Post-lesion plasticity of the Olivocerebellar pathway : molecular mechanism underlying the climbing fibre re-innervation of Purkinje cells

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Abstract

In the olivocerebellar pathway (OCP) the afferent climbing fibres (CFs), which are the terminal axon projections of the inferior olivary nucleus (ION), innervate cerebellar Purkinje cells (PCs) in a highly organized topographic manner. Following unilateral transection of mature OCP, the local addition of brain-derived neurotrophic factor (BDNF) into the denervated hemicerebellum induces functional CF reinnervation of PCs. The aim of my thesis was to understand the BDNF-activated mechanisms underlying this plastic response in the mature OCP and assess whether they recapitulated developmental processes.

Using an optimized *ex vivo* model of the mouse OCP, we have shown that even at mature states this pathway intrinsically activates a plastic response in presence of denervated PCs by locally extending transverse branches (TBs) from intact CFs. However, this response was not enough to induce CF-PC reinnervation in the denervated hemicerebellum. In contrast, BDNF injection into the denervated hemicerebellum increases the growth of these TBs, suggesting that the addition of exogenous BDNF to denervated PCs potentiates the intrinsic post-lesion plasticity of the mature OCP to induce, or permit, CF reinnervation of denervated target PCs.

At the molecular level, we found that the addition of BDNF in the deafferented hemicerebellum rapidly activates a transcription response in the intact ION; up-regulating the expression of Pax3. Lentivirus-mediated changes to Pax3 expression in the ION further confirmed a novel afferent role for this transcription factor in functional CF-PC reinnervation in the mature OCP: olivary *pax3* over-expression itself induced reinnervation and siPax3 knockdown abolished the effect of BDNF. A proposed effector of this new Pax3 function is the neuroplasticity biomarker polysialic acid-neural cell adhesion molecule (PSA-NCAM). We found that BDNF treatment to the denervated hemicerebellum increases the expression of PSA-NCAM both in the hemicerebellum and the ION, and that olivary *pax3* also increased cerebellar PSA-NCAM. Overexpression of PSA-NCAM synthetic enzyme, ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase (ST8Sia2), in the ION induced extensive CF reinnervation of denervated PCs, confirming a role for PSA-NCAM in post-lesion plasticity in the mature OCP. This effect is probably mediated by its function of axon outgrowth and pathfinding: specifically elongation of CF TBs and their appropriate recognition of denervated PCs.

It is known that, during development, the OCP shows a high degree of intrinsic post lesion plasticity following lesion, which is able to compensate anatomically and functionally for early PC denervation. Using our *ex vivo* model of the OCP early in development, we demonstrated similar spontaneous post-lesion plasticity, and that it activates and depends on the expression of Pax3 and PSA-NCAM. These results suggest that BDNF-induced reinnervation of mature OCP reactivates at least some developmental plasticity mechanisms.

Résumé

La voie olivocérébelleuse (OCP) comprend les fibres grimpantes (CFs), terminaisons axonales des neurones de l'olive inferieure (ION) et leurs cellules cibles, les cellules de Purkinje (PCs). Les projections olivocérébelleuses suivent une topographie hautement organisée. A la suite d'une transection unilatérale de la OCP mature, l'application locale du "brain-derived neurotrophic factor" (BDNF) dans le hemicervelet dénervé induit la reinnervation fonctionnelle des PCs par les CFs. L'objectif de ce travail de doctorat était de comprendre les mécanismes activés par le BDNF et permettant la plasticité post-lesionnelle au stade mature dans le OCP.

Avec un modèle *ex vivo* de la OCP chez la souris, nous avons montré que dans le système mature les CFs intacts émettent des branchements transverses (TBs) en réponse à la présence de PCs dénervées. Cette réponse n'est pas suffisante pour permettre la réinnervation CF-PC, sauf si le BDNF est injecté dans le hemicervelet dénervé. Ceci suggère que le BDNF exogène ajouté aux cellules cibles dénervées augmente la plasticité post-lésionnelle intrinsèque de la OCP mature pour induire, ou permettre, la réinnervation olivocérébelleuse.

Au niveau moléculaire, nous avons trouvé que l'injection de BDNF dans le hemicervelet dénervé active rapidement l'expression de Pax3 dans l'ION intact. La modification de l'expression de Pax3, par l'injection des lentivirus dans l'ION post-lésion, confirme son rôle dans la réinnervation fonctionnelle des PCs par les CFs dans la OCP mature : la surexpression de *pax3* dan l'ION induit cette réinnervation et la diminution de l'expression du *pax3* empêche l'effet du BDNF. Un effecteur proposé pour Pax3 est le PSA-NCAM, biomarqueur de neuroplasticité important pour la croissance et la guidage axonal. Nous avons trouvé que l'injection de BDNF dans le hemicervelet dénervé augmente l'expression de PSA-NCAM dans ce tissu mais aussi dans l'ION. La surexpression de l'enzyme de synthèse de PSA-NCAM dans l'ION induit la réinnervation des PCs par les CFs. Ce résultat confirme un rôle pour PSA-NCAM dans la plasticité post-lésionnelle dans la OCP mature, probablement en augmentant la croissance des TBs à partir des CFs, et la reconnaissance des PC cibles.

