reinnervation, the former studies clearly demonstrate the involvement of inferior olive and cerebellar BDNF during CF terminal plasticity.
3.3 Relevance to neural circuit repair and project aims

In conclusion, it has been described that axonal regeneration is limited by both an inhibitory extracellular milieu and a low growth potential of injured neurons. In cases where sprouting has been induced in the adult central nervous system (e.g. via myelin blockers), this is usually non-specific or specific to only nearby target neurons and does not compensate for skilled movements or complex tasks. We have studied another form of post-lesion repair system, which involves using spontaneous neonatal collateral reinnervation after injury aiming to recapitulate this process from surviving neurons in the mature system in order to provide more target-specific reinnervation that may improve complex functions and cognitive deficits. We have used the olivocerebellar pathway of the rat as it has the capacity to grow a transcommissural projection over a long distance post-injury and synapses onto specific target neurons with associated compensation of skilled motor synchronisation.

The broad aims of this thesis are (i) to characterise the extent of behavioural recovery on complex neural functions associated with transcommissural olivocerebellar reinnervation in young and juvenile animals (induced via BDNF) and (ii) to examine the long-term function of these reinnervating synapses to see whether accurate synapse replacement has taken place. As the recovery of function after injury requires a number of steps, notably a sprouting response of remaining/injured axons, the choice of the correct target neurons and electrophysiologically functional synaptic contacts; we have taken this approach using our model to provide a complete description of the functional accuracy of injury-induced olivocerebellar reinnervation.
RESULTS
1.0 The Functional Compensation Provided by Transcommissural Olivocerebellar Reinnervation in a Spatial Learning Task

Article I. Developmental neuroplasticity and its cognitive benefits: olivocerebellar reinnervation compensates for spatial function in the cerebellum.

The olivocerebellar projection to the cerebellum is required for motor learning and control and spatial function (Dahhaoui et al., 1992b; Rondi-Reig et al., 1997; Rondi-Reig et al., 2002). Furthermore, each of these functions appears to be linked to a discrete anatomical region of the cerebellum, in which the medial hemicerebellum (vermis) mediates motor control, i.e. postural and equilibrium, while the lateral (hemisphere) zone is involved in movement planning and spatial orientation. Given the apparent functional differences within the cerebellar system, this allowed us to examine the functional benefit provided by post-lesion transcommissural olivocerebellar reinnervation. Dense reinnervation in the vermis (87% of Purkinje cells reinnervated, Sugihara et al., 2003) is associated with improved motor function (Dixon et al., 2005). However, reinnervation is partial in the hemisphere (44% of Purkinje cells reinnervated, Sugihara et al., 2003) which is the region associated with spatial function (Joyal et al., 1996). Therefore by using this model of reinnervation, we were able to examine to what extent partial reinnervation could allow spatial function.

To do this, we assessed the spatial learning abilities of control animals, animals with transcommissural olivocerebellar reinnervation (pedunculotomised on P3, Px3) and animals without reinnervation (pedunculotomised on P11, Px11). After behavioural tests, we used retrograde tract tracing techniques (injected into the left hemisphere) to indicate the degree of reinnervation in each animal. This allowed us to compare the degree of reinnervation and its relation to spatial function.

The results of this work are presented as an article (Article I).
Developmental neural plasticity and its cognitive benefits: olivocerebellar reinnervation compensates for spatial function in the cerebellum

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Keywords: cerebellum, climbing fibres, plasticity, rat, spatial learning

Abstract
The adult mammalian central nervous system displays limited reinnervation and recovery from trauma. However, during development, post-lesion plasticity may generate alternative paths, thus providing models to investigate reinnervation and repair. After unilateral transection of the neonatal rat olivocerebellar path (pedunculotomy), axons from the remaining inferior olive reinnervate the denervated hemicerebellum. Unfortunately, reinnervation to the cerebellar hemisphere is incomplete; therefore, its capacity to mediate hemispheric function (navigation) is unknown. We studied sensorimotor control and spatial cognition of rats with and without transcommissural reinnervation using simple (bridge and ladder) and complex (wire) locomotion tests and the Morris water maze (hidden, probe and cued paradigms). Although pedunculotomized animals completed locomotor tasks more slowly than controls, all groups performed equally in the cued maze, indicating that lesioned animals could orientate to and reach the platform. In animals pedunculotomized on day 3 (Px3), which develop olivocerebellar reinnervation, final spatial knowledge was as good as controls, although they learned more erratically, failing to retain all information from one day to the next. By contrast, animals pedunculotomized on day 11 (Px11), which do not develop reinnervation, did not learn the task, taking less direct routes and more time to reach the platform than controls. In the probe test, control and Px3, but not Px11, animals swam directly to the remembered location. Furthermore, the amount of transcommissural reinnervation to the denervated hemisphere correlated directly with spatial performance. These results show that transcommissural olivocerebellar reinnervation is associated with spatial learning, i.e. even partial circuit repair confers significant functional benefit.

Introduction
Cognitive function involves the interaction of precise neuronal circuits. Therefore, to minimize deficits following brain injury any circuit re-formation must be equally precise (Lund, 1978; Kolb & Whishaw, 1998). Unfortunately, in the adult mammalian central nervous system, neither specific circuits nor behavioural deficits are always compensated for by the limited axonal regeneration (Dunlop et al., 2004) or terminal sprouting of remaining axons (Azmitia et al., 1978; Raineteau & Schwab, 2001). In contrast after injury to the developing nervous system, surviving neurons can develop alternative axonal projections to correct targets (Leong, 1977; Angaut et al., 1982; Spear, 1995) that reduce functional deficits (Weber & Stelzner, 1977; Dixon et al., 2005). The compensation provided by these paths correlates with the specificity with which they recreate the original circuit (Finlay et al., 1979; Schneider, 1979; Gramsbergen & Ijksma-Paassen, 1982). However, it remains unknown whether such alternative circuitry displays the accuracy and adaptability to facilitate complex neuronal tasks, such as learning.

The developing olivocerebellar pathway of the rat is one example that generates an alternative pathway following injury, thus presenting a model for studying functional compensation provided by developmental neural plasticity. In the adult, olivocerebellar axons enter the cerebellum via the contralateral inferior peduncle and terminate as climbing fibres (CFs) on Purkinje cells (PCs) with precise parasagittal topography (Sugihara et al., 2001). This path regulates motor learning (McCormick et al., 1985; Thompson, 1989; Apps & Lee, 2002) and the procedural aspects of spatial learning (Rondi-Reig et al., 2002; Rondi-Reig & Burguière, 2005) and memory (Dahhaoui et al., 1992; Gasbarri et al., 2003). Following unilateral CF transection early in development, the contralateral (axotomized) inferior olive degenerates and new axons, arising from the remaining inferior olive, grow into the denervated hemicerebellum, reinnervate denervated PCs and partly recreate the olivo-cortico-nuclear circuit (Fig. 1; Zagrebelsky et al., 1997; Sugihara et al., 2003). Reinnervation to the medial hemicerebellum is dense (Angaut et al., 1985; Sugihara et al., 2003) and improves motor function (Dixon et al., 2005). However, reinnervation to the lateral hemisphere is incomplete (Sugihara et al., 2003), so it is uncertain whether such partial reinnervation can compensate for hemispheric function, i.e. spatial learning (Joyal et al., 1996, 2001; Noblett & Swain, 2003).

In this study, we used the Morris Water Maze (MWM) to assess spatial function (Leggio et al., 1999; Gandhi et al., 2000; Lalonde &
with food and water available

birth date of pups was designated as P0 followed by weaning at P25, own colony, and housed under a 12-h light
guidelines of the NIH. Animals were obtained from the University's
of animals for scientific purposes
which is in accordance with the
Cook University Animal Experimentation Ethics Committee (A732),
Experiments were performed on Wistar rats under licence from James

**Materials and methods**

**Animals**

Experiments were performed on Wistar rats under licence from James
Cook University Animal Experimentation Ethics Committee (A732),
which is in accordance with the Code of practice for the care and use of animals for scientific purposes of the NH MRC of Australia and the
guidelines of the NIH. Animals were obtained from the University's own colony, and housed under a 12-h light/dark reversible cycle. The
birth date of pups was designated as P0 followed by weaning at P25,
with food and water available ad libitum. All behavioural experiments occurred during the dark phase cycle in order to complement the
active time of the animals.

**Transection of olivocerebellar axons**

Seven litters of Wistar rats (67 pups) were assigned to two
experimental groups: (1) animals pedunculotomized (Px) on P3
(Px3) to induce the growth of a new transcommisural olivocerebellar
pathway and (2) animals pedunculotomized on P11 (Px11) in which
the transcommisural path does not develop. Pups were anaesthetized with
diethyl ether (BDH, Poole, UK) and underwent unilateral
transsection of the left inferior cerebellar peduncle either at P3 or at
P11, as previously described (Bower & Waddington, 1981; Fig. 1).
Some pups in each litter underwent a sham operation in which the
peduncle remained intact. All animals were allowed to recover and
mature to P30 after which their motor performance and spatial
learning were assessed.

**Motor assessment**

Three motor tests were used to identify any motor dysfunction that
may affect the spatial learning data. Animals aged between 30 and
35 days underwent daily tests (three per day for 6 days) assessing
simple and complex locomotor skills (Molinari et al., 1990;
Petrosini et al., 1990; Dixon et al., 2005). The tests were to cross a
crossroad, ascend a ladder (17 steps, 25° tilt) and progress
along a wire to reach a goal escape platform at either end (Dixon
et al., 2005). For each test, the dependent variables were success or
failure of completing the task within the upper time limit of 3 min
(Dixon et al., 2005) and time taken. For each animal, their scores
during the 5th training day were used to calculate intergroup
comparisons.

**Spatial learning and swimming assessment**

**Apparatus and protocol**

The MWM (Morris, 1984) consisted of a circular pool (120 cm
diameter) filled with water (21 °C) containing a clear plexiglass escape platform (15 cm diameter) centred in one (north-west)

In this test, the escape platform was above the water in the opposite

Spatial memory was assessed 2 h after the last hidden platform trial.

**Probe test**

Spatial memory was assessed 2 h after the last hidden platform trial. The
platform was removed and the rat given two trials to find the platform’s former (‘test’) location. The time spent in the test quadrant, the
percentage of direct swims and success to locate the goal. A direct swim was analysed on hand-drawn trajectories, being defined as a path that did not deviate outside a 20-cm radius from the rat’s entry point to the hidden platform (Day et al., 1999;
Fig. 2). An entry into a quadrant required all four limbs to have
crossed the quadrant line. The searching behaviour (e.g. thigmotaxis
or random swimming routes) was also recorded.

**Visible (cued) platform test**

In this test, the escape platform was above the water in the opposite
(south-west) quadrant and a globe-shaped flag was attached to the
platform (proximal cue). The variables measured were the number of

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total quadrants crossed, escape latency and percentage of direct swims to the platform.

Injection of an axonal tracer

After behavioural tests, the number of inferior olive neurons which develop reinervating CFs to the cerebellar hemisphere was identified by retrograde axonal tracing using an intracerebellar injection of Fast Blue (Illing, Germany) as previously described (Sherrard & Bower, 2003). Under Avertin anaesthesia (2.5%, 10 mL/kg i.p.), 1 µL Fast Blue (2% in 0.1 M PB, pH 7.4) was injected, in 12–16 aliquots, into the left cerebellar hemisphere lateral to the paravermal vein.

Histological analysis

Two days after injections, animals were re-anaesthetized with sodium pentothal (60 mg/kg i.p.) and transcardially perfused with heparinized saline (5 IU/mL) followed by 10% phosphate-buffered formaldehyde (pH 7.4). The cerebellum and brainstem were dissected free, post-fixed and cryoprotected in 30% buffered sucrose. Two parallel sets of serial 30-µm coronal sections were cut of the cerebellum and inferior olive. One set was stained with 0.5% methylene blue and analysed for (1) a complete pedunculotomy according to established criteria, histological analysis of the lesion and total degeneration of the right inferior olive (Angaut et al., 1985; Sherrard et al., 1986); and (2) the presence of the left deep cerebellar nuclei to provide outflow from the left hemicerebellum. On the second set, the cerebellar injection site was located and brains were cut and stained with 0.5% methylene blue and analysed for (1) a complete pedunculotomy or total degeneration of the left deep nuclei.

Histological analysis

Two sets were stained with 0.5% methylene blue and analysed for (1) a complete pedunculotomy, residual deep nuclei and transcommissural CF reinnervation, and their number and location noted. Only animals with a complete Px, residual deep nuclei and restriction of the injection to the left hemicerebellum, were included in the study. Thus, 18 animals were excluded due to either incomplete pedunculotomy or total degeneration of the left deep nuclei.

Data analysis

Motor tests

For each test, motor performance had plateaued by day 5, and therefore the three trials on day 5 were averaged to calculate the mean time performance for each animal. These values were used to calculate the average time of each group for statistical analyses.

MWM tests

The data (escape latency/quadrants) for all trials within one day were averaged to calculate the mean daily performance of each animal, which was then used to calculate the mean group performance on each day.

Correlation of reinnervation and behaviour

To correlate the behaviour data to reinnervation, individual animal scores were calculated for each motor test and the escape latency/total quadrants crossed on the last day of MWM training. The mean time/quadrants crossed by the control group was defined as a maximum score (10) and the standard error of the mean (SEM) of the control group as an arbitrary bin size. Using this information, each animal’s performance (time/quadrants crossed) was converted to a score depending on how far it differed from controls, i.e. time equal to control = 10, time within 2× SEM from control = 8, etc. A behavioural score was obtained for each animal in each test and this was correlated with the animal’s corresponding number of labelled inferior olive neurons.

Statistics

Neither motor nor MWM data attained homogeneity or normality of variance; therefore, all dependent variables were analysed using the Kruskal–Wallace test followed by post-hoc Mann–Whitney U test for intergroup comparisons, and Friedman plus post-hoc Wilcoxon analyses for intragroup comparisons. ANOVA on transformed data, in which homogeneity but not normality of variance was attained, replicated the intergroup comparisons. The percentage success of a task was assessed using the χ² test. Pearson’s correlation was used to test the relationship between the number of retrogradely labelled (reinnervating) olivary neurons and behavioural scores. For comparisons with other studies and clearer graphical presentation, all values were stated as mean ± SEM and the alpha level was set at α = 0.05.

Results

Although transcommissural olivocerebellar axons partially recreate the olivo-cerebello-nuclear circuit (Sugihara et al., 2003) with associated motor benefit (Dixon et al., 2005), the effect of this reinnervation on complex neural function required for cerebellar cognitive processing was unknown. To examine this question, we compared spatial learning between sham-operated controls (n = 9), animals with transcommissural olivocerebellar reinnervation (pedunculotomized on P3: Px3, n = 8) and animals without this reinnervation (pedunculotomized on P11: Px11, n = 14).

Pedunculotomized and normal animals perform differently in basic motor tasks

Because sensorimotor disturbance impairs locomotor control and water maze performance (Cain et al., 1996), we analysed whether...
pedunculotomy-induced motor deficits impede swimming in the maze and hence confound spatial learning data. Therefore, we examined the animals' performances in simple and complex motor coordination tasks. Simple quadruped locomotion was tested by the ability to cross a narrow bridge and ascend a ladder, and trunk-limb coordination by traversing a horizontal wire (Dixon et al., 2005). In the simple locomotion tests, all animals in each group were able to complete the task. However, in accordance with previous results showing a broad-based gait (Dixon et al., 2005), both Px3 and Px11 animals took longer than controls to cross the bridge (Px3, \( P < 0.01 \); Px11, \( P < 0.001 \); Fig. 3) and ascend the ladder (Px3, \( P < 0.01 \); Px11, \( P < 0.01 \); Fig. 3). By contrast, the complex task was impaired only in Px11 animals (Fig. 3): fewer animals successfully completed this task (86%, \( P < 0.05 \)) and those that did took longer to traverse the wire (\( P < 0.05 \)).

**Pedunculotomized and normal animals can swim directly to a visible target**

As unilateral pedunculotomy is associated with impaired motor tests, all groups were tested in the visible (cued) platform MWM to differentiate sensorimotor and spatial deficits (Morris, 1984; Goodlett et al., 1992), i.e. to ensure that pedunculotomized animals could orientate to and reach the platform. For this test a separate group of animals in identical experimental groups were used (control, \( n = 9 \); Px3, \( n = 4 \); Px11, \( n = 5 \)) because prior non-spatial swimming experience can mask subtle water maze learning deficits (Saucier et al., 1996).

For the escape latency, total quadrants crossed and percentage of direct swims, there were no significant differences between training days or groups (Fig. 4A and B; Table 1). To ensure that average daily performance did not mask transient intergroup differences, individual trials were also compared (data not shown), again without significant intergroup differences. Thus, the presence of some motor deficits following pedunculotomy did not impair the animals’ abilities to orientate to and swim to a target location.

**Px3 animals acquire a spatial task better than Px11 animals**

Spatial learning of control, Px3 and Px11 animals was tested over 6 days in the hidden platform MWM task. As a group, the control animals replicated previous findings (Petrosini et al., 1996; Le Marec et al., 1997), i.e. they initially circled the pool wall (thigmotaxis; Fig. 5A) and by day 3 their swimming trajectory became more direct and did not subsequently change (Fig. 5B and C).