Pendant le développement de la OCP, la plasticité post-lésionnelle intrinsèque est plus importante, permettant la compensation anatomique et fonctionnelle de la dénervation des PCs. Dans notre modèle ex vivo au stade immature, nous avons vu cette même plasticité spontanée, et nous avons montré que cette plasticité implique l'expression de Pax3 et de PSA-NCAM. L'ensemble de ces résultats suggère que la reinnervation post-lésionnelle dans la OCP mature active certains mécanismes de la plasticité développementale.

List of figures and tables

Abbreviations

3-AP	3-acetylpyridine
AKT	Protein kinase B
BDNF	Brain-derived neurotrophic factor
CAMs	Cell adhesion molecules
cAMP	Cyclic adenosine monophosphate
ChABC	chondroitinase ABC
CFs	Climbing fibres
CGRP	Calcitonin gene related-peptide
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CREB	cAMP response element-binding protein
CSPG	Growth-inhibitory chondroitin sulphate proteoglycan
CST	Corticospinal tract
DAO	Dorsal accessory olive
DCN	Deep cerebellar nuclei
DRG	Dorsal root ganglia
ECM	Extracellular matrix
EndoN	Endoneuroaminidase
ERK	Extracellular signal-regulated kinases
GABA	Gamma amino-butyric acid
GAP	Growth-associated protein
GCs	Granule cells
GSK-3	Glycogen synthase kinase 3
IgCAMs	Immunoglobulin superfamily CAMs
IGF-1	Insulin-like growth factor-1
ION	Inferior olivary nucleus
IO	Inferior olivary
KLFs	Kruppel-like factors
MAG	Myelin-associated glycoprotein
MAO	Medial accesory olive
MFs	Mossy fibres
mTOR	Mammalian target of rapamycin
NCAM	Neural cell adhesion molecule
NCCs	Neural crest cells
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NT	Neurotrophin
OC	Olivocerebellar
OCP	Olivocerebellar pathway
Pax3	Paired box 3 transcription factor
PCs	Purkinje cells
PFs	Parallel fibres
PI3K	Phosphatidylinositol-3 kinase
PKA	cAMP-dependent protein kinase
PNS	Peripheral nervous system
PO	Principal olive
PSA	Polysialic acid
PSA-NCAM	Polysialic acid neural cell adhesion molecule
PST	Polysialyltransferase ST8SiaIV

PTEN	Phosphatase and tensin homologue
Px	Pedunculotomy
RAF	Ras-activated rapidly accelerated fibrosarcoma
RAGs	Regeneration associated genes
RGCs	Retinal ganglion cells
SCI	Spinal cord injury
Sem	Semaphorin
SOCS3	Suppressor of cytokine signalling 3
STX	Polysialyltransferase ST8SiaII
TB	Transverse branches
Trk	Tropomyosin-related kinase

CHAPTER 1

Introduction

Chapter 1 – Introduction

I- Plasticity in the mature central nervous system following lesion

Disrupting connectivity in the mammalian CNS by injury activates a compensatory plastic mechanism by growing neurites either from intact fibres or regenerating damaged axons (Maier and Schwab, 2006). Both types of growth are developmental state-dependent, being more abundant in damaged neonatal CNS for a determined period of time. Afterwards, there is a decline in the re-growth ability as the CNS matures, so that the capacity to regenerate injured axons becomes limited. The failure of mature axons to regenerate has been linked to a lack of intrinsic neuronal growth capacity, plus an extrinsic inhibitory environment (Chen & Zheng, 2014).

However, mounting evidence has shown extensive axonal sprouting occurring spontaneously after injury in the adult CNS (Geoffroy & Zheng, 2014), and different pharmacological and genetic approaches have been used to induce re-growth from severed axons, and/or promote axon sprouting, in some cases with mild functional recovery (Liu *et al.*, 2011). Both collateral sprouting and induced axon regeneration are evidence of structural reorganization occurring in the adult CNS following injury, and more importantly, shows that this intrinsic post lesion plasticity can be boosted by different external stimuli.

Despite the current general acceptance of a plastic adult brain, little is known about its molecular components, which allow such plasticity following injury, nor whether the mechanisms involved recapitulate developmental growth processes or development-related plasticity.