### Table 1: Percentage (over all trials) of direct swims to the platform during the hidden or visible platform and probe tests

<table>
<thead>
<tr>
<th></th>
<th>Direct swims to the platform (%)</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Visible platform</td>
<td>30</td>
</tr>
<tr>
<td>Spatial training</td>
<td>48</td>
</tr>
<tr>
<td>Probe</td>
<td>33</td>
</tr>
</tbody>
</table>

Significant differences between control and Px11: *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \). Significant differences between Px3 and Px11: \( \dagger P < 0.05 \); \( \ddagger P < 0.01 \).
The Px3 group displayed a similar searching behaviour (Fig. 5A) to controls, with the same percentage of all swims with direct trajectories (Table 1) and decreasing escape latencies and total quadrants crossed (Fig. 5B and C). Post-hoc analyses showed their improvement continued until day 5, vs. day 3 for controls, so that on day 4 the total quadrants crossed was greater than control \( (P < 0.01; \text{ Fig. 5C}) \). In order to clarify whether the day 3–4 performance of the Px3 group indicated motivational difficulties or a difference in learning the MWM, we analysed individual training trials. Whereas the control animals steadily improved their performance between trials (Fig. 5D and E), the Px3 group showed a daily fluctuation so that on days 2–4 was worse than the last trial of the same day. Also, performance on the first trial of days 1–3 is worse than for the last trial of the previous day. Significant differences between control and pedunculotomized groups: \(* * * P < 0.01; \quad * * * * P < 0.001\). Significant differences between Px3 and Px11: \( * P < 0.05; \quad ** P < 0.01; \quad *** P < 0.001\). Significant differences between trials by Px3 animals: \( * P < 0.05, \quad ** P < 0.01\).

By contrast, the Px11 group searched more randomly persevering with prolonged searching in all pool quadrants up to the last training day (Fig. 5A). They displayed fewer direct swims (Table 1; \( P < 0.05 \)), longer escape latencies (Fig. 5B; \( P < 0.05 \)) and more total quadrants crossed (Fig. 5C; \( P < 0.01 \)) than both the control and the Px3 groups on days 2–6. To clarify whether these deficits were primarily motor or learning impairment, we analysed the relative performance differences to control and Px3 groups at the start (untrained animals showing motor differences only) and end (showing motor and learning differences) of training (Rondi-Reig et al., 1997). On day 1, when no group had learned the maze, the performance difference to controls was \( \sim 18\% \) \((\text{control} - \text{Px11})/\text{control}: \ 	ext{time} = [31.1 - 36.7]/31.1 = -0.18; \text{quadrants} = [11.8 - 13.9]/11.8 = -0.17; \text{Fig. 5B and C}, \ \text{‘a’}), \ \text{which increased to} \sim 100\% \text{on day 6 (time} = [9.8 - 22.7]/9.8 = -1.31; \text{quadrants} = [3.6 - 7.0]/3.6 = -0.94; \text{Fig. 5B and C}, \ \text{‘a’}). \ Also in comparison with Px3 the deficit increased from about 35\% on day 1 \((\text{Px3} - \text{Px11})/\text{Px3}: \ 	ext{time} = [27.7 - 36.7]/27.7 = -0.32; \text{quadrants} = [10.4 - 13.9]/10.4 = -0.37; \text{Fig. 5B and C}, \ \text{‘c’}) \ \text{to around} 100\% \text{for escape latency and} 50\% \text{for total quadrants crossed at the end of testing (time} = [10.5 - 22.7]/10.5 = -1.16; \text{quadrants} = [4.9 - 7.0]/4.9 = -0.43; \text{Fig. 5B and C}, \ \text{‘d’}), \ \text{suggesting a learning impairment. However, overall the performance of the PXX11 group did improve, reducing escape latency and total quadrants crossed} \ (P < 0.001; \text{ Fig. 5B and C}). \ Post-hoc analysis showed this occurred between days 3 and 6 \( (P < 0.01; \quad \text{quadrants,} P < 0.05 \), \text{i.e. during a different time course to the control \( (\text{days 1–3}) \) or Px3 \( (\text{days 1–5}) \) groups.}

**Px3 animals remember the spatial task**

To test whether the animals knew the location of the platform, they underwent two probe trials in which the platform was removed. Irrespective of the probe trial number, all groups demonstrated a similar spatial bias to the relevant ‘test’ quadrant (t-test; P > 0.05; data not shown). However, intergroup differences were found in the number of direct swims to the ‘test’ site, whereas the Px11 animals made significantly fewer than either control (P < 0.05) or Px3 (P < 0.01) animals.

These data suggest that all animals have some spatial memory of the platform’s location. Whereas Px3 animals appear to remember similarly to controls, Px11 animals remember less well by taking fewer direct swims and maintaining searching patterns.

**Relationship between reinnervation and function**

The presence/absence of retrogradely labelled neurons in the left inferior olive was used to verify whether reinnervation into the left cerebellar hemisphere occurred. Inferior olive analysis confirmed previous studies (Angaut et al., 1985; Sherrard et al., 1986): retrogradely labelled neurons were found in the right inferior olive of control animals (Fig. 6A), the right inferior olive had degenerated in pedunculotomized animals (Fig. 6B and C) and retrogradely labelled neurons were localized in the left inferior olive in Px3 animals only (Fig. 6B). These neurons were found predominately in the medial accessory olive, close to the midline but also in the principal and dorsal accessory olives.

In order to examine whether there was any correlation between reinnervation and function, we counted the number of retrogradely labelled neurons in the left inferior olive in each Px3 rat and calculated their scores on the motor and MWM tests. Retrograde tracing from the left cerebellar hemisphere was used because intracortical sprouting of CF terminals within the denervated molecular layer (Rossi et al., 1991a,b) is not associated with functional benefit (Rondi-Reig et al., 1997, 2002) whereas transcommissural CF reinnervation is (Dixon et al., 2005). In all but one animal, which was excluded due to very poor injection and dye transport, the cerebellar injection was confined to and filled crus 1 and 2. In the motor tests, only the ladder test correlated with number of labelled neurons (Pearson’s coefficient = 0.89, P < 0.01; Fig. 7). In contrast, in the MWM both escape latency and total quadrants crossed correlated with the number of retrogradely labelled neurons (time: Pearson’s coefficient = 0.98, P < 0.01; quadrants, Pearson’s coefficient = 0.90, P < 0.01; Fig. 7).

**Discussion**

Spatial learning was used to examine whether partial re-formation of a neural circuit could compensate for cognitive function. We have shown that transcommissural olivocerebellar reinnervation to the left cerebellar hemisphere correlates with spatial function and that animals devoid of reinnervation (i.e. Px11) showed impaired spatial learning.

**Olivocerebellar reinnervation: sensorimotor or cognitive improvement?**

Previous studies show that spatial learning is adversely affected by cerebellar dysfunction, and this is associated with sensorimotor disturbance (Goodlett et al., 1992; Loyal et al., 1996; Gandhi et al., 2000; Martin et al., 2003). The cerebellar (procedural) component of spatial navigation involves the acquisition of specific motor patterns.
behaviours that encode optimal (direct) paths to a goal within a learned spatial context (Schenk & Morris, 1985; Leggio et al., 1999; Rondi-Reig & Burguie`re, 2005). Thus, spatial learning necessarily includes motor learning and is subserved by the same cellular mechanisms (Ito, 2001; Burguie`re et al., 2005). As transcommissural CF reinnervation ameliorates motor learning (Dixon et al., 2005), it has to be considered that the better spatial function observed in the Px3 group is due to sensorimotor rather than spatial cognitive recovery.

Although intergroup differences in sensorimotor function will contribute to the observed behaviours, we propose that a significant part of the Px3 group performance reflects compensation of spatial function. There are several reasons for this. First, animals with sensorimotor impairment (vs. cerebellar lesion) are unable to orientate correctly to a goal, making fewer direct trajectories in both the visible (cued) and the hidden MWMs (Cain et al., 1996). In our study all animals, including the Px11, could orientate to a target, as demonstrated by the number of direct swims in the cued test. Second, animals with cerebellar learning deficit (absent PC long-term depression [LTD]) but no sensorimotor loss have impaired spatial function in the normal hidden MWM, but not in the visible or Star maze in which the procedural challenge is lessened by taxic cues (Burguie`re et al., 2005). Our Px11 group, without CF reinnervation, was similar: impairment in the hidden MWM but not in the visible platform test. Third, sensorimotor abnormalities secondary to cerebellar lesions have less effect on swimming than terrestrial locomotion (Federico et al., 2006) and swimming in the cued MWM differentiates sensorimotor from spatial disturbance (Morris, 1984; Goodlett et al., 1992; Cain et al., 1996; Martin et al., 2003). Although both Px3 and Px11 groups displayed similar motor deficits, swimming in the cued maze was the same for all groups despite different CF innervation. This supports spatial rather than sensorimotor differences in the hidden MWM. Fourth, we find a strong correlation between the number of olivary neurons involved in reinnervation and spatial but not motor function. Although this may reflect that the retrograde tracing evaluated reinnervation to the left hemisphere (spatial function: Joyal et al., 1996, 2001) not the left hemivermis (motor control: Joyal et al., 1996), the correlation of spatial function and reinnervation supports the proposal that CF reinnervation provides some recovery of spatial performance.

In addition, it is possible to differentiate sensorimotor and learning dysfunction by comparing the relative performance in naive (sensorimotor differences) and trained (sensorimotor and learning differences) animals (Rondi-Reig et al., 1997). In our study, all animals performed the hidden MWM equally on day 1, confirming that motor dysfunction did not impair the animals’ ability to search the maze. With training the control and Px3 animals improved more than the Px11s, so that at the end of training, the relative deficit of Px11 animals to controls had increased from ~18% on day 1 to ~100% on day 6, and compared with Px3 from 35% to ~100% (see Results), indicating a learning deficit (Rondi-Reig et al., 1997). Although the results of this study cannot differentiate the relative contribution of CF reinnervation to motor learning vs. spatial cognition, spatial navigation requires a link between learned motor responses (direct trajectory) and specific inputs from the environment (cues), which depends upon PC LTD (Burguie`re et al., 2005) for which CF innervation is essential (Ito, 2001). Our data suggest that CF reinnervation not only aids motor learning, but also enables this link so animals can spatially navigate.

**Olivocerebellar reinnervation is associated with spatial learning**

Whereas the cerebellum contributes to navigation, the olivocerebellar path is thought to promote the procedural aspects (Rondi-Reig & Burguie`re, 2005) and retention (Dahhaoui et al., 1992; Meignin et al., 1999) of spatial knowledge.

In our study, CF reinnervation is associated with spatial function, as Px3 animals acquired the same scores and percentage of direct swims as control animals during both training and probe tests. This is consistent with CFs reinnervating PCs in the spatially related cerebellar hemisphere (Sugihara et al., 2003), enabling the animal to orientate itself and find a direct route to the hidden target (Burguie`re et al., 2005). However, the compensation was not complete: the learning in the Px3 animals was more variable than in controls with longer searching and poor performance at the start of each training day. This partial recovery concurs with the incomplete
nature of the reinnervation only partially recreating the olivo-Purkinje-nuclear circuit (Sugihara et al., 2003). Normal PC function is central to spatial learning (Gandhi et al., 2000; Lalonde & Strazielle, 2003; Burguérié et al., 2005). Also, CF-deprived PCs have abnormal firing patterns (Batini et al., 1985), an abnormality that rapidly extinguishes spatial memory (Gandhi et al., 2000). Therefore, the presence of some PCs lacking CF reinnervation (Sugihara et al., 2003) is consistent with disturbed cerebellar adaptation of searching strategy (Leggio et al., 1999; Mandolesi et al., 2003; Burguérié et al., 2005) and altered retention of spatial knowledge (Dahhaoui et al., 1992).

In contrast, lack of olivocerebellar reinnervation in the Px11 animals was associated with impaired navigation, few correctly orientated direct swims during training and probe test, and circuitous swimming routes. These behaviours are consistent with the absence of the olivo-Purkinje-nuclear circuitry failing to mediate left/right discrimination (Petrosini et al., 1998), select effective searching (Leggio et al., 1999; Mandolesi et al., 2003; Burguérié et al., 2005) and spatial memory (Dahhaoui et al., 1992). Furthermore, these behaviours are also observed in other models where CF modulation of PC outflow is lost: hemicerebellectomy (Petrosini et al., 1999), an abnormality consistent with greater intergroup differences in spatial than sensorimotor function are with training and correlation of reinnervation with spatial function are consistent with greater intergroup differences in spatial than sensorimotor function. Previous studies show the pivotal role of PC function for spatial learning (Goodlett et al., 1992; Gandhi et al., 2000; Burguérié et al., 2005). Importantly, normal PC function is recreated by CF reinnervation (Batini et al., 1985; Sugihara et al., 2003), thus suggesting that transcommissural olivocerebellar axons provide compensation to spatial cognition.

Acknowledgements

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Abbreviations

CF, climbing fibre; LTD, long-term depression; MWM, Morris Water Maze; PC, Purkinje cell; Px, pedunculotomy; SEM, standard error of the mean.

References


2.0 The Role of BDNF to Increase Transcommissural Olivocerebellar Reinnervation and its Associated Behavioural Outcomes

Article II. BDNF repairs neural circuits: increasing olivocerebellar reinnervation and associated complex skills.

*Article under review.*

The transcommissural olivocerebellar reinnervation induced after an early lesion appeared to be associated only with partial spatial compensation concordant with the partial olivocerebellar reinnervation. We next wanted to examine whether improving the incomplete transcommissural olivocerebellar reinnervation in the left hemisphere would improve spatial function. Furthermore, as those animals without olivocerebellar reinnervation (Px11) were impaired on the spatial task, we chose to induce reinnervation and test whether this enabled better motor and/or spatial function.

Previous studies have shown that growth factors such as BDNF are involved in axonal growth and sprouting post-lesion (Mamounas *et al*., 2000; Tucker *et al*., 2001; Alsina *et al*., 2001). Significantly, neurotrophins increase neuronal cAMP levels, which can overcome myelin-associated inhibition (Cai *et al*., 1999; Gao *et al*., 2003), and induce neurite outgrowth at more advanced stages of axonal maturation (Lu *et al*., 2004). A single injection of BDNF in older-pedunculotomised animals (i.e. at P15 and P30) induces olivocerebellar axons to sprout into the denervated hemicerebellum and reinnervate denervated Purkinje cells (Sherrard and Bower, 2001; Dixon and Sherrard, 2006; Letellier *et al*., 2007). Therefore in the present study, we investigated the efficacy of BDNF treatment in improving motor and spatial learning and memory tasks by increasing spontaneous olivocerebellar reinnervation in animals lesioned at P3, or inducing this reinnervation in animals lesioned at P11. In addition, the same animals which were treated with BDNF or vehicle and tested on behavioural tasks, were examined with semi-quantitative analysis of the amount and distribution of reinnervation. This enabled us to evaluate BDNF’s ability to optimise olivocerebellar reinnervation and the associated behavioural outcomes. This work is presented in the form of an article currently under review.
BDNF repairs neural circuits: increasing olivocerebellar reinnervation and associated complex skills

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Running title: Functional BDNF-induced neuronal repair
Keywords: Climbing fibres; Gait; Reinnervation; Spatial function
ABSTRACT
Recovery of complex neural function following injury to the adult central nervous system (CNS) is limited by minimal spontaneous regrowth of injured or sprouting from remaining pathways. In contrast, the developing CNS displays spontaneous plasticity following lesion, in which uninjured axons can develop projection pathways to the right target neurons and provide partial functional recovery. These compensatory pathways can be induced in the mature CNS by neurotrophic factors, providing models to optimise recovery of complex motor and cognitive skills. After unilateral transection of a developing olivocerebellar path (pedunculotomy), remaining olivary axons topographically reinnervate the denervated hemicerebellum. Such reinnervation can be induced in the mature cerebellum by injection of brain-derived neurotrophic factor (BDNF); however the restitution of complex olivocerebellar-associated functions has not been evaluated. We used a set of simple and complex motor (i.e. gait synchronisation; rotarod test) and cognitive (i.e. spatial learning and memory; water maze) tests in lesioned Wistar rats. These rats were either lesioned on day 3 (Px3; with reinnervation) or 11 (Px11; without spontaneous reinnervation) and treated with BDNF.

BDNF treatment did not affect simple motor function. However on the gait synchronisation task, BDNF-treated Px11 animals equalled the control group, outperforming vehicle-treated lesioned animals. Moreover, BDNF-treated lesioned animals achieved spatial learning and memory tasks as good as controls. Notably BDNF-treated Px11 animals exhibited significantly better orientation in the spatial task than vehicle-treated Px11 animals. All groups performed similarly in the cued water maze confirming that any lesion-induced motor dysfunction did not confound swimming or spatial skills. BDNF treatment also increased the density and distribution of olivocerebellar reinnervation in both Px3 and Px11 groups. Furthermore, the amount of reinnervation in BDNF-treated and vehicle-treated Px3 groups was significantly greater than in BDNF-treated Px11 animals, which suggests that even sparse reinnervation provided functional compensation on complex (rotarod and water maze) tasks. These data imply that pharmacological-induced compensatory sprouting of surviving neurons after injury in older-lesioned animals can facilitate appropriate complex motor and spatial skills.
INTRODUCTION
The adult mammalian central nervous system (CNS) has limited repair after injury due to intrinsic neuronal properties (Neumann and Woolf, 1999) and inhibitory extracellular molecules (De Winter et al., 2002; Silver and Miller, 2004) preventing effective axonal regeneration. However, uninjured axons can develop collaterals to reinnervate denervated neurons (Rossi et al., 1991; Dellar and Frotscher, 1997; Bareyre et al., 2004; Ballermann et al., 2006) and this is increased by neurotrophins (Coumans et al., 2001; Sherrard and Bower, 2001; Zhou et al., 2003; Zhou and Shine 2003; Chen et al., 2006; Dixon and Sherrick, 2006; Vavrek et al., 2006). However, such collateralisation either arises from axons already within the target region which synapse onto appropriate neurons (Rossi et al., 1991; Dellar and Frotscher, 1997), or involves axon growth through grey matter that does not reach appropriate targets (Vavrek et al., 2006; Chen et al., 2006). Furthermore, this reconnectivity may be detrimental (McCouch et al., 1958; Cotman et al., 1981), not compensate behavioural impairment (Rondi-Reig et al., 1997), or improve function, but via adaptive movements using inappropriate muscles (Ballermann et al., 2006) secondary to non-specific and inaccurate anatomy (Vavrek et al., 2006). To recover complex behavioural functions e.g. fine motor skills or learning, the re-formed neuronal circuitry has to be both accurate and appropriate.

Axon collateral sprouting through the white matter tracts to re-form specific projection pathways (Hicks and D’Amato, 1970; Sharp and Evans, 1982; Angaut et al., 1985; Sherrard et al., 1986; Naus et al., 1987; Spear, 1995) with appropriate afferent-target connections (Zagrebelsky et al., 1997; Sugihara et al., 2003) can occur in the injured neonatal CNS. The new circuits confer motor (Weber and Stelzner, 1977; Dixon et al., 2005) and cognitive (Levine et al., 1987; Willson et al., 2007) improvement in proportion to the specificity with which they recreate the original circuit (Finlay et al., 1979; Gramsbergen and Ijkema-Paassen, 1982; Willson et al., 2007). Thus recreating developmental plasticity in the mature CNS may improve recovery following injury.

We have studied the behaviour associated with neurotrophin-induced alternate paths using the rat olivocerebellar projection as a model. In the adult, olivocerebellar axons enter the cerebellum via the contralateral inferior peduncle and terminate as climbing fibres (CFs) on Purkinje cells (PCs) organised in narrow parasagittal microzones (Sugihara et al., 2001). This path regulates motor learning (Apps and Lee, 2002), the
procedural aspects of navigation (Rondi-Reig et al., 2002; Rondi-Reig and Burguiere, 2005), and spatial memory (Meignin et al., 1999). After unilateral CF transection (pedunculotomy) early in development, the contralateral (axotomised) inferior olive degenerates and new axons, arising from the intact inferior olive, reinnervate PCs of the denervated hemicerebellum and partly recreate the olivo-cortico-nuclear circuit (Sugihara et al., 2003; Fig 1A). Dense reinnervation of the medial hemicerebellum (Angaut et al., 1985), which coordinates movement, improves motor function (Dixon et al., 2005). In contrast, sparse reinnervation to the intermediate and lateral hemicerebellum (Sugihara et al., 2003) only partially restores the function of that region, spatial learning (Willson et al., 2007). In the young adult, similar olivocerebellar reinnervation with normal morphology is recreated by brain-derived neurotrophic factor (BDNF) injection into the denervated target hemicerebellum (Dixon and Sherrard, 2006; Letellier et al., 2007)

Because the olivocerebellar system provides us with a well-defined model (from synapse to behaviour), we investigated motor and spatial functions associated with BDNF-induced olivocerebellar reinnervation in relatively mature system. By examining the relationship between the behaviour and underlying anatomy of BDNF-treated lesioned animals, we identified that increasing compensatory sprouting commensurately improves appropriate complex neural functions, specifically fine motor and cognitive skills.
MATERIALS AND METHODS

Olivocerebellar Axonal Transection

One hundred and fourteen Wistar rat pups (12 litters) were used in this study. Experiments were performed under license from James Cook University (A732) and The University of Western Australian (AEC 04/100/359) in accordance with the regulations of the NH&MRC of Australia and the NIH. The birthdate was designated P0 and pups were allocated to 2 experimental groups: (1) pups lesioned on P3, after which transcommissural olivocerebellar reinnervation occurs spontaneously; and (2) juveniles lesioned on P11 in which the transcommissural path does not develop. After diethyl ether anaesthesia (BDH, Poole UK), pups underwent unilateral transection of the left inferior cerebellar peduncle at P3 (Px3) or P11 (Px11), as previously described (Bower and Waddington, 1981; Dixon et al., 2005). The skin over the neck was incised longitudinally and the muscles retracted to expose the atlanto-occipital membrane. A capsulotomy knife (MSP, 3mm blade) was inserted parallel to the brainstem into the 4th ventricle and rotated to the left to cut the left inferior cerebellar peduncle. Some pups in each litter underwent a sham operation in which the peduncle remained intact. After recovery from the anaesthetic, animals were returned to the dam. Food and water were provided ad libitum.

Intracerebellar BDNF Treatment

To increase (Px3 group) or induce (Px11 group) reinnervation, animals were treated with recombinant human BDNF (r-metHu BDNF, Amgen Inc, CA USA) in 0.1% bovine serum albumin in phosphate buffer. The dose is 3.5nmol/mL of cerebellar tissue, which is the optimal dose for inducing transcommissural reinnervation (Sherrard and Bower, 2001). The amount of BDNF for each age group is calculated according to the predetermined volume of the left hemicerebellum at each injection age (Heinsen, 1977). The vehicle contained cytochrome C (Sigma-Aldrich), which has a similar charge and molecular weight as BDNF (Caleo et al., 2003). Under ether anaesthesia, a craniotomy was performed to expose the left hemicerebellum and 1µL containing the appropriate concentration of BDNF/vehicle was injected, in multiple (12-14) aliquots (Sherrard and Bower, 2001). In the Px3 pups, BDNF/vehicle was injected into the left lateral hemicerebellum from the lobulus simplex rostrally to crus II caudally, 72 hours after lesion (i.e at P6; Fig 1A) when reinnervating axons first reach this region (Zagrebelsky et al., 1997). In Px11 pups, BDNF/vehicle was injected in the left medial/intermediate hemicerebellum (lobules VI-VIII) up to 1.5 mm from the midline at 24 hours post-lesion.
Basic Motor Skills

Three motor tests were used to identify motor dysfunction that could affect the spatial learning data. From P30 to P35, animals underwent daily tests (3x/day for 6 days) assessing relatively simple locomotor skills (Petrosini et al., 1990; Dixon et al., 2005). The tests were to cross a narrow bridge (60 x 3 cm, 60 cm above foam), ascend a ladder (17 steps, 25° tilt) and progress along a narrow wire (140 x 0.3 cm, 50 cm above foam) to reach an escape platform at either end (Dixon et al., 2005). For each test, the time taken and success or failure to complete the task within a 3 min session were noted.

Complex Motor Synchronisation Behaviour

The rotarod was used to test the animals’ ability to correctly match stepping frequency and stride length to the speed of rotation, which relies *inter alia* on an intact CF path (Chen et al., 1995; Rondi-Reig et al., 1997). The rotarod is a horizontal cylinder (50 cm long and 5 cm diameter), which rotates about its long axis at 10, 20 or 30 revolutions per minute (rpm). The rat was placed on the rotating cylinder so it had to walk forward synchronising its gait to the rotation speed to maintain its position on top of the rod. At each speed, there were 3 trials/day for 9 days with an inter-trial interval of 3 min (Dixon et al., 2005). For each trial, the error latency (falling or clinging to the rod) or the upper limit of 180 s was recorded. This limit was chosen as animals that reach this time can walk for much longer (Auvray et al., 1989).

Spatial Training and Tests

The water maze (Morris, 1984) is a circular pool (120 cm in diameter) filled with water (21°C). A clear Plexiglas escape platform (15 cm in diameter) was positioned in one (northeast) quadrant and submerged 2 cm below the water surface in the hidden platform test. The animal’s starting position was randomly selected from one of 4 entry points (N, S, E and W) and the rat was released facing the pool wall. The maze was in a room with numerous extra-maze cues (Rondi-Reig et al., 2002) and white noise. Three versions of the spatial task were carried out in the following order: hidden platform training over 3
sessions (10 trials/session), probe test over 2 trials (removal of escape platform) and a retrieval test seven days after the probe test (4 trials).

For hidden platform training, the animal was given 120 s to find the escape platform, as previously described (Willson et al., 2007). The measured variables in each trial were: (i) escape latency (ii) total quadrants crossed (iii) percentage of direct swims, defined as a swim path that did not deviate outside a 20cm wide corridor from the rat’s entry to the platform (Day et al., 1999; Willson et al., 2007), and (iv) success to locate the goal. From this data, a search score was calculated to quantify the swim trajectory (Burguière et al., 2005). The probe test was conducted 2 hours after the last hidden platform training trial in which the escape platform was removed and the rat was given 60 s to search the platform’s former (‘test’) location. The time spent in the test quadrant and the same measures as training were taken. A learned spatial position was recorded if the swim time in the test quadrant was greater than chance (ie 1:4 =15 s). Seven days after the probe test, animals underwent a retrieval test (i.e. the hidden platform was returned to its original position) and the same procedure as the hidden platform protocol was used.

Swimming Assessment
Although cerebellar lesions have less effect on swimming than terrestrial locomotion (Federico et al., 2006), to ensure that motor dysfunction did not impair the animal orientating and swimming to the platform, animals were subjected to 4 training sessions (5 trials/session) in a visible platform test. The escape platform was 2 cm above the waterline in the south-west quadrant with a globe shaped flag attached (proximal cue). Measured variables were escape latency, total quadrants crossed and search score.

Olivocerebellar Axonal Tracing
After behavioural testing, the presence or absence of CF reinnervation was revealed by either injection of a retrograde or anterograde tract tracer. Specifically, after motor testing (basic skills and rotarod), Fast Blue (2% in distilled water, Illing, Germany) was injected into the left cerebellar hemisphere (Sherrard and Bower, 2001). After spatial learning tests (basic motor and water maze), animals were injected in the left inferior olive with Fluoro-Emerald (4% in distilled water; dextran-FITC conjugate; 10 000 MW, Molecular Probes, OR USA) to reveal reinnervating CFs, as described previously (Dixon and Sherrard, 2006).
Histology

Seven days after injections, animals were reanaesthetised with Lethobarb (365mg/Kg) and transcardially perfused with heparinised saline (5 units/mL) and 4% paraformaldehyde in phosphate buffer (pH7.4; Dixon and Sherrard 2006). The cerebellum and brainstem were dissected free, post-fixed and cryoprotected in 30% buffered sucrose. Three parallel sets of serial coronal or sagittal cerebellar sections were cut at 30µm and 40µm, respectively. Two parallel sets of serial 30µm coronal sections of the brainstem were also taken.

To confirm complete pedunculotomy, one set of coronal brainstem and cerebellar sections was stained with 0.5% methylene blue. Only animals with: (i) total degeneration of the right inferior olive (Angaut et al., 1985; Sherrard et al., 1986) (ii) separation of the left hemicerebellum from the brainstem at the level of the inferior cerebellar peduncle (Sherrard et al., 1986) and (iii) residual left interpositus deep cerebellar nuclei, which provides cerebellar output via the intact superior cerebellar peduncle (Altman and Bayer, 1997), were retained. Forty animals were excluded due to either incomplete pedunculotomy, degeneration of the left deep cerebellar nuclei or inadvertent brainstem damage, e.g. to the vestibular nuclei.

Quantification of Labelled Olivocerebellar Reinnervation

Depending on the tracer injected, a second set of sections was used to visualise either retrogradely Fast-Blue labelled olivary neurons or Fluoro-Emerald filled reinnervating CF arbors and CF terminals labelled by vesicular glutamate transporter VGLUT2 immunohistochemistry (Hioki et al., 2003; Miyazaki et al., 2003). To label VGLUT2, sections were washed in phosphate-buffered saline containing 0.25% Triton-X100 (T-PBS) followed by blocking solution 0.2% gelatin in T-PBS (T-PBS-G) for one hour. Sections were then incubated overnight with guinea pig polyclonal anti-VGLUT2 (Euromedex, 1:3000) in T-PBS-G. The VGLUT2 was revealed for 2 h with Cy3-conjugated donkey anti-guinea pig (Beckman, 1:200) in T-PBS-G. After washes in T-PBS, sections were mounted in Mowiol.

In animals injected with Fast Blue, CF reinnervation was quantified by counting all the retrogradely labelled neurons in the left inferior olive. In animals injected with Fluoro-
Emerald and processed for VGLUT2 immunohistochemistry, the cerebellum of pedunculotomised animals was divided into a series of parasagittal zones (500µm wide) extending from the midline to the left lateral hemicerebellum. Within each zone, the amount of VGLUT2 positive CF reinnervation in each lobule was scored using an arbitrary scale i.e. 1 = few strands of CFs, 2 = ¼ CF-filled lobule, 3 = ½ lobule, 4 = ¾ lobule and 5 = completely CF-filled lobule (examples shown in Fig 2). Lobule scores in each 500µm zone were summed to generate a reinnervation value for each cerebellar cortical zone and functional region. The functional regions were designated as medial (0 to 1500µm), intermediate (1500µm to 2500µm) and lateral hemicerebellum (> 2500µm; Sugihara et al., 2003) based on the longitudinally-orientated olivo-cortico-nuclear zones (Voogd and Glickstein, 1998).

Data Analyses

Motor Tests: For each basic motor test, performance plateaued by day 5, thus the 3 trials on day 5 were averaged to calculate each animal’s mean time for inter-group comparisons. For the rotarod, at each speed the 3 trials on each day were averaged to obtain each animal’s mean performance for intra-group comparisons and those from the last (9th) day were used for inter-group analyses.

Spatial/Swim Tests: For each test the data for all trials within one session were averaged to calculate the mean performance (escape latency/quadrants/search score) of each animal, which was then used to calculate the mean group performance during each session.

Correlation of Reinnervation and Behaviour: To correlate behaviour to reinnervation, each animal’s behavioural performance on the motor tests and escape latency/total quadrants crossed the last session of the visible platform, hidden platform and retention tests were converted to a score depending on how far performance differed from control animals, as previously described (Willson et al., 2007). Thus a behavioural score was obtained for each animal in each test. For the reinnervation data, the reinnervation score in each functional region was normalised to a theoretical value if all the lobules in the region were completely CF-filled (5 x number of lobules). A score of the amount of reinnervation was then calculated for each zone. This reinnervation score and the animal’s corresponding behavioural score were tested for a correlation.

Statistics: Transformed (logged) motor and water maze data revealed homogeneity of variance and were analysed using analysis of variance (ANOVA) and either Bonferroni’s
or Dunnet’s T3 (if normality was not attained) post-hoc tests, for both inter and intra-group comparisons. For the probe trials, the time spent in the ‘test’ quadrant was compared to that predicted by chance according to $t$ -tests. The percentage success of a task and frequency of direct swims was assessed using the $X^2$ test with Fisher’s exact test. Pearson’s correlation was used to test the relation between reinnervation and behavioural scores. All values were stated as mean ± SEM and the alpha level was set at $\alpha=0.05$. 

- 108 -
RESULTS

Minor effects of BDNF on simple locomotor tasks

We examined whether BDNF treatment in Px3 and Px11 animals affected their ability to perform simple locomotor tasks. Compared to age-matched vehicle-treated animals, BDNF treatment did not change the performance of sham-operated controls nor significantly improve the time taken by either pedunculotomised group in the bridge, ladder or wire tests. The only exception being that the Px3-BDNF animals improved their performance on the ladder \((F_{5,31}=6.9, P>0.05)\). Vehicle-treated groups essentially replicated previous findings with lesioned groups (i.e. Px3 and Px11) being slower on the bridge and ladder tests while Px11-Vehicle animals took longer on the wire than controls (Fig 3A, B & C: Willson et al., 2007).

BDNF improves synchronisation of gait to rotarod rotation in juvenile-lesioned animals

As a more sensitive test of CF function (Rondi-Reig et al., 1997), some animals (Control-Vehicle, \(n=6\); Px11-Vehicle, \(n=10\); Px11-BDNF, \(n=6\)) had their gait tested on the rotarod. We did not analyse a set of BDNF-treated controls because, with an upper time limit of 180 s, they had no opportunity to walk for longer than vehicle-treated controls who reach the maximum time (Dixon et al., 2005, this study, Fig 3D-G). There were no inter-group differences at a slow speed (10 rpm). At 20 rpm, the Px11-BDNF group had significantly longer latency to error compared to Px11-Vehicle \((F_{3,27}= 11.26, P<0.05)\) and were not significantly different from the control group. Furthermore at 30 rpm, when vehicle-treated Px11 animals were worse than control \((F_{3,24}= 25.3, P<0.01)\), the Px11-BDNF were not (Fig 3D); showing an intermediate performance different neither from control nor vehicle-treated lesioned groups.

We also examined how the animals learned this task because CFs are required for motor learning and the same cellular mechanisms are thought to underlie the learning of both motor and spatial tasks (Ito, 2001; Burguière et al., 2005). Learning was examined at 10 rpm, since skills learned at one speed are transferred to the next; (Rondi-Reig et al., 1997). The Px11-BDNF group displayed learning at 10rpm improving over the first 2 days \((F_{8,53}= 3.82, P<0.01\): Fig 3E-G), which contrasts with controls, who learned within the first day, and Px11-Vehicle animals that did not demonstrate learning on any day.
These data suggest that BDNF-treatment in juvenile rats allows learning in the rotarod task and better matching of gait with rotarod speed than rats without reinnervation. This is consistent with BDNF inducing transcommissural olivocerebellar reinnervation.

**BDNF treatment does not alter swimming ability in the cued water maze**

Since prior sensorimotor training (e.g. the rotarod) can mask subtle water maze abnormalities (Cain *et al.*, 1996), we used a different set of animals for spatial learning tasks (Control-Vehicle, *n*=6; Control-BDNF, *n*=3; Px3-Vehicle, *n*=4; Px3-BDNF, *n*=6; Px11-Vehicle, *n*=7; Px11-BDNF, *n*=10). We tested all groups in the visible platform task as this controlled for any effect of motor impairment or BDNF on the animal’s ability to orientate to and reach a target platform. All groups, irrespective of lesion or BDNF treatment, had similar escape latencies and total quadrants crossed (Fig 4). Moreover, escape latency correlated with search score (i.e. swimming trajectories) indicating that all animals swam at a similar speed. Therefore, the same correlation values (R²=0.79, *P*<0.01; R²=0.79, *P*<0.01 respectively; Fig 4C) for vehicle and BDNF-treated groups reveal similar search strategies for both groups, confirming that BDNF did not affect how the animal searched the maze.