I.1- Regulators of axonal growth and re-growth

During development, growing axons extend over long distances throughout the nervous system before reaching their post-synaptic target, in a process supported by a set of trophic factors, extracellular matrix molecules, and guidepost cells (**Figure 1.1**; Liu *et al.*, 2011). After target contact, the elongating process of developing axons must stop to allow the motile growth cones to produce pre-synaptic terminals, thus switching from a growth state to synapse formation and maturation. In accordance, most CNS neurons at these developmental

stages show a decrease in the expression of several axon growth-related genes (Maier & Schwab, 2006), whereas molecular programs related to synaptic assembly are activated (Hökfelt *et al.*, 1994). These support the hypothesis that an axon's capability to grow long distances is likely to be developmentally programed (Skene & Willard, 1981) and that this developmental growth program may interact with a genetic program of synapse formation (Liu *et al.*, 2011).

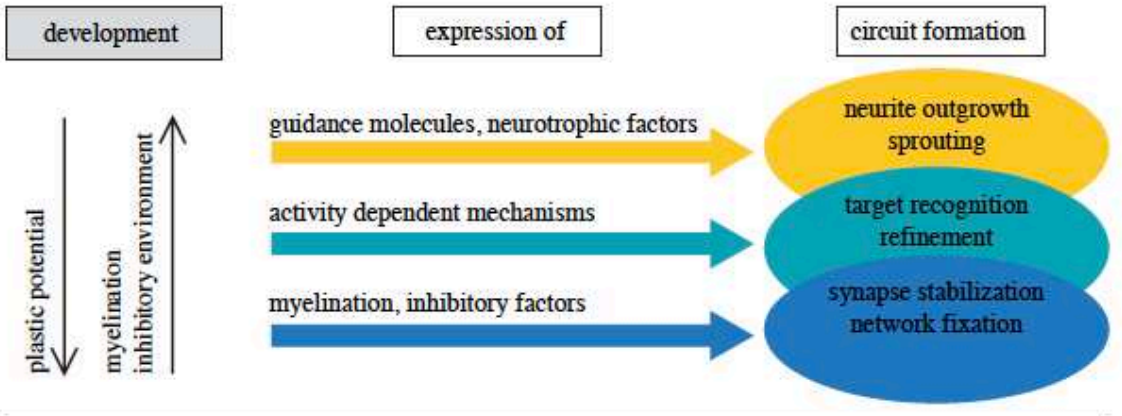

Figure 1.1: Scheme of developmental circuit formation and the extrinsic modulators involved. The development of axons and the final synapse formation with its corresponding target requires differential steps, such as guidance target recognition, and fine-tuning and stabilization. Adapted from Maier & Schwab, 2006.

I.1.1- Developmental axon growth, molecular mechanisms

I.1.1.1 - Neurotrophins

The developing axon requires intracellular machinery that assembles cytoskeletal elements and membrane components into the new axon. It has been suggested that activation of this elongation program requires extracellular signals (Goldberg *et al.*, 2002; Zhou & Snider, 2006). Among the most studied extracellular factors inducing axonal growth are the neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). They induce robust axon growth in different cell populations by activating their corresponding receptor tyrosine kinases (TrkA, TrkB, and TrkC; Belliveau *et al.*, 1997; O'Keeffe *et al.*, 2008; Liu *et al.*, 2011). Downstream from neurotrophin-mediated Trk activation, two classical pathways play an important role in stimulating axon growth: the phosphatidylinositol-3 kinase (PI3K)/serine/threonine protein kinase B (AKT) pathway, and the Ras-activated rapidly accelerated fibrosarcoma (RAF)/extracellular signal-regulated kinases (ERK) kinase pathway (**Figure 1.1**; Polleux & Snider, 2010). Both cascades are thought to have a role in the regulation of the axon assembly

at different levels, including direct control upon cytoskeletal filaments (microtubules and actin filaments), and local control of protein synthesis in the growth cone (see Zhou & Snider, 2006).