**BDNF-treated lesioned animals show improved acquisition of a spatial task**

The hidden platform water maze was used to assess whether reinnervation induced by BDNF improved spatial learning. In addition to confirming previous results for vehicle-injected groups (Willson *et al.*, 2007), with Px3-Vehicle animals reaching control performance and Px11-Vehicle animals performing poorly in the maze, our study revealed new data that BDNF facilitated spatial learning performance in pedunculotomised animals without significantly altering controls.

The BDNF-treated control and Px3 groups improved their performance in the maze between all sessions with decreased escape latencies (*p*<0.05; Fig 5A), total quadrants crossed (*p*<0.01; Fig 5B) and better search strategies (*p*<0.01; Fig 5C). This improvement continued for longer than in vehicle-treated control and Px3 groups which reached their learning asymptotes by session 2 (Fig 5A & B). To ensure that the BDNF groups had reached their optimal performance within the testing period, we analysed individual training trials within each session (i.e. 10 trials per session) for each parameter. The Px3 group plateaued their performance by the last 2 trials of session 2 (trials 19-20).
while the control group reached a plateau at the mid-point of session 3 (trials 22-28). As
the control-BDNF animals continued to improve in session 3, they became better than
BDNF-treated Px11 animals (escape latency $F_{5,66} = 6.7$, $P<0.01$; quadrants crossed $F_{5,66} =
6.5$, $P<0.05$; search score $F_{5,66} = 5.7$, $P<0.05$). This is because the BDNF-treated Px11
group improved its performances until session 2 and thereafter plateaued. While this
learning-curve was the same as Px11-vehicle animals, the BDNF group developed better
maze performance overall. This is shown by the Px11-BDNF group having the same
learning scores as vehicle-treated controls and both Px3 groups during training sessions 2
and 3 (Fig 5A & B), while the Px11-vehicle animals remained significantly impaired
(session 2: escape latency $P<0.05$, quadrants crossed $P<0.05$; session 3: escape latency
$P<0.05$, quadrants crossed $P<0.05$).

The effect of BDNF on spatial function was further demonstrated in the number of direct
swims to the platform. Animals in both BDNF-treated pedunculotomised groups made
more direct swims to the hidden platform compared to their vehicle-treated counterparts
($\chi^2$ test: Px3, $p<0.05$; Px11, $p<0.01$) and equalled the control (Control-Vehicle) group
(Table 1). In addition, the Px11-BDNF animals also made as many direct swims as both
Px3 groups (Table 1). Comparisons were made to the control-vehicle groups since the
BDNF-treated controls also increased their direct swims ($\chi^2$ test, $p<0.01$).

To discriminate whether the better motor performance (e.g. on the rotarod) of the BDNF-
treated Px11 animals contributed to their improved spatial function, we made 2 further
analyses. First we tested for correlations between the learning parameters in the visible
and hidden platform mazes (Cain et al. 1996). No correlations were observed for any
group between these two versions of the water maze. Correlations existed only between
learning parameters within each test for each group (e.g. hidden platform maze: escape
latency–total quads, $p<0.01$; escape latency–search score, $p<0.01$; total quads–search
score, $p<0.05$). Second, we re-compared our groups using the time taken in the basic
motor tests as a covariate of the escape latency in the hidden platform (Martin et al.,
2003). In the bridge and wire tests there was a significant effect of the covariate
(RANCOVA covariate: bridge, $F_{1,5} = 8.75$, $P<0.01$; wire, $F_{1,5} = 5.23$, $P<0.05$) but a group
effect persisted (RANCOVA group: bridge, $F_{1,5} = 3.19$, $P<0.01$; wire, $F_{1,5} = 2.48$, $P<0.05$), whereas for the ladder there was no covariate effect so the group effect remained
(RANCOVA group, $F_{1,5} = 3.8$, $P<0.01$). These data indicate that motor ability does affect
the performance in the hidden platform, but that disparity in spatial learning accounts for
the residual inter-group differences. This was further confirmed when comparisons were
made between only BDNF and vehicle-treated Px11 groups, in which the covariate was
significant (RANCOVA covariate: \( F_{1,2} = 5.7, P<0.05 \)) as too the group effect
(RANCOVA group, \( F_{1,2} = 4.3, P<0.05 \)).

**BDNF treatment of lesioned-animals facilitates spatial memory**

A probe test, in which the escape platform is removed, evaluates how accurately animals
learned the spatial task. There was rapid extinction during this task in most groups from
trial 1 to 2, thus only trial 1 data were analysed. While all groups showed a similar spatial
bias to the test quadrant (~19 s), only the BDNF-treated groups (p<0.05 for each) and
vehicle-treated control (p<0.05) group stayed there significantly longer than chance.
However, the number of quadrant entries, total quadrants crossed and direct swims were
not significantly different between any groups.

A retrieval test was next undertaken to test the ability of animals to remember previously-
learned strategies. In this test, the platform was replaced into its original location and
performance was compared to the last hidden platform training session. The intervening
week between the probe and retrieval test did not impair escape latency, total quadrants
crossed or search strategies for any group. However, BDNF-treated animals remembered
better as the number of direct swims were similar for all BDNF-treated groups (Table 1),
whereas vehicle-treated lesioned animals made fewer direct trajectories than their controls
(Px3, p<0.01; Px11, p<0.01).

**Olivocerebellar reinnervation and its relation to behaviour**

To examine the presence or absence of reinnervation we used Fast Blue retrograde tracing
or Fluoro-Emerald anterograde tracing with VGLUT2 immunohistochemistry. Animals
tested for appropriate gait synchronisation (simple motor tests and rotarod) received Fast
Blue into the left cerebellar hemisphere. Retrogradely-labelled olivary neurons were
observed only in the left olive of the Px11-BDNF group (~9-31 neurons), confirming
transcommissural olivocerebellar reinnervation.

In animals tested for spatial function (simple motor tests and water maze), anterograde
tracing revealed the expected reinnervation in Px3 and Px11-BDNF groups and that its
morphology was as previously described (Dixon and Sherrard, 2006; Letellier et al., 2007). However, the present study adds information on the distribution and density of this reinnervation. Reinnervating CFs were distributed in the left hemicerebellum up to 8 mm from the midline, predominately in vermal lobules III-VIII and in the hemisphere from lobulus simplex to copula pyramidis (Fig 6A - C). In BDNF-treated Px3 animals, reinnervation appeared denser in the intermediate and lateral hemicerebellum compared to vehicle-injected animals. In addition, in Px11 animals BDNF-induced reinnervation appeared much less extensive and dense than in either vehicle or BDNF-treated Px3 groups (p<0.05).

To examine whether olivocerebellar reinnervation was related to an animal’s behaviour, we correlated reinnervation scores for each functional region of the left hemicerebellum (medial, intermediate or lateral) and behaviour scores for each motor and water maze test. For the basic motor and rotarod tests, reinnervation (either by VGLUT2 mapping or retrograde olivary analysis) did not correlate to motor score in any group. In contrast, in the hidden platform and retention tests, the total quadrants crossed and escape latency correlated with the reinnervation score in the intermediate and lateral hemicerebellum of Px3 groups (n= 5 animals, hidden platform: total quadrants R²=0.81 p<0.01; escape latency R²=0.63 p<0.05; retention test: total quadrants R²=0.64 p<0.05; escape latency R²=0.67 p<0.05). Furthermore, when all groups are considered, the reinnervation score of the left hemicerebellum directly correlated with the escape latencies in the hidden platform task (R²=0.40 p<0.05). These results suggest that increasing olivocerebellar reinnervation aids spatial learning responses.
DISCUSSION
In this study, we examined the function of BDNF-induced circuit repair using motor and spatial learning tests. We observed that, irrespective of the age of treatment, BDNF increased transcommissural CF reinnervation and this was associated with enhanced spatial learning, memory and gait synchronisation. As navigation (Dahhaoui et al., 1992; Rondi-Reig et al., 2002), spatial memory (Gasbarri et al., 2003) and gait synchronisation (Rondi-Reig et al., 1997) are related to CF function, in contrast to other aspects of cerebellar motor activity (i.e. muscular strength, equilibrium and postural control; Petrosini et al., 1990; Caston et al., 1995; Rondi-Reig et al., 1997), our data suggest that improved rotarod and spatial functions are related to the increased reinnervation.

BDNF promotes transcommissural olivocerebellar reinnervation
The growth promoting effect of BDNF on alternate connectivity is demonstrated by the increased transcommissural CF reinnervation in neonatal (Px3) animals and induced reinnervation in juvenile (Px11) animals. In both age groups, reinnervating CFs were observed not only at the BDNF injection sites but also along the whole rostro-caudal plane of the cerebellum (Fig 7), replicating the distribution of spontaneous neonatal reinnervation (Angaut et al., 1985; Sugihara et al., 2003). Furthermore, the lack of transcommissural reinnervation in vehicle-injected Px11 animals (our data; Sherrard and Bower, 2001; Dixon and Sherrard, 2006), confirms that the reinnervation was due to BDNF. The few CF terminals adjacent to the midline in these animals is consistent with terminal sprouting in the molecular layer, which occurs after neurotoxin-induced CF deprivation (Rossi et al., 1991) but provides no functional benefit (Rondi-Reig et al., 1997).

The facilitation of transcommissural reinnervation by BDNF concurs with its chemotactic effects (Ming et al., 1997) increasing axonal growth (Tucker et al., 2001), arborisation (Alsina et al., 2001) and synaptogenesis (Vicario-Abejon et al., 2002). BDNF-Trk receptor signalling activates intracellular cascades (e.g PKC, ERK and Mst3b: Irwin et al., 2006; Reichardt, 2006; Zhou and Snider 2006) involved in axon growth and collateralisation (spinal cord, Sivasankaran et al., 2004; hippocampus, Dinocourt et al., 2006). TrkB is abundantly expressed in the inferior olive from P0 to P6 (Riva-Depaty et al., 1998), when normal innervation (Crepel et al., 1976; Mariani and Changeux, 1981) and neonatal reinnervation occur (Sugihara et al., 2003; Lohof et al., 2005). Since Trk...
receptors are anterogradely transported (Bhattacharyya et al., 2002) to growth cones (Gomes et al., 2006), the greater CF reinnervation in BDNF-treated cerebella is consistent with BDNF-TrkB signalling in reinnervating CFs. Moreover, BDNF induced transcommissural reinnervation in the maturing system of juvenile animals despite less favourable conditions. Amongst these factors are PC expression of truncated TrkB (Lindholm et al., 1997) which will sequester injected BDNF (Bramham and Messaoudi, 2005), reduced olivary TrkB expression (Riva-Depaty et al., 1998) decreasing the capacity for CF response to BDNF, and cerebellar myelin (Reynolds and Wilkin, 1991) inhibiting neurite outgrowth. However, transcommissural reinnervation is still concordant with BDNF activating TrkB growth-associated cascades (discussed above) in CF terminals, because BDNF also elevates cAMP in maturing neurons (Cai et al., 1999) which in turn, increases the intrinsic growth state of neurons (Cai et al., 2001) and blocks growth cone retraction in response to myelin to facilitate axonal growth.

Therefore, our data suggest that BDNF activates developmental growth mechanisms that permit olivocerebellar axonal growth into the denervated hemicerebellum when it begins to be myelinated.

**Behavioural improvement associated with BDNF-induced reinnervation**

In our study, transcomissural olivocerebellar reinnervation that takes place in the presence of BDNF was associated with improved CF-mediated behaviours.

In the spatial learning and memory tests, BDNF-treated pedunculotomised animals performed better than their vehicle-injected lesioned counterparts. The densely reinnervated Px3-BDNF group attained control performance, whilst the moderately reinnervated vehicle-injected group (Px3-Vehicle) had an intermediate phase of poor spatial learning. Furthermore, the lightly reinnervated Px11-BDNF animals made more direct trajectories than the non-reinnervated vehicle-injected group (Px11-Vehicle) but remained worse than controls. Incomplete recovery in Px3-Vehicle and Px11-BDNF animals may be explained by the imperfect nature of the reinnervation, only partially recreating the olivo-Purkinje-nuclear circuit (Sugihara et al., 2003; Willson et al., 2007). CF-deprived PCs have altered firing rates (Montarolo et al., 1982; Batini et al., 1985), which induce abnormal activity in their target neurons (Batini et al., 1985) and disrupt sensorimotor processing (Hoebeek et al., 2005). Therefore, increased CF reinnervation in
the Px3-BDNF group reduces the number of CF-deprived PCs and minimises altered PC discharge. This is consistent with better adaptive learning strategies (Leggio et al., 1999) and retention of spatial knowledge (Dahhaoui et al., 1992). Our hypothesis is supported by the correlation between hemispheric (Px3 groups), but not vermal (Px11-BDNF), CF reinnervation and spatial function (Fig 8).

In addition CF-related motor function was also improved in BDNF-treated animals. The rotarod task showed that groups with CF reinnervation (Px3-Vehicle, Px11-BDNF) could synchronise their gait with rotational speed better than those without reinnervation (Px11-Vehicle). As skilled movements require groups of PCs to fire synchronously in response to olivary activation (Welsh et al., 1995), we propose that CF reinnervation re-established olivary-PC synchrony to provide gait coordination. Thus better rotarod function in the Px11-BDNF group compared to Px11-Vehicle is consistent with BDNF-induced reinnervation being similar to spontaneous neonatal reinnervation (Px3-Vehicle). In addition, since retrograde tracing only assessed olivocerebellar reinnervation to the left hemisphere (spatial function: Joyal et al., 1996), the lack of correlation between olivary labelling and rotarod data is consistent with reinnervating CFs showing relatively normal olivocerebellar topography (Buisseret-Delmas and Angaut, 1993). In contrast, all lesioned animals, irrespective of BDNF treatment, performed similarly on the simpler motor co-ordination tests (bridge, ladder, wire), in keeping with their regulation by cerebellar cortical and deep nuclear circuits (Caston et al., 1995) rather than CFs.

Sensorimotor vs. spatial improvement: BDNF-induced reinnervation assists recovery in a complex cognitive function

Although behavioural improvement occurs in BDNF-treated animals, treatments to increase neural circuit repair are beneficial if they are associated with recovery of appropriate functions, i.e. those subserved by the repaired circuit. In the cerebellum, deafferentation or dysfunction of PCs adversely affects both sensorimotor and spatial functions (Dahhaoui et al., 1992; Le Marec et al., 1997; Meignin et al., 1999; Martin et al., 2003). As transcommissural olivocerebellar reinnervation improves motor function (Dixon et al., 2005), sensorimotor (vs. spatial) deficits in lesioned groups with little or no reinnervation may impair spatial acquisition in the hidden platform test, i.e. worse spatial navigation in the Px11-Vehicle versus Px11-BDNF animals reflects sensorimotor rather than spatial dysfunction. Our data do not support this interpretation. First, sensorimotor
and spatial dysfunction are differentiated in the visible maze (Martin et al., 2003): animals with sensorimotor disturbance cannot correctly orientate to a goal in the visible and hidden water maze (Cain et al., 1996). In our study, all groups orientated to the target in the visible maze, as demonstrated by the same search scores. Second, contributions of sensorimotor dysfunction to spatial impairment are detected by correlations across visible and hidden maze tests (Cain et al., 1996). However, there was no correlation for any measure between the visible and hidden mazes. These data indicate that sensorimotor recovery in BDNF-treated groups does not explain better spatial function.

In addition, the cerebellar (procedural) component of spatial navigation involves learning the motor patterns required to make a direct path to a target within a learned environment (Leggio et al., 1999; Burguiere et al., 2005). Since transcommissural olivocerebellar reinnervation also facilitates motor learning (Dixon et al., 2005; rotarod this study), apparent changes in spatial learning may simply reflect better motor learning. Sensorimotor impairment can be distinguished from learning deficiency by comparing the relative performance in naive (sensorimotor differences) and trained (sensorimotor and learning differences) animals (Rondi-Reig et al., 1997). In our study, all groups performed equally on day 1 of the hidden water maze (Fig 5), confirming that motor dysfunction did not impair the animal’s ability to search the maze. With training, the control and reinnervated animals (Px3-Vehicle, Px3-BDNF, Px11-BDNF) improved more than those without reinnervation (Px11-Vehicle), so that at the end of training the Px11-Vehicle animals were significantly worse than controls, indicating learning deficits (Rondi-Reig et al., 1997). Although the results of this study cannot delineate how much CF reinnervation facilitates motor vs. spatial learning, navigation requires a link between learned motor responses (direct trajectory) and specific inputs from the environment (cues), which depends on PC long-term depression (LTD; Burguiere et al., 2005) for which CF innervation is necessary (Ito, 2001). Therefore, our data suggest that CF reinnervation not only aids motor learning (i.e. Px11-BDNF group), but also enables this link so animals can spatially navigate. As compensation of spatial function occurs in both neonatal (Px3) and juvenile (Px11-BDNF) animals, BDNF appears to reproduce developmental plasticity to compensate both fine motor synchronisation and spatial cognition.
Conclusions
Although increasing post-lesion neuroplasticity is associated with some functional recovery (Coumans et al., 2001; Roy et al., 2006; Vavrek et al., 2006), poor correlation of function with structure suggests that behavioural outcome represents adaptation (Bareyre et al., 2004) vs. real recovery. Moreover, axonal sprouting that recruits functionally-related contralateral motor pathways (e.g. rubrospinal instead of corticospinal) is associated with recovery of skilled movements (Smith et al., 2007). Our data show that adding growth-promoting agents into the denervated target (Sherrard and Bower, 2001) induces an alternate projection whose structure correlates with appropriate complex motor and cognitive recovery. Since such alternate paths can be induced in the mature system (Sherrard and Bower, 2003; Dixon and Sherrard, 2006) and BDNF is widely distributed throughout the CNS (Conner et al., 1997; Das et al., 2001), this recreation of developmental plasticity has significant potential therapeutic strategies after injury involving axonal damage.

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ABBREVIATIONS
BDNF, brain-derived neurotrophic factor
CF, climbing fibre
ERK, extracellular signal-regulated kinases
Mst3b, mammalian Ste20-like protein kinase-3b
PC, Purkinje cell
PKC, protein kinase C
Px, pedunculotomy
LTD, long-term depression
Trk, tropomyosin-related kinase
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TABLE 1
Percentage of direct swims (over all trials) for each group

<table>
<thead>
<tr>
<th>Maze task</th>
<th>Control</th>
<th>P3 lesion</th>
<th>P11 lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>BDNF</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Training</td>
<td>27</td>
<td>44**</td>
<td>16**</td>
</tr>
<tr>
<td>Probe</td>
<td>67</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Retention</td>
<td>58</td>
<td>50</td>
<td>13**</td>
</tr>
</tbody>
</table>

Significant differences between control and Px groups: **p<0.01.
Significant differences between Px3 and Px11 groups: #p<0.05.
Significant differences between BDNF and vehicle-treated groups: ^p<0.05, ^^p<0.01.
Figure 1

(A & B) Schematic diagrams representing the normal olivocerebellar pathway (filled-line) that originates in the inferior olive, crosses the midline in the medulla and ascends to the contralateral hemisphere via the inferior cerebellar peduncle.

(A) If the peduncle is transected (•) on P3, the contralateral olive degenerates and new transcommissural fibres grow to reinnervate Purkinje cells densely in the vermis (bold dotted line) and more sparsely in the hemisphere (fine line; Sherrard et al., 1986). BDNF was injected at P6 lateral to the paravermis vein and into the intermediate/lateral hemicerebellum in an attempt to augment reinnervation in the partially reinnervated zone (grey dotted line).

(B) When the peduncle is transected (•) on P11 no spontaneous reinnervation occurs. BDNF was administered into the left medial/intermediate hemicerebellum at P12 to induce transcommissural axonal sprouting into the denervated region (grey dotted line: Sherrard and Bower, 2001).
Figure 2
Arbitrary scale used to define the amount of reinnervation in each lobule of the left hemicerebellum within 500µm bands from the cerebellar midline. The VGLUT2 positive scores in the molecular layer were graded as: 1 = strands of CFs, 2 = ¼ lobule CF-filled lobule etc until 5 = completely CF-filled lobule. Asterisks designate regions with reinnervation. WM=white matter, GCL=granule cell layer, ML=molecular layer. Bar = 100µm.
Figure 3
Bar charts represent the time taken (mean ± SEM) by vehicle and BDNF-treated groups to complete the bridge (A), ladder (B) and wire (C) during the 5th day of training and the error latencies on the rotarod (D – G). All lesioned groups were slower than control groups on the bridge (A), whilst only the Px3-Vehicle (Px3V) group performed worse than controls on the ladder (B). On the rotarod (D), at 20rpm and 30rpm the Px11-Vehicle (Px11V) group failed more quickly than control or Px11-BDNF (Px11B). Furthermore, control and Px11-BDNF groups learned the task at 10rpm by the 2nd and 3rd days respectively while the Px11V did not (E). At higher speeds (F & G) no group significantly improved their error latency over the 9 days of training. Significant differences between control and lesioned: *p<0.05, **p<0.01; #p<0.01; Significant difference between Px11V and Px11B groups: ^p<0.05.
Figure 4

(A & B) Line graphs represent the total mean number of quadrants crossed by all vehicle-treated (A) and BDNF-treated (B) groups to reach the visible platform. There were no inter-group differences. (C) Correlation graph of each animal’s search score (quantified swim trajectory) and the escape latency in the last training session. Vehicle and BDNF-treated animals had the same correlation value.
Figure 5
Graphs showing the total quadrants and escape latency to reach the hidden platform during each training session (10 trials/session).

(A) Line graphs represent the mean escape latency for vehicle and BDNF-treated control, Px3 and Px11 groups. Note the Px11-Vehicle (Px11V) group was consistently slower than control animals. The sketches on the right Y-axis illustrate typical search trajectories for the calculated search scores (Petrosini et al., 1996).

(B) Line graphs represent mean total quadrants crossed for vehicle and BDNF-treated control, Px3 and Px11 groups. The BDNF-treated Px3 group (Px3B) performed as controls, whilst the Px3-Vehicle (Px3V) animals were intermittently worse performers crossing more quadrants than controls in session 2. The Px11V group consistently crossed more quadrants than controls whereas the Px11-BDNF group improved performances until the last session.

(C & D) Regression line graphs showing that high escape latencies of any lesioned group correspond to poor search strategies. Each Px3 (C) and Px11 (D) group had a positive and significant correlation (**p<0.01) between escape latency and search strategy used. Significant differences between control and lesioned animals: *p<0.05, **p<0.01.
Figure 6
The amount of CF reinnervation was quantified using VGLUT2 positive staining in each lobule within 500µm bands and represented on an unfolded cerebellum (adapted from Buisseret-Delmas and Angaut, 1993). (A) & (B) Illustrate the difference in vehicle (A) and BDNF (B) treated Px3 animals, with an observed increase in the density of reinnervation. (C) Reveals the less dense but efficient reinnervation induced by BDNF in Px11 animals and also confirms a medio-lateral gradient of BDNF-induced CF reinnervation.
3.0 The Synaptic Function of Spontaneous and BDNF-Induced Transcommissural Olivocerebellar Reinnervation in the Adult Cerebellum

As BDNF-induced olivocerebellar reinnervation in the older-lesioned (i.e. Px11) animals appeared to provide significant behavioural improvements, it was important to confirm whether the synaptic function of the re-formed connections was also normal. It is known that following an early lesion (at P3), the reinnervating axons re-form climbing fibre-Purkinje cell synapses in the vermis with normal electrophysiological parameters (Sugihara et al., 2003); however, whether those synapses which form at later ages, i.e. in the hemisphere (at ~6 days post-lesion) would also attain normal function remained to be verified. As these later reinnervating synapses develop from axons which ramify into more climbing fibres (~58 climbing fibres) than their early reinnervating counterparts (~22 climbing fibres, Sugihara et al., 2003), their presynaptic function may be altered by more pronounced synaptic fatigue. In addition, the excess number of CF terminals, which form these later reinnervating synapses, could overwhelm the inferior olivary neuron’s capacity to provide neurotransmitter and proteins (e.g. cadherins linked to actin cytoskeleton) to regulate normal synapse function after activation. In animals lesioned at P11, olivocerebellar reinnervation was induced by BDNF after the period of spontaneous reinnervation (Sherrard and Bower, 2001) and afferent selection (Mariani and Changeux, 1981) which may also have affected synaptic parameters. Furthermore, synapses that have formed after a lesion at P15 do not display all of the normal developmental stages of CF-PC synaptogenesis (i.e. no multi-innervation; Letellier et al., 2007), therefore the long-term function at these synapses may not be normal and currently remains unknown.

The following study assessed the synaptic function of later forming and BDNF-induced climbing fibre-Purkinje cell synapses in the adult cerebellum. In addition to a potential effect of target age, an examination of the electrophysiological characteristics of these re-formed synapses was essential, as this would indicate whether any long-term effects of BDNF activity (refer to Introduction, section 1.4.2) changed synapse function. In parallel, the area of contact between the reinnervating climbing fibre terminal arbor and...
its reinnervated Purkinje cell dendritic tree were analysed. This study allowed us to test whether the correct morphology and synaptic function of olivocerebellar reinnervation depended upon the age at which climbing fibre-Purkinje cell synaptogenesis occurred, and whether the presence of BDNF during the reinnervation period altered these parameters.

These data are presented in the format of an article in preparation.
Effect of BDNF in olivocerebellar system repair: moderate modifications of synaptic function at adult climbing fibre-Purkinje cell synapses after lesion

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Running title: BDNF-induced synapse re-formation

Key words: BDNF, Climbing fibre, Paired-pulse depression, Purkinje cell, Reinnervation
ABSTRACT

Functional neural circuitry requires the formation of precise neuronal connections with appropriate synaptic activity. While post-lesion reinnervation may be promoted by growth factors such as brain-derived neurotrophic factor (BDNF), it remains unknown whether BDNF-induced reinnervation, involving synaptogenesis between abnormally mature partners, generates synapses with normal function. We used an animal model of reinnervation to cerebellar Purkinje cells (PCs) after unilateral transection of their climbing fibre (CF) afferents (pedunculotomy: Px). After pedunculotomy on postnatal day (P) 3, CFs densely reinnervate medially located PCs within 2 days then fewer hemispheric PCs by 6 days post-lesion. In contrast, reinnervation does not occur after CF transection at P11 unless induced by exogenous BDNF. To examine the function of BDNF-associated reinnervating synapses, BDNF/vehicle was injected into the denervated cerebellum either in the hemisphere 72 hrs after Px3, or in the vermis 24 hrs after Px11 and the amplitude, paired-pulse depression (PPD) and synaptic fatigue of reinnervating synapses, plus CF and PC morphology were measured. In controls, BDNF had no effect on the gross morphology or synaptic parameters of normal CF-PC synapses. Also, reinnervating CF-PC synapses forming between 2-6 days after Px3 (i.e. between P5-9) exhibited normal synaptic parameters and CF-PC morphology. But after BDNF treatment, reinnervating synapses forming 6 or more days post-lesion (i.e. P9-11) had smaller CF arbors, reduced synaptic amplitude and greater PPD. Moreover, later reinnervation induced by BDNF after Px11 had normal morphological properties but greater PPD. Overall, reinnervation associated with BDNF generates synapses with minor modifications and would be likely to function normally in vivo. Our results suggest that despite neuronal age, once pre and post-synaptic neurons are in contact post-lesion, relatively normal function will be re-established.
INTRODUCTION

Appropriate synaptic formation and function requires that axon-target interactions be correctly coordinated to allow for normal structure and physiology. However, after adult central nervous system (CNS) axonal injury, the factors that regulate correct synapse formation and stability limit functional recovery (Pizzorusso et al., 2002; McGee et al., 2005; Liu et al., 2006). Axonal growth after injury is restricted by a poor cell-autonomous intrinsic growth program and the presence of extracellular inhibitory molecules (Chen et al., 1995; Fournier et al., 2001; Goldberg et al., 2002; Mingorance et al., 2004). Repair strategies to overcome these limitations have included priming the environment with inductive signals (such as trophic factors) or reducing inhibitory molecules in order to induce axons to grow and reinnervate denervated targets (Coumans et al., 2001; Moon et al., 2001; Vavrek et al., 2006). Nonetheless, it remains rare that axon projections grow and reach the correct target neurons to replace the missing connections with functional synapses. Furthermore, the anatomical and physiological interaction formed at these new synapses after injury is poorly defined.

Brain-derived neurotrophic factor (BDNF) is one secreted factor that induces normal axonal growth and arborisation (Cohen-Cory and Fraser, 1995; Alsina et al., 2001; Tucker et al., 2001), regrowth after injury (Vavrek et al., 2006) and dendritic growth and remodelling (Lom and Cohen-Cory, 1999; McAllister 2000; Xu et al., 2000; Horch and Katz, 2002). Exogenous BDNF treatment to promote post-lesion reinnervation could also affect the target neuron’s dendritic arborisation. This in turn may influence the number and pattern of presynaptic axonal contacts received by the target neuron (Hume and Purves, 1981; Purves et al., 1986) and hence presynaptic structure. Therefore, it is important to define whether a post-lesion treatment using BDNF allows us to recreate correct synaptogenesis with normal axon-target morphology and physiological function.

We have used the olivocerebellar projection of the rat cerebellum to investigate the effects of BDNF on the formation of new functionally beneficial synaptic connections post-lesion in early and late postnatal development. In the adult, olivocerebellar axons enter the cerebellum via the contralateral inferior cerebellar peduncle and terminate as climbing fibres (CFs) on Purkinje cells (PCs) (Sugihara et al., 2001). After unilateral transection of the olivocerebellar path early in development (before postnatal day (P) 7),
the contralateral (axotomised) inferior olive degenerates, and new axons arising from the remaining inferior olive grow into the denervated hemicerebellum and reinnervate denervated PCs (Fig 1A; Angaut et al., 1982). After lesion at P3, reinnervating CFs densely reinnervate vermal PCs within 2 days post-lesion (Lohof et al., 2005) and display normal CF arborisations with functional synapses on the PCs in the adult (Sugihara et al., 2003). Fewer paravermal and hemispheric PCs become reinnervated in this model, and the process begins 4 to 6 days post-lesion (Zagrebelsky et al., 1997), a period when PCs have primary dendrites and begin to bear secondary branches (Altman 1972; Berry and Bradley, 1976). In contrast, after unilateral transection at P11, no PC reinnervation occurs unless induced by BDNF or another growth factor (Fig 1B; Sherrard and Bower, 2001). The effect of different developmental stages on new synapse formation, as well as the effect of exogenous BDNF, is not known.

In this study, we assessed the synaptic function and CF-PC morphological interaction at early- and later re-forming synapses in the adult cerebellum for several reasons. First, reinnervating axons do not always exhibit normal electrophysiological responses (Finlay et al., 1979), hence it cannot be presumed that older reinnervating CFs and those induced by BDNF will function correctly. Second, BDNF has long-lasting effects on neurotransmitter vesicle release probability (Lessmann and Heumann, 1998; Tyler et al., 2006), which may alter synaptic function of reinnervating CFs in our model. Third, PCs reinnervated after early lesion, which exhibit normal CF synaptic function (Sugihara et al., 2003), have slightly abnormal dendritic morphologies (Lohof et al., 2005). Therefore, for those PCs reinnervated later during development, normal PC anatomy cannot be guaranteed. This study will address the nature of the synaptic transmission of re-formed synapses induced by BDNF and indicate the extent to which these synapses are functionally normal.
MATERIALS AND METHODS

Different ages of synapse re-formation induced by unilateral olivocerebellar transection

Experiments were performed according to the regulations of the Comité National d’Éthique pour les Sciences de la Vie et de la Santé, which are in accordance with the European Communities Council Directive (86/609/EEC). To examine the synaptic function of BDNF-induced reinnervating fibres at different maturational ages, 117 pups were assigned to 2 experimental groups: (i) pups pedunculotomised on P3, in which CFs begin to reinnervate vermal PCs within 2 days (Lohof et al., 2005) and paravermal and hemispheric PCs 4 and 6 days after lesion, respectively (Zagrebelsky et al., 1997; Fig 1A) and (ii) those lesioned on P11, in which no reinnervation occurs without exogenous neurotrophic factor (Sherrard et al., 1986; Dixon and Sherrard, 2006; Fig 1B). Under diethyl ether (Prolabo, FR) anaesthesia, pups underwent unilateral transection of the left cerebellar peduncle, as previously described (Bower and Waddington, 1981). Some pups in each litter underwent a sham operation in which the inferior cerebellar peduncle remained intact. After recovery from the anaesthetic, animals were returned to the dam for care and nourishment.

Intracerebellar BDNF injections

Recombinant human BDNF (r-metHu BDNF: Amgen Inc, CA USA; Alomone, France) was administered at ~12ng/mg cerebellum in 0.1% bovine serum albumin in phosphate buffer. Under diethyl ether anaesthesia, a craniotomy was performed to expose the left hemicerebellum (Sherrard, 1997). Using a glass micropipette (diameter ~50µm), a total of 1µL BDNF solution or vehicle was injected, at multiple (12-14) sites. For P3-lesioned pups, BDNF or vehicle was injected into the left lateral hemicerebellum (lobulus simplex to crus II), 72 hours after lesion (i.e at P6; Fig 1A) when spontaneous reinnervation of paravermal PCs begins (Zagrebelsky et al., 1997). For P11-lesioned pups, BDNF or vehicle was injected into the left hemivermis (lobules VI to VIII) and paravermis (lobulus simplex to crus II) 24 hours post-lesion (i.e P12; Fig 1B) to induce reinnervation (Dixon and Sherrard, 2006). Some animals in both sham and lesioned groups were injected with vehicle solution (bovine albumin serum in 0.1% phosphate buffer) to control for any effect of the injection alone on normal developing synapses.
Slice preparation
Animals aged P23 –37 (P3-lesion + Vehicle, n = 11; P3-lesion + BDNF, n = 16; P3-sham + Vehicle, n = 10; P3-sham + BDNF, n = 15; P11-lesion + BDNF, n = 9; P11-sham + Vehicle, n = 8; P11-sham + BDNF, n = 9) were anaesthetised with isoflurane (Baxter, France) and decapitated. Sagittal slices (300μm) were cut from the left cerebellar vermis, pars intermedia and hemisphere using a vibratome (VT 1000S; Leica, Germany). Each cerebellar zone was identified based on lobular formation and distance from the midline (vermis: 0-1500 μm, pars intermedia: 1500-2400 μm and hemisphere: > 2400 μm; Sugihara et al., 2003). This division allowed us to analyse synapses re-formed at different neuronal ages in each animal. The slices were cut and maintained in a bicarbonate-buffered saline solution containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 25 glucose, bubbled with 95% O₂/5% CO₂. The tissue was incubated for 1 hour at 37°C before recording at room temperature.

Electrophysiology
Whole cell recordings of PCs were obtained as previously described (Llano et al., 1991) using an internal solution containing (in mM): 120 CsD-gluconate, 10 HEPES, 10 BAPTA, 4 MgATP, 3 TEA Cl, 0.2 NaGTP, and 13mM biocytin; adjusted to pH 7.3 with CsOH, 290-300mOsm. Recording pipettes with resistances of 3-6 MΩ were used and the range of series resistance was 4-16 MΩ, routinely compensated at 70%. Synaptic currents were monitored at a depolarised membrane potential of 0 to +30 mV to inactivate voltage-gated sodium and calcium channels and to prevent voltage clamp escape of large synaptic currents. Picrotoxin (100μM) was added to the external solution to block post-synaptic inhibitory currents. The access resistance and leak current were monitored continuously and PCs were excluded from analysis if either value increased significantly during recording.