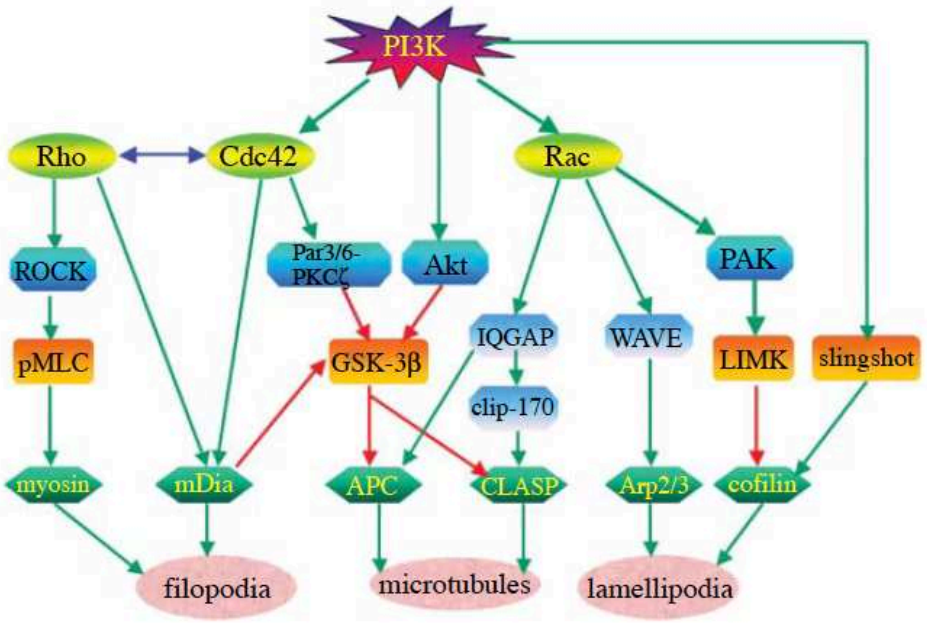

Figure 1.2: PI3K signalling involved in the regulation of axon cytoskeleton. Main downstream effectors including Rac, Cdc42, and Rho regulate different cytoskeletal proteins that participate in the final axon growth. Modified from Zhou & Snider, 2006.

I.1.1.2- ECM and trans membrane proteins (Integrin and cell adhesion molecules)

In addition to neurotrophic factors, extracellular matrix (ECM) proteins (e.g. laminin and heparan sulphate proteoglycans) and cell adhesion molecules (CAMs), such as cadherins, L1 and neural-CAM (NCAM), participate in the axon growth process during development (Goldberg, 2003) by providing a critical substrate along which axons elongate (Goldberg, 2003). Although *in vivo* blockade of their function impairs axon growth (Riehl *et al.*, 1996), *in vitro* studies have shown that ECM proteins require trophic factors to induce proper neurite outgrowth (Goldberg *et al.*, 2002); thus it is not clear whether ECM molecules have a direct growth-promoting effect or simply provide a permissive substrate (Zhou & Snider, 2006). Receptors for ECM molecules and CAMs, including integrins and immunoglobulin superfamily CAMs (IgCAMs), are highly expressed during development and are down-regulated in adult neurons (Goldberg, 2003).

Integrin surface receptors are expressed in the growth cone of developing neurons, where they interact with intracellular actin filaments. After binding to their ECM ligands, integrins

directly link the ECM to cytoskeletal elements, in what is thought to be a mechano-chemical regulation of growth cone motility (Kerstein *et al.*, 2015). Furthermore, integrins can also mediate axon growth by binding with semaphorin 7A (sem7A), a member of the semaphorin family of guidance molecules (Pasterkamp *et al.*, 2003). In vitro studies have shown that the sem7A-integrin interaction activates downstream signalling through focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and ERK pathways (Pasterkamp *et al.*, 2003).

The trans membrane IgCAMs are recognition molecules with critical roles in axon outgrowth, and also in axon fasciculation, neural migration, synaptic and post lesion plasticity (Pollerberg *et al.*, 2013). One of the most studied IgCAMs is NCAM, which is expressed in growth cones and has a pivotal role in axonal growth by mediating cell-cell interaction and activating intracellular signalling pathways. After homophilic trans- and cis-interactions, several axon growth-related downstream effectors are activated, including Ras/ERK kinase pathway, cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent protein kinase type II alpha chain (CAMKIIα; see Maness & Schachner, 2007). Activation of these signalling cascades is independent of each other, where some of them require the proper distribution and organization of NCAM into plasma membrane compartments called lipid raft. The ensemble of these intermediaries mediates a co-signalling mechanism that is required for the cytoskeletal rearrangement and transcriptional changes to induce the axon outgrowth (Maness & Schachner, 2007).

Additionally, NCAM has an important role in axon guidance (elongation and target recognition) mediated by posttranscriptional modifications on its extracellular portion. The addition of polysialic acid (PSA), a long, negatively charged and linear homopolymer, to NCAM confers a bigger volume that is likely to reduce the contact between cells (Rutishauser, 2008), interrupting homophilic NCAM interaction and heterophilic NCAM-IgCAMs binding (Zhang *et al.*, 2008). The selective elimination of PSA from NCAM by the enzyme endoneuroaminidase (EndoN) has shown in different neural systems that PSA-NCAM participates in nerve fasciculation, axon branching, target recognition, and in synapse