CF-induced excitatory post-synaptic currents (CF-EPSCs) were evoked using a glass stimulation pipette (tip diameter ~10μm) filled with Ringer’s solution and placed in the granule cell layer near the PC soma. The stimulation pipette (connected to a Digitimer stimulator, DS2; SEGA) was repositioned and the stimulus intensity adjusted (1-100mV, 0.1 ms pulses) until an all-or-none response (Eccles et al., 1966) exhibiting synaptic depression after paired stimulations (Paired Pulse Depression, PPD; Konnerth et al., 1990) was elicited. Stimulation of CF-EPSCs in the granule cell layer can also activate
PF-EPSCs, which leads to underestimation of the PPD (Dittman and Regehr, 1998). In addition, antidromic activation of the PC axon can occur. To minimise this problem, the stimulus threshold was slowly adjusted and/or the stimulation electrode was repositioned. Currents were recorded with an Axopatch 200 (Axon Instruments). Data acquisition and offline analysis were done using Acquis1 and Elphy software, respectively (Biologic, Grenoble, France).

The synaptic function of induced CFs was examined with three different approaches. First, the synaptic amplitude of the CF was measured in PCs held at 0mV, in order to estimate basal neurotransmission. Second, the PPD was examined by paired stimulations of the CF using a range of inter-stimulus intervals (50 to 3000ms). The PPD ratio gives an indication of neurotransmitter release properties of the reinnervating CFs. Third, synaptic fatigue of CF-EPSCs was tested by sustained trains of stimulation (0.033Hz to 10 Hz).

**Criteria for inclusion of lesioned animals**

Gross inspection of the lesion site and histological analysis of the brainstem allowed us to determine whether the transection of the left inferior cerebellar peduncle was complete. For histology, the brainstem was post-fixed in 4% paraformaldehyde and cryoprotected in 30% buffered sucrose. 30µm coronal sections were cut and stained with 0.2% cresyl violet and analysed using well-established criteria (Angaut et al., 1982; Sherrard et al., 1986). Only animals with complete degeneration of the right inferior olive (Angaut et al., 1982) and separation of the left hemicerebellum from the brainstem at the level of the inferior cerebellar peduncle (Sherrard et al., 1986) were included in the study. In total, 39 animals were excluded due to incomplete degeneration of the right inferior olive, total degeneration of the left hemicerebellum or inability to evoke CF-induced synaptic responses.

**Biocytin revelation and VGLUT2 immunocytochemistry**

Cerebellar slices used for electrophysiology were subsequently treated to reveal biocytin-filled PCs and CF terminals using the immunocytochemical localisation of the glutamate transporter VGLUT2 (Hioki et al., 2003). VGLUT2 immunocytochemistry specifically labels CFs in the molecular layer (Hioki et al., 2003; Miyazaki et al., 2003). Slices were fixed in 4% paraformaldehyde overnight and stored in phosphate-buffered saline (PBS).
for 1 to 2 days. Subsequently, slices were washed in PBS containing 0.25% Triton-X100 (T-PBS), incubated with methanol (70%)-PBS for 30 mins and followed by blocking non-specific binding with 0.2% gelatin in T-PBS (T-PBS-G) for 40 mins. Slices were incubated overnight with guinea pig polyclonal anti-VGLUT2 (Euromedex, 1:3000) in T-PBS-G. The VGLUT2 and biocytin-filled neurons were revealed for 2 h with a mixture containing Cy3-conjugated donkey anti-guinea pig antibody (Beckman, 1:200) and streptavidin conjugated to Alexa Fluor 350 (Molecular Probes, 1:200), in T-PBS-G. After washes in T-PBS, slices were mounted in Mowiol. Images at several focal planes were acquired with a CCD camera (DFC 300FX, Leica) on a Nikon E800 microscope (Nikon, Japan) or an inverted confocal microscope (DM IRBE, Leica).

Morphological analysis of labelled Purkinje cells and climbing fibres

To examine the effects of BDNF and age of denervation-reinnervation on PC morphology and climbing fibre arborisations, we measured the (i) PC soma size, (ii) dendritic density, (iii) total dendritic territory, and (iv) CF territory as a proportion of the PC dendritic tree. Several focal planes of each PC’s dendritic structure and CF arborisation were acquired and flattened with Stack Focuser (Image J, NIH, USA). We applied Fast Fourier Transform to each flattened image, followed by contrasts and a binary threshold in order to calculate the total number of pixels in the desired anatomical zone (MetaMorph Imaging System, Universal Imaging Corporation, USA; Adobe Photoshop 6). The PC dendritic density was calculated as the proportion of the total PC dendritic territory occupied by labelled dendritic branches. To measure the extent of CF arborisation, the VGLUT2 puncta most distal on the PC dendritic tree were connected with straight lines and the enclosed area was measured and compared to the area of the total PC dendritic tree (Hisatsune et al., 2006).

Statistical Analyses

Electrophysiological and morphological data showed homogeneity of variance and in most cases normality. Therefore we used one-way ANOVAs with post-hoc Tukey’s test and the alpha level was set at α=0.05 with the values stated as means ± SEM.
RESULTS

In early reinnervated CF-PC synapses (P5-7), the synaptic properties and anatomical interaction observed in the adult were normal (Sugihara et al., 2003) but whether synapses re-forming later during development would have appropriate form and function remained unknown. We compared to control synapses the parameters of reinnervating CF-PC synapses formed at intermediate (P7-P9), later (P9-11) and relatively mature (P14-15) stages by measuring their synaptic amplitude, paired pulse depression, and synaptic fatigue, as well as the anatomical relationship between the CF and its PC.

The presence of exogenous BDNF during synapse re-formation modified the basal synaptic amplitude at some climbing fibre – Purkinje cell synapses

We first used whole-cell patch clamp recording to measure the amplitude of the CF-mediated excitatory postsynaptic current (CF-EPSC) in PCs. This gave an indication whether the basal synaptic transmission of reinnervating CFs was the same as normal CFs. Control CF-EPSCs were from both vehicle and BDNF-treated sham-operated animals, as there was no effect of BDNF on a range of tested CF-PC synaptic properties in non-lesioned animals (data not shown).

CF-EPSC amplitudes for synapses reforming between P5 and P11 in the absence of BDNF did not significantly differ from controls (Fig 2: control and P3 lesion + vehicle). These data show that although later re-forming CFs synapsed onto more mature PCs, these synapses had normal basal synaptic transmission. In addition, for the relatively mature reinnervating CFs induced to develop from P14 to P15 via BDNF application, CF-EPSC amplitudes did not differ from age-matched control CFs (Fig 2: control and P11 lesion + BDNF).

By contrast, the presence of BDNF during earlier periods of reinnervation altered CF-EPSC amplitudes: CFs that reinnervated PCs in the presence of BDNF from P7 until P11 had smaller EPSC amplitudes (p<0.05; Fig 2).

These observations suggest an effect of BDNF at some stages of PC denervation-reinnervation. Notably, the amount of time the PC remained denervated may be an
important factor: in the case of synapses re-formed between P14 and P15 (with normal basal synaptic transmission), the PCs had been denervated for only 2 days, while CFs re-forming synapses between P7 to P11, with BDNF present, took 4 to 8 days to reach their denervated PC. The suboptimal synaptic transmission in these cases may thus be partly due to the length of time the PC remained denervated.

**Morphological analyses of reinnervating climbing fibre – Purkinje cell synapses in the presence and absence of BDNF**

Since these observations suggested some modifications of reinnervating CF-PC synapses, and denervation has been reported to modify PC dendritic tree development (Lohof *et al.*, 2005), we next analysed the anatomical relationship between CFs and PCs at normal and reinnervated synapses. There were no morphological differences observed between sham-operated vehicle and BDNF-treated CFs or PCs, thus these control groups were combined for each age (data not shown). Three morphological characteristics of PCs were measured: soma size, dendritic density and total dendritic territory. We observed no differences in the soma size of PCs reinnervated from P5 to P11 in the presence or absence of BDNF but an enlarged soma size at PCs reinnervated from P14 to 15 in the presence of BDNF with respect to control PCs (p<0.05). Moreover, PCs reinnervated at any neuronal age had unchanged dendritic density irrespective of BDNF.

Most PCs reinnervated at different neuronal ages, with or without BDNF, also had total dendritic territories similar to control, consistent with the absence of a BDNF effect on PC dendritic morphology previously described *in vitro* (Shimada *et al.*, 1998; Adcock *et al.*, 2004). An exception was the group of PCs reinnervated after ~6 days of denervation (i.e. P9 to P11; Fig 3A-D). With or without BDNF treatment, this latter group of PCs had smaller total dendritic territories than control PCs (p<0.001). Therefore a delay in PC reinnervation of 6 days or more (P3 lesion, hemispheric cells; Fig 3B-D) compared to only 2 days (P11 lesion + BDNF, vermal cells; Fig 3I - K) appeared to induce a lasting effect on PC dendritic morphology.

At these synapses where the PC had a long period of denervation, BDNF treatment also affected the arborisation of the reinnervating CFs. The extent of CF arborisation over the PC dendritic tree was calculated by comparing the CF territory to the PC dendritic surface.
area. Early-reinnervated PCs (2 to 6 days post-lesion, i.e. P5-P11 and P14-P15) had CFs extending over the same area as age-matched control animals (e.g. Fig 3I-K). In contrast, PCs reinnervated after 6 days post-lesion and exposed to BDNF (i.e. P9 to P11 + BDNF) had a smaller CF reinnervation territory than age-matched control animals (p<0.005; Fig 3E-H).

Thus, the period between PC denervation and reinnervation appeared to affect PC dendritic tree size. Those PCs denervated for 6 days post-lesion had a reduced PC dendritic size, consistent with the fact that CFs influence PC dendritic development (Sotelo and Arsenio-Nunes, 1976; Rossi and Strata, 1995). In cases where exogenous BDNF was present, CF territory on the PC was also reduced, which suggests that CF branching is partly responsive to modifications of the PC (Rossi et al., 1993) and BDNF.

**BDNF produced minimal alterations in short-term synaptic plasticity at early re-forming synapses and greater alterations at later re-forming synapses**

The remaining experiments were undertaken to further examine the electrophysiological characteristics of postlesional CF-PC synapses which developed at different maturational stages. PCs were voltage-clamped at holding potentials of 0 to +30 mV and CF-EPSCs were recorded following paired stimuli with interstimulus intervals ranging from 50 to 3000 ms (Sugihara et al., 2003). This test was selected in order to detect any modifications in presynaptic transmitter release as PPD is a function of release probability (Silver et al., 1998; Hasimoto and Kano, 1998). Figure 4F shows examples of CF-induced EPSCs during paired-pulse stimulation.

For CFs which reinnervated PCs from P5 until P11 in the absence of BDNF, PPD values were the same as control CFs (P5-7, data not shown, Sugihara et al., 2003; Fig 4A & C). Similarly, the presence of BDNF during PC reinnervation from P7 to P9 had no effect on PPD compared to age-matched control animals (Fig 4B). However, CFs which reinnervated PCs later (P9 to P11) in the presence of BDNF seemed to have moderately-altered properties (Fig 4D). PPD was greater at these later formed synapses, indicating that the presence of BDNF during synapse re-formation altered some aspect of normal neurotransmitter release.
More consistent alterations of short-term plasticity were found at synapses formed from P14-P15. These synapses can only form in the presence of BDNF. Figure 4Fii shows an example of BDNF-induced reinnervating CF-EPSCs in response to paired stimuli. These reinnervating synapses had significantly increased PPD (i.e. greater depression) compared to the age-matched control animals (Fig 4E).

Therefore, those CFs which reinnervated PCs from 6 days post-lesion (i.e. P9 to P11) had altered basal neurotransmission, smaller arbors and increased PPD. This suggested that these older re-forming synapses were more susceptible (compared to those that re-formed synapses from 4 to 6 days post-lesion) to changes in BDNF levels in the extracellular milieu. Moreover, although BDNF allows CFs to reinnervate maturing PCs, these new CF synapses have altered presynaptic transmitter release properties.

**Similar synaptic fatigue at all re-formed synapses during repetitive stimulation**

Synaptic depression produced by sustained repetitive stimulation was another test used to examine presynaptic CF function. The stimulation frequencies ranged from 0.03 to 10 Hz, as previously described (Silver *et al.*, 1998). Synaptic depression was analysed by averaging the current amplitudes of the final 10 CF-EPSCs (to give an indication of the depressed response) and normalising to the amplitude of the first EPSC (program designed by Dr G.Sudoc, France). There were no significant differences in the synaptic fatigue found at reinnervating CFs in the presence of BDNF as compared to control CFs (Fig 5).

We also recorded the number of response failures at the end of the high-frequency stimulation protocol (the final 10 stimulations). Failures are considered to reflect either failure of the axon to propagate an action potential or depletion of vesicles in the immediately releasable pool in the presynaptic terminal. Comparisons were first made between the two age groups of sham-operated controls (at P3 and P11) wherein we found that the frequency of failed responses was similar for both groups, ranging from 2.5% to 27.7% at 1 to 10 Hz stimulation frequencies. This failure level for CF-EPSCs in control animals has been previously seen under periods of sustained climbing fibre activation at moderate and high frequencies (Andersson and Hesslow, 1987).
Notably, BDNF-induced CFs reinnervating PCs from P14 to P15, which exhibited increased PPD, had no failed response during all stimulation frequencies. By comparing the frequency of failed responses between control CFs and these late-reinnervating CFs, we observed a significant difference at 0.2, 5 and 10 Hz (p<0.01; Table 1). In contrast, no consistent significant differences in the frequency of failed responses between other ages of PC reinnervation (irrespective of BDNF treatment) and control synapses were found. Therefore, failed responses during sustained stimulation seemed to be normal at control synapses and it was solely PCs reinnervated from P14 to P15 that had no EPSC failures; these are also the reinnervated synapses that show the most consistent PPD modifications.
DISCUSSION

We have investigated the physiological function of post-lesion axon-target connections which form at different ages of maturation. In this study, the olivocerebellar projection was used as a model to examine the effects of BDNF and neuronal age on neosynaptogenesis. We show that both of these factors influenced axonal (CF) function but not the gross morphology of the PC target.

In contrast to the physiologically normal CF-PC synapses which develop spontaneously at different neuronal ages after a P3 lesion (Sugihara et al., 2003; Fig 4A & C), the increased availability of BDNF, when combined with a longer period of PC denervation, resulted in modified anatomical and physiological properties of the reinnervating CF. The effect of exogenous BDNF on induced CF-PC synapses in animals lesioned at P11 (when the PC denervation period is relatively short) was less pronounced, with normal morphology but modified electrophysiological properties.

BDNF increases reinnervation to the cerebellar hemisphere with smaller CF arbors and altered synapse function

The effect of BDNF on inducing axons to grow into a denervated region is revealed by the increased hemispheric reinnervation in neonatal animals (P3 lesion + BDNF; refer to Article II) and induced functional synapses recorded in the juvenile animal (P11 lesion + BDNF; Fig 4E). This result is consistent with BDNF’s positive effects on axonal outgrowth (Tucker et al., 2002), branching (Alsina et al., 2001), and synaptogenesis (Vicario-Abejon et al., 2002). The action of BDNF is complex, however, as during one period of synapse re-formation (~P9 to P11) the increased availability of BDNF reduced the extent over reinnervating CFs onto the PC dendritic tree.

We have considered several possible explanations for our observation of less extensive CF arborisations when later reinnervation occurs in the presence of BDNF. First, CF innervation appears to be regulated by cues from PCs both during development (Rabacchi et al., 1992; Ichise et al., 2000) and in structural plasticity in the mature cerebellum (Rossi et al., 1993; Rossi et al., 1995). Therefore, a modification in the PC by BDNF could affect the CF response; however, we found no gross morphological changes
between PCs treated with BDNF compared to those without BDNF. An alternative explanation is that non-CF synaptic inputs were rapidly established on the previously CF-dedicated zone of the PC following deafferentation and prevented reinnervating CFs from reoccupying the original CF territory. It has been reported that parallel fibres, stellate cell axons and ascending collaterals of basket cells innervate the ectopic spines which emerge on proximal PC dendrites after CF ablation (Sotelo and Arsenio-Nunes, 1976; Rossi et al., 1991). However, CFs can displace ectopic parallel fibre innervation (Morando et al., 2001; Cesa and Strata, 2005) and we found that CFs of age-matched vehicle-treated lesioned animals occupied the same area on the PC as normal unlesioned CFs. Despite this, we cannot discount the possibility that abnormal, if temporary, invasion of CF territory takes place and impedes proper CF reinnervation. This is conceivable given that increased availability of BDNF accelerates the maturation of inhibitory interneuron development in the cerebellum (Bao et al., 1999).

A third explanation, which indirectly involves BDNF, is that CF modifications reflect a limitation in the plasticity of the olivocerebellar system. In this lesion model, olivocerebellar axons which terminate in the lateral hemisphere (i.e. from P9 to P11) ramify with more CFs than those axons in the vermis (i.e. from P5 to P7; Sugihara et al., 2003). In light of the increased CF outgrowth observed after BDNF treatment (refer to Article II) and modified synapses formed, the inferior olive may be limited in further growth. As inferior olive neurons increase their somatic size to respond to changes in their target field (Neppi-Modona et al., 1999), it is possible that the inferior olive no longer can produce the additional raw materials in sufficient quantity to maintain the growth of axons to their full extent and sufficient neurotransmitter to sustain activity at the extra synapses. Therefore, the increased exogenous BDNF may have exhausted the plastic potential of olivary neurons post-lesion.

**Functional relevance of modifications at reinnervating synapses**

Functional synaptic recovery post-lesion depends not only on the morphological organisation of axon-target connections but also appropriate synaptic function. We examined the synaptic properties of reinnervating CFs after lesion. In the presence of BDNF, those CFs which reinnervated PCs after > 6 days of denervation (discussed above) also had altered synaptic function. The basal CF-EPSC amplitude was smaller than at a
normal CF synapse. Because the initial amplitude is related to the extent of synaptic contact and CFs form extensive synaptic contacts along the PC dendrite (Palay and Chan-Palay, 1974; Strata and Rossi, 1998), our results suggest that in the presence of BDNF, CFs do not reinnervate the PC as extensively as normal. This is consistent with their smaller extent on the PC dendritic tree as measured morphologically, compared to either normal CFs or reinnervating CFs in the absence of BDNF.

Another functional property of synaptic transmission observed at the CF-PC synapse is short-term synaptic plasticity. Depending on the age of PC reinnervation, in the presence of BDNF, reinnervating CFs demonstrated increased PPD. This effect was more pronounced at a mature age when BDNF is necessary to induce reinnervation. As normal CFs release multiple vesicles per site after stimulation (Wadiche and Jahr, 2001), the reduced CF-mediated EPSC amplitude after the second of a paired pulse suggests that these reinnervating synaptic terminals have decreased neurotransmitter release. This could be due to a reduction in the number of vesicles released by an action potential, in the amount of neurotransmitter per vesicle or other processes involved in synapse form and function.

Taken together, these results show that later re-forming CFs show most of the functional properties of a normal CF, but with altered short-term plasticity. Over time, this difference could modify the signalling processes and temporal coding of the postsynaptic neuron (Zucker et al., 1989; Silver et al., 1998). It is known that CF activation of PC exerts a powerful inhibitory effect on the PC target neurons, the deep cerebellar nuclei (Llinas and Muhlethaler, 1988). Therefore, if PC activity is modified by altered CF release properties, the altered inhibition onto the deep cerebellar nuclei (which in turn project to other motor systems) might affect these complex systems. However given that the presynaptic modifications are relatively small, we hypothesise that the effects of these modifications on PC synaptic integration and output would be functionally negligible. Moreover, animals with BDNF-associated reinnervation had improved behavioural performance in complex motor tests (refer to Article II) which is coherent with their functional re-formed synapses.
Conclusion

Our results show that all synapses re-formed post-lesion, despite their neuronal age, were functional. The presynaptic modifications, seen predominately as synapses re-formed later during development, were relatively small and thus unlikely to change the function of the post-synaptic PC. The post-synaptic PC and the highly plastic reinnervating CF terminal seem to establish synaptic contacts with nearly normal synaptic function. This suggests that once both elements of a synapse are in close proximity, it is likely that the synapses re-formed can functionally replace the lost connections.

ABBREVIATIONS

BDNF, brain-derived neurotrophic factor
CF, climbing fibre
CNS, central nervous system
EPSC, excitatory post-synaptic current
P, postnatal day
PC, Purkinje cell
PPD, paired pulse depression
Trk, tropomyosin-related kinase
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**TABLE 1.**

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Significant differences between control and reinnervated synapses:
*\( p < 0.05 \), **\( p < 0.01 \)
Figure 1

(A & B) Schematic diagrams representing the normal olivocerebellar pathway (solid-line) that originates in the inferior olive, crosses the midline in the medulla and ascends to the contralateral hemisphere via the inferior cerebellar peduncle.

(A) If the peduncle is transected (*) on P3, the contralateral olive degenerates and new transcommissural fibres grow to reinnervate Purkinje cells densely in the vermis (black bold-dotted line) and more sparsely in the hemisphere (black fine-dotted line line; Angaut et al., 1985; Sherrard et al., 1986). BDNF was injected at P6 into the intermediate/lateral hemicerebellum in an attempt to augment reinnervation in the partially reinnervated zone (grey fine-dotted line).

(B) When the peduncle is transected (*) on P11 no spontaneous reinnervation occurs. BDNF was administered into the left medial/intermediate hemicerebellum at P12 to induce transcommissural axonal sprouting into the denervated region (grey bold-dotted line; Sherrard and Bower, 2001).
Figure 2
Bar graph indicating the basal synaptic transmission at normal and reinnervated CF-PC synapses, which form during different developmental stages. Notably, reinnervating synapses which re-formed in the presence of BDNF after several days of denervation (from P7 – P11) had smaller amplitudes than control synapses recorded in the same region of a different animal. In contrast, BDNF-induced synapses developing after a short period of denervation (from P14 – P15) had basal synaptic amplitude similar to controls. Figures within the bar graphs indicate the sample size of each group.

* Significant differences between control and reinnervating synapses, p<0.05.
Figure 3

Morphological analysis of the CF-PC synapse interaction at re-formed and normal synapses (A – K).

(A – D) Anatomical features of PCs reinnervated from P9 to P11 in the lateral hemicerebellum were compared to normal innervated PCs. Both groups of PCs reinnervated relatively late (6 days post-deafferentation) had reduced total dendritic territories in contrast to normal PCs (A). All PCs were filled with biocytin during recording and revealed with AF350 (shown in green for increased clarity) including control (B), reinnervated synapses in the absence of BDNF (C) and reinnervated synapses in the presence of BDNF (D).

(E – H) The relative area occupied by the CF on its corresponding PC. Only VGLUT2-positive puncta located directly on the labelled PC were only used in this measure. CFs reinnervating PCs from P9 to P11, in the presence of BDNF, had a smaller territory on the PC dendrite compared to normal CFs (E). Illustrations of control CFs (F) and reinnervating CFs without (G) and with (H) BDNF treatment.

(I – K) CF-PC anatomical relationship of CF arbors formed from P14 to P15 after BDNF application in juvenile animals. (I) Illustrates the similar total surface area of PCs and proportion of the PC covered by the CF arbor in both reinnervated and control cases. Anatomical similarity depicted between control synapses (J) and BDNF-induced reinnervated (K) arbors.

Figures within the bar graphs indicate the sample size of each group.
Bar = 50µm.

**Significant differences between control and reinnervated synapses, p<0.01.
Figure 4

Paired-pulse depression exhibited at normal and reinnervating synapses at different ages of synapse neosynaptogenesis (A – F).

(A – B) CF-PC synapses which re-formed from P7 to P9 in the intermediate hemicerebellum, displayed similar PPD to control synapses, irrespective of BDNF treatment.

(C – D) Synapses developing from P9 to P11 had similar PPD to control synapses (C) while the presence of BDNF (D) is associated with greater PPD at certain inter-stimulus intervals compared to control synapses.

(E – F) BDNF-induced synapses reinnervating from P14 to P15 consistently displayed increased PPD compared to age-matched control synapses (E). Examples of PPD from a control synapse is shown in Fi and a reinnervating synapse in Fii.

*Significant differences between control and reinnervating synapses: p<0.05.
Figure 5
Effect of high-frequency stimulation at control and BDNF-induced reinnervating synapses. No consistently significant differences between control and new synapses were detected.
*Significant difference between control and reinnervating synapses, p<0.05.
DISCUSSION
I will begin this general discussion by briefly summarising the main results of my thesis. This will be followed by an integration of these findings into the context of other neural systems, a description of the short-comings of the presented work and also the possible future experiments which could be undertaken to improve our understanding of appropriate repair after a lesion.

We have used the rat olivocerebellar pathway as a model to examine the function of spontaneous and BDNF-associated collateral axonal growth from remaining fibre tracts after an injury in order to further characterise how well these remaining intact pathways can be induced to remodel and compensate for loss of neural circuitry. An assessment of the function associated with the re-formed olivocerebellar pathway in younger and older animals was made by anatomical, electrophysiological and behavioural methods.

We have shown that developmental olivocerebellar reinnervation, which involves the recreation of the projection by a topographically organised alternate pathway (Zagrebelsky et al., 1997; Sugihara et al., 2003), conferred behavioural improvement in a specific cognitive task (spatial learning) in comparison to those animals without reinnervation. In addition, as neurotrophic factors such as BDNF can directly promote axonal sprouting after a lesion, not only in the cerebellum but also after injury to the spinal cord, the cortex and visual system (Mansour-Robaey et al., 1994; Sawai et al., 1996; Mamounas et al., 2000; Coumans et al., 2001; Logan et al., 2006), we examined the quantity, distribution and function of BDNF-induced reinnervation. A single application of BDNF increased the amount of reinnervation in regions that were previously sparsely reinnervated (i.e. Px3 hemisphere) and provided a small difference in the behavioural performance of these animals on spatial learning tasks compared to those animals without BDNF-treatment.

In addition, BDNF promoted reinnervation in older animals (i.e. Px11), as previously shown (Sherrard and Bower, 2001; Dixon and Sherrard, 2006; Letellier et al., 2007), and was associated with better gait synchronisation and spatial learning than those animals without reinnervation. Finally, we assessed a repertoire of electrophysiological properties of reinnervating synapses which formed at different developmental ages during spontaneous neonatal olivocerebellar reinnervation and that induced by BDNF after the critical period of reinnervation. This allowed us to determine whether
replacement of the injured olivocerebellar system with another but homologous projection pathway was associated with the establishment of new synapses with functional capacities similar to normal. Despite certain presynaptic modifications (i.e. primarily PPD), all reinnervating synapses irrespective of PC developmental age or BDNF treatment had appropriate synaptic function. Given that both spontaneous and BDNF-associated olivocerebellar reinnervation accurately replaces synapses following denervation, the results of this thesis highlight a conservation of correct afferent-target interactions despite pharmacological induction of axonal growth in the olivocerebellar system.

1.0 The Functional Accuracy of Olivocerebellar Repair

The re-formation of a projection pathway that occurs in response to target denervation is not part of the normal developmental process and thus cannot be presumed to have the same function as the normal pathway. In the olivocerebellar system, which regulates motor learning (Llinas et al., 1975) and synchronises repetitive movements such as gait (Rondi-Reig et al., 1997), the partial reinnervating projection pattern created following lesion in young animals was associated with improved sensorimotor (i.e. postural and equilibrium) control, simple coordination and gait synchronisation compared to animals without reinnervation (Dixon et al., 2005). After early lesions in other regions of the CNS, functional recovery is generally associated with extensive regeneration or greater reorganisation (e.g. collateral or terminal sprouting) in the immature CNS (i.e. Kennard principle; Finger and Wolf, 1988). Despite this, anatomical and physiological investigations have shown that such compensatory responses in developing or adult systems can also be disadvantageous. Sprouting of spared dorsal root projections has been associated with the development of hyperactivity and reflex spasticity in the spinal cord (McCouch et al., 1958; Cotman et al., 1981). Furthermore, even the re-establishment of topographically organised projections to the retino-recipient visual brain centre after unilateral optic nerve damage (Beazley, 1984; Jacobson, 1991) did not fully restore normal visuo-motor response of these animals (Dunlop et al., 1997). That study clearly showed that it was better to have only one intact optic nerve rather than one regenerated and one intact nerve.
Transcommissural olivocerebellar reinnervation changes postural asymmetry

Although animals with olivocerebellar reinnervation do not appear to display maladaptive motor skills, qualitatively they exhibited postural asymmetry contralateral to the lesion side (unpublished observations, in Article I). In contrast, animals without reinnervation had a postural tilt ipsilateral to the lesion side. Postural tilt reflects muscle hypotonia (Petrosini et al., 1990). Such hypotonia ipsilateral to a cerebellar lesion in adult animals (Petrosini et al., 1990) and humans (Holmes, 1939) involves reduced cerebellar influence (via its DCN) on the contralateral thalamus, which decreases activity of motor cortical cells that in turn lessens spinal motor neuron discharge and therefore muscle tone (Manni and Dow, 1963). In Px11 animals, the loss of CF input would cause increased PC simple spike activity and hence inhibit DCN output to other motor paths. Therefore, the Px11 animals displayed cerebellar hypotonia ipsilateral to the lesion. The contralateral hypotonia seen in the Px3 animals is similar to rats hemicerebellectomised at P1 which showed a postural tilt contralateral to the lesion side (Petrosini et al., 1990). A possible explanation is that in neonatal cerebellar-injured hemicerebellectomised animals (hemicerebellectomised prior to P10), there is compensatory sprouting in several paths, including ipsilateral cerebello-thalamic projections (Molinari et al., 1986), the cerebello-rubral path (Leong, 1977) and an associated increase in neuronal number in the remaining DCN (Gramsbergen and Ijkema-Paassen, 1982). Therefore, neonatal reorganisation of other central motor paths may account for the postural differences between Px3 and Px11 groups.

The posture tilt in reinnervated animals suggests a limitation of the function of transcommissural olivocerebellar reinnervation. In these animals, one inferior olive is integrating peripheral sensorimotor information from two sides of the body, and providing synaptic input to both hemicerebella. The neural basis of muscle hypotonia (above) suggests that providing a ‘compensatory’ response to the left hemicerebellum causes an adverse effect on the right hemicerebellum and thus other components of the motor system. Potentially, PC-DCN outflow from the right hemicerebellum is compromised, diminishing contralateral (left) thalamic and cortical neuronal activity (Petronsini et al., 1990), and thus reducing spinal cord discharge would presumably produce hypotonia on the right side.
Olivocerebellar reinnervation and spatial learning: cerebellar, motor or hippocampal function

Transcommissural olivocerebellar reinnervation was also associated with improved spatial function but some retention deficits were present during the first 3 days of training in the water task (Article I). To further characterise the retention impairment from one training day to the next, we decided to test whether this deficit was evident 7 days after the last spatial training trial (Article II). However, we observed no differences between control or Px3 animals at this later 7 day test which could be explained by the timing of the retention test. By the end of the spatial training task, the Px3 animals performed similarly to the control animals (Article I and II), suggesting that once the task was learned there was no long-term spatial memory deficit.

In comparison to the animals with reinnervation, the Px11 animals did not appear to demonstrate clear spatial learning. However, from trials 8 to 30 our Px11 group showed a 17% improvement. This is consistent with animals whose CFs have been ablated bilaterally by 3-AP neurotoxin: initially they cannot learn the water maze, but with continued practice (~20 trials) they finally achieve the task (Dahhaoui et al., 1992). Since neither our Px11 animals nor animals treated with 3-AP develop transcommissural olivocerebellar reinnervation, it cannot be excluded that the residual learning exhibited by Px11 animals may be due to the interactive function of the cerebellum with other networks of spatial processing areas. Brain regions critical for the acquisition and storage of spatial information include the hippocampus, limbic system, superior colliculus, parietal cortex and dorsolateral prefrontal cortex (Middleton and Strick, 1994; McCarthy et al., 1994; Gandhi et al., 2003). A compensatory or even normal process at the level of any of these structures might have occurred when the cerebellar circuit was compromised. A previous study has proposed that cerebellectomised rats can learn the spatial task via thigmotaxis which enables them to learn the spatial environment through hippocampal processing (Petrosini et al., 1998). Alternatively, animals subjected to behavioral tests which involves increased motor activity, spatial learning and/or explorative behaviour, also upregulates the expression of neurotrophic factors, growth-associated protein (e.g. GAP-43) and synaptophysin in the brain, particularly in the cerebellum (Gomez-Pinilla et al., 2001; Ickes et al., 2000; Klintosova et al., 2004) and hippocampus (Gomez-Pinilla et al., 1998; Kesslak et al., 2003).
1998). These factors can induce neurite outgrowth and the formation of new synapses (Chen et al., 1998). However, only minimal terminal sprouting into the deafferented hemicerebellum was seen in the Px11 group (via VGLUT2 immunohistochemistry within 500 µm from the midline, Article II) supporting the hypothesis of complementary hippocampal function allowing navigation in this group.

A limitation of the present study (Article II) and the majority of hippocampal and cerebellar-lesion studies is the lack of sensorimotor description in the spatial version of the water maze. In this task, the effects of maze learning and sensorimotor processes would be inter-related and few studies, with the exception of Cain et al., (1996), have thoroughly examined this link. In our study, it was noted that some animals in each group made swimovers in the hidden platform test, which means that instead of stopping once they had reached the platform, they continued to swim. The incidence of this behaviour (i.e. either at the start or end of training) was inconsistent and therefore, it remained speculative whether the animal knew where the platform was positioned or not, which in turn, could have confounded the measured variables. These observations were noted in the data collected for Article II. Other animals temporarily floated in the maze randomly throughout the testing period (6 days) suggesting some differences in excitability and motivation between animals. The swimover and floating factors could have affected semi-quantitative measures (used in Article II). A correlation analysis between the frequency of these sensorimotor variables (particularly swimovers) and the escape latency or number of quadrants crossed would have potentially provided a more sensitive indicator of any sensorimotor disturbances in the visible and hidden platform paradigms.

To further assess the degree of compensatory function provided by transcommissural olivocerebellar reinnervation, we also undertook a pilot study (undescribed data) to examine the specific components of spatial function i.e. short term and reference memory test (Hodges, 1996; Mandolesi et al., 2001). Briefly, we used the radial arm maze consisting of eight arms radiating from a circular central platform, with a recessed food well positioned at the end of each arm so that the food was not visible to the animal from the central platform. The maze was baited with food in 3 out of the 8 wells (fixed positions throughout training) and the rat had 10 minutes to find the rewards in the food wells. We measured the time taken to visit the three baited arms, the number
of re-entries into earlier visited baited arms (i.e. working memory error) and entries into an unbaited arm (i.e. reference memory error). Although time differences were observed between groups (control, Px3, Px11) in this task, there were no differences in working or reference memory errors. The results suggested that neither transcommissural olivocerebellar reinnervation nor unilateral CF loss impaired the accuracy of the task in comparison to normal animals. This finding contrasted with the only other cerebellar lesion study using the radial arm maze, in which hemicerebellectomy led to deficits in spatial working memory as demonstrated by numerous spatial errors (Mandolesi et al., 2001). In contrast, our Px3 and Px11 groups had the same number of spatial errors as the control group. These findings suggested that either the radial arm maze was unable to detect the differences in CF loss or that the cerebellum was not involved in the working and reference components of spatial memory tasks. This latter proposition has been suggested by Nixon et al., (1999). In their study, monkeys with lesions of the lateral cerebellar nuclei relearned a spatially delayed alternating task in as many trials as control animals. However, comparisons in the response times of monkeys with cerebellar lesions to that of control animals revealed that lesioned animals were slower to make a decision as to where to respond (Nixon et al., 1999), which agrees with the longer radial arm maze times for both the Px3 and Px11 animals.

Transcommissural olivocerebellar reinnervation and its downstream synaptic consequences

Additional aspects of the functional significance of transcommissural olivocerebellar reinnervation include the amount of reinnervation and the accuracy of synaptic function. Part of this question has been previously addressed by Sugihara et al., (2003). We confirm their findings by recording the same percentage of reinnervated PCs in each region of the denervated hemicerebellum (undescribed observations in Article III). In addition, we extended the findings in Sugihara et al., (2003), testing synaptic function at those synapses re-formed in the paravermis and hemisphere (i.e. ~4 to 6 days post-injury) vs CF-PC synaptic parameters in the vermis (Sugihara et al., 2003), to see whether these later reinnervating synapses displayed similar synaptic parameters to earlier reinnervating ones in the vermis (i.e. ~2 days post-injury). The electrophysiological work described (Article III) also allowed us to generate the
hypothesis that the long-term functional properties of reinnervating synapses in the olivocerebellar system were independent of the age of CF-PC synaptogenesis. As PPD is a mechanism which ensures that presynaptic terminals (which exert a powerful effect on their target neurons) provide reliable neurotransmission at low firing rates in vivo, the similar PPD at reinnervated and control synapses would ensure normal PC function. Functional PC outflow to their DCN is consistent with the improved behavioural function seen in the reinnervated animals.

The hypothesised normal and functional integration of the PC at reinnervated synapses leads to the interesting point of the role of the remaining DCN in both reinnervated and denervated PCs and their functional implications for behaviour. The DCN neurons project their axons onto the contralateral thalamic nuclei, red nucleus, vestibular nuclei and reticular formation. Therefore this link can be seen as a point where the cerebellum influences a range of other motor systems. In animals with reinnervation in the lateral hemicerebellum, a proportion of PCs (~60%; and their corresponding DCN) are without CF-induced complex spike activity (Sugihara et al., 2003; undescribed observations in Article III). CF-driven complex spike activity induces a 5 to 15 millisecond period of ‘pause’ in PC axonal firing that in turn relieves inhibition at the PC-DCN synapse and allows rebound firing of the DCN (Llinas and Muhlethaler, 1988; Monsivais et al., 2005). Inactivation of the inferior olive causes a marked increase in PC simple spike discharge (Montarolo et al., 1982; Savio and Tempio, 1985). As the effects of the CF directly influences PC and DCN activity, the loss of CF input to some PCs in both partially reinnervated and denervated animals will affect the DCN neuronal activity and their downstream synapses. In the case of CF deafferentation, the remaining increased PC activity will be driven by MF-PF simple spike activity (Batini et al., 1985). The constant inhibition at PC-DCN synapses (due to no CF-induced ‘pause’) would presumably alter the modulating activity of the DCN onto other motor systems such as the contralateral ventrolateral thalamus. Therefore, in animals with partial reinnervation, the glutamatergic synaptic investment from the DCN to the thalamus (Aizenmann et al., 2000) would presumably be closer to normal than those animals without innervation into the left hemicerebellum. This will affect the consequent ascending flow to the motor cortex and subsequent descending outflow onto the precerebellar nuclei and the spinal motor neurons. The difference in synaptic
modulation outside the cerebellum could potentially influence the ability of the animal to execute the correct motor actions required for a spatially-demanding task.

2.0 The Role of BDNF as a Stimulator of Growth and Repair in the Olivocerebellar System

After a lesion to the spinal cord, delivery of a single neurotrophin into the injured area only enhances limited axonal sprouting, and growth beyond the lesion site is rare (Tuszynski et al., 1996; Ramer et al., 2002; Lu et al., 2004). Even sustained application via adeno-associated viral vector (AAV) mediated gene transfer to overexpress BDNF after an optic nerve crush only promotes RGC survival but does not significantly improve RGC axonal regrowth (Leaver et al., 2006). In contrast, ciliary neurotrophic factor (via an AAV vector) increased the regeneration of RGC axons across the lesion site (Leaver et al., 2006), highlighting that a single neurotrophic application in certain neuronal populations does not always produce a significant regenerative response; this response depends on the neurotrophic factor applied. This is further complicated by the fact that the site of neurotrophin application, whether at the site of axotomy or at the soma, influences the neural response. It has been shown that exogenous neurotrophin at the site of axotomy can lead to the formation of the ‘neurotrophic sink’ which entails the knotting of growing axons into the lesion site and thus stopping their effective regeneration (Logan et al., 2006).

In the work presented in Article II and Dixon and Sherrard (2006), anterograde tract tracing revealed axons coursing through the white matter in BDNF-treated Px11 animals, which has been previously reported in animals with spontaneous reinnervation (Sugihara et al., 2003). The concentration or site of BDNF injection used in Px3 and Px11 animals did not appear to create a neurotrophin sink and, in both age groups, increased CF reinnervation. As a related approach, instead of injecting BDNF into the denervated hemicerebellum, we could have delivered neurotrophins to the somata of the degenerating right inferior olive to attempt to promote neuronal survival and/or neurite outgrowth through the ipsilateral peduncle (which occurs during development; Bower and Payne, 1987; Lopez-Roman et al., 1993). However, in a number of studies involving optic nerve injury in both neonate and adult animals, even if RGC survival is promoted via the over-expression of certain anti-apoptotic transgenes (e.g. bcl-2), cell
survival does not automatically lead to RGC axonal regeneration (Chierzi et al., 1999; Cho et al., 2005; Jiao et al., 2005). The work in the visual system seems to suggest that the two processes of neuronal survival versus axonal regeneration are regulated differently and therefore probably use independent intracellular signalling (Harvey et al., 2006).

Notably, the role of BDNF as a promoter of the ingrowth of olivocerebellar axons and correct target choice has been previously considered in older-pedunculotomised animals (i.e. Px15 and Px30; Dixon and Sherrard, 2006; Letellier et al., 2007). However, in those studies the distribution of reinnervation associated with BDNF had not been quantified and it was relevant to do so in light of the behavioural improvement in the Px11 animals. We showed that the reinnervation associated with BDNF in Px11 animals extended lateral to the paravermal vein and along the rostral-caudal plane. It should also be noted that a pedunculotomy can also induce mossy fibre sprouting into the molecular layer (Murase, 1995). However, such sprouting is not predominately visible until a survival period of 3 months post-pedunculotomy. Given that the anatomical analysis in the present study was conducted at approximately P60 and mossy fibre sprouting is distinguishable from CFs (i.e. less branched), the likelihood that mossy fibres were included in the semi-quantitative analysis of CF reinnervation is low.

Furthermore, in BDNF-treated Px11 animals (undescribed observations in Article III), only 20% of recorded PCs in the left hemivermis showed CF-EPSCs. This result supports the relatively sparse PC reinnervation semi-quantified anatomically (Article II). Despite this low amount of reinnervation, it did provide significant functional improvement in the gait synchronisation task (Article II). Also, the percentage of reinnervated PCs, calculated via electrophysiological technique (undescribed observations in Article III), was much lower than the percentage of PC reinnervation presented by Letellier et al., (2007) due to a different sampling technique. Overall, the reinnervation density from Article II and III indicates a large difference between reinnervation in the same region of younger animals (86% of PCs reinnervated) compared to older animals (20% of PCs reinnervated).
Proposed mechanisms for BDNF-associated olivocerebellar reinnervation

Possible mechanisms for the difference in the amount of reinnervation between the two age groups (i.e. Px3 and BDNF-treated Px11 animals) are the development of myelination in the cerebellum and its consequent growth-suppressive effects. As previously mentioned (refer to Introduction 3.0, Nogo and p75 interaction), myelin contains many axon growth inhibitory molecules (i.e. MAG, OMgp, NgR). As the myelin receptor NgR is linked to the p75 signal-transducing receptor (Yamashita et al., 2002), it is hypothesised that exogenous BDNF in the older-pedunculotomised animals results in less effective activation of the neurite-outgrowth enhancing Trk second messenger system (e.g. MAP kinase and cAMP). This could be due to the competition of the neurotrophin between Trk and NgR-p75. Furthermore, myelin-p75 signalling leads to the activation of the Rho intracellular pathways (which stimulates actin depolymerisation) and the depression of Trk-mediated cAMP elevation. This in turn, would make the incoming growth cones more susceptible to the environmental inhibitory molecules in the older animals as cAMP levels are an indicator of the ‘growth responsive’ state of neurons. In addition, myelin in the cerebellum expresses specific axon pathfinding proteins such as the semaphorins (Moreau-Fauvarque et al., 2003) which are known to inhibit axon growth (optic nerve; Goldberg et al., 2004). Potentially these molecules could also suppress transcommissural olivocerebellar axon outgrowth in the older pedunculotomised animals. In addition to these processes, several other factors could be participating in the low reinnervation induced in BDNF-treated Px11 animals. These include the short duration of action of the injected BDNF to provide a substrate for outgrowth and the changing receptivity to BDNF by reinnervating axons.
3.0 Behavioural and Synaptic Function Correlate with BDNF-associated Olivocerebellar Reinnervation

Despite only 20% of PCs being reinnervated in BDNF-treated Px11 animals, these animals showed behavioural improvement in the complex motor task (i.e. rotarod) and in the spatial learning task (i.e. hidden platform) compared to animals without reinnervation. Furthermore, the performance of BDNF-treated Px11 animals was as good as Px3 animals on the rotarod and in the spatial learning task (Article II). These results point out two important components of the olivocerebellar reinnervation model: the amount of reinnervation required for behavioural improvement and the functional relevance of the region reinnervated.

We first consider the relevance of the amount of reinnervation that takes place and its relation to functional recovery. Previous studies have shown that even a small increase in plasticity after a lesion provides functional improvement after spinal cord lesions (Bareyre et al., 2004). In these cases, functional improvement is associated with compensatory sprouting of spared fibres onto neurons that have not been denervated and contain no vacant synaptic sites (Thallmair et al., 1998; Z’graggen et al., 1998). In parallel with the results of spinal cord lesion studies, the behavioural data of the BDNF-treated Px11 animals seems to suggest that the compensatory role of reinnervation (with its functionally-appropriate synapses), although sparse, is sufficient to provide a level of normalised outflow from the PC-DCN to other motor systems and in turn allows the recruitment of appropriate muscle groups to perform complex tasks (Lang et al., 1999). Furthermore, it could be hypothesised that if the olivo-cortico-nuclear circuit was recreated in BDNF-treated Px11 animals (as occurs in Px3 animals), the direct input of reinnervating CFs onto the DCN could potentially contribute to normalising the DCN outflow from the cerebellum.

It should also be mentioned that the behavioural effect of BDNF-treatment in Px3 animals was less pronounced than seen in BDNF-treated Px11 animals. The semi-quantitative increase in reinnervation in the Px3 group (Article II) did not appear to significantly improve performance on motor or spatial tasks. This result is not surprising given that the untreated Px3 animals were able to achieve the spatial tasks;
we thus propose that the small behavioural effect seen in BDNF-treated Px3 animals is due to a ceiling effect of the behavioural test.

We next consider whether the reinnervated cerebellar region significantly affects the behavioural performance on a specific task. The BDNF-treated Px11 animals had improved spatial abilities even with little reinnervation in the lateral hemicerebellum, the region associated with spatial function. As plasticity of other motor systems, (e.g. the cerebello-thalamic projections), is much less likely to occur at this late lesion age (Gramsbergen and Ijkema-Paassen, 1982), compensatory processes by other brain regions involved in navigation may account for the functional improvement seen in these BDNF-treated animals. Furthermore, it could be potentially due to a combined effect: improved sensorimotor abilities plus partial reinnervation in the cerebellar region for the spatial task. It must be said that the improvement in this group during the spatial task was not significantly different from untreated Px11 animals because of the high variability within each group. Therefore, BDNF’s effect in the spatial task is relatively small compared to that observed in the rotarod. This is congruent with the minimal reinnervation to the cerebellar hemisphere and denser reinnervation to the vermis that further indicate the significance of this new olivocerebellar path in recreating the olivocortico-nuclear circuit and compensating complex neural tasts.

Although BDNF application resulted in increased reinnervation, its effects also appeared to be quite complex when examining the reinnervating CF arbor morphology. In the cerebellar hemisphere BDNF-treated Px3 animals had a reduced CF terminal arbor size on the PC and decreased basal synaptic transmission (i.e. synaptic amplitude). BDNF application has only been shown to decrease axonal growth and branching in vitro in cultured Xenopus spinal neurons (Wang and Zheng, 1998) and sympathetic neurons (Kohn et al., 1999) so a similar effect on the CF arbor would be unusual. However, BDNF could have less direct consequences on CF arborisation by increasing the maturation of GABAergic interneuron systems (as seen in the visual cortex; Huang et al., 1999) which then could impede normal CF arborisation. Furthermore, the altered PPD at these synapses could be due to modifications in presynaptic mechanisms which manoeuvre the vesicles (e.g. cadherin-β-catenin interactions). The cadherins appear to be involved in synapse formation as they are localised at synapses adjacent to active zones and manoeuvre synaptic vesicles to
presynaptic compartments. BDNF has recently been found to change the motility of cadherin-β-catenin interactions (Bamiji et al., 2006), therefore exposure to differing BDNF concentrations could modify the localisation of synaptic vesicles (i.e. fewer vesicles available). Examination of the processes at these presynaptic terminals could include loading of the ready-releasable vesicle pool with the fluorescent styryl dye FM1-43 in cerebellar slice (using dual-photon laser scan microscopy) in order to detect any differences in motility of synaptic vesicles at reinnervating and control synapses.

The requirement for appropriate CF-PC reinnervation to improve associated complex behaviour is further supported by experiments in the spinal cord. After unilateral pyramidotomy in the adult rat, transcommissural neurite outgrowth has to course through high levels of myelin in the white matter. After application of a myelin-neutralising antibody in vivo, long distance regeneration of cortico-spinal axons occurs (Schnell and Schwab, 1990; Schnell and Schwab, 1993; Bregman et al., 1995), but these axons do not terminate on a specific target. Furthermore, increased sprouting of cortico-spinal axons onto undamaged propriospinal interneurons (i.e. aberrant intraspinal circuit) via application of BDNF does not significantly improve lesioned-animals performance in a motor task (Vavrek et al., 2006). This suggests that the recovery of skilled movements requires appropriate cortico-spinal tract repair (Smith et al., 2007) otherwise movements will remain crude post-injury. Given this, the role of specific synapse replacement onto an appropriate target which has relatively normal long-term synaptic function (as viewed in BDNF-treated Px11 animals, Article III) is one important step in repair systems. Transcommissural olivocerebellar reinnervation seems to have a greater range of specificity than the spinal cord for repair as target accuracy appears to improve complex neural function. However, its limitations are seen both at the synapse (i.e. PPD) and behavioural level (i.e. postural asymmetry and retention deficit). Although this reinnervation can also be considered as aberrant in terms of being an ipsilateral projection, it does provide us with a useful model to examine post-lesion repair in maturing systems.
4.0 Future Experiments

Based on the findings of this project, there are a number of possible experiments that could be undertaken to further examine BDNF-associated olivocerebellar reinnervation. First, the genes that are implicated in the initiation of BDNF-associated reinnervation could be identified and compared to when no reinnervation occurs. RNA could be extracted from the left vermis approximately 2 days post-BDNF injection at a time concurrent to the onset of reinnervation. A comparison between the transcriptomes in these 2 groups will identify which gene clusters in the cerebellum are expressed during the initial stages of reinnervation as a starting point. Once verification of the identified set of genes (at the mRNA and protein levels) in single PCs has taken place, transfection of these genes in PCs in vitro would then show whether or not the specific set of genes drive long distance neurite outgrowth during olivocerebellar reinnervation.

Second, although BDNF appears to be one neurotrophic factor involved in CF-PC synaptogenesis, a mix of NT-3 (previously observed to induce reinnervation in older animals; Sherrard and Bower, 2001) and BDNF could be applied to the denervated hemicerebellum in older pedunculotomised animals. This would allow us to identify whether there is a synergistic and additive effect on reinnervation in this system by using multiple growth factors. Third, BDNF could be supplied over a longer period of time through adeno-associated viral (AAV) viral vectors. AAV viral vectors are non-enveloped single-stranded DNA viruses which are both non-pathogenic and non-toxic (Harvey et al., 2006). Such vectors could be injected within the denervated hemicerebellum which would supply constant and high quantities of BDNF. An anatomical and behavioural analysis of pedunculotomised animals with AAV-BDNF treatment after several months would indicate whether sustained BDNF in a relatively mature system also produces topographically specific projection pathways. Fourth, the significant behavioural improvement of BDNF-treated Px11 animals should be further explored. These animals illustrated a high level of recovery on the rotarod. Further kinematic analysis of their hindlimb usage during locomotion and stepping patterns by using an electromyography or Basso-Beattie-Bresnahan locomotion rating scale would be an important step to see if their hindlimb movements are quantitatively normal. Furthermore, inducing reinnervation in older animals and undertaking the same movement analysis would indicate the effectiveness or crudeness of movements.
associated with transcommissural olivocerebellar reinnervation in aging animals. Next, in terms of the long-term synaptic properties observed in the present study, it would be interesting to examine whether reinnervating and normal synapses have similar long-term synaptic depression (LTD) after CF-sustained stimulation (e.g. homosynaptic CF-LTD). Because LTD is a marker of motor and spatial learning in the cerebellum (Burguiere et al., 2005) and BDNF-associated reinnervation improves motor and spatial abilities, it is hypothesised that those PCs which receive CFs would also attain normal LTD. Lastly, in the present study we observed that the application of BDNF did not appear to affect the dendritic density of PCs. However, as BDNF has been previously reported to increase the number of spines on dendrites (Jing et al., 2005), BDNF could have affected PC dendritic spine number. Quantitative analysis of confocal images presented in the results (section 3.0) may be a useful step to closely examine the effect of BDNF on post-synaptic target neurons.
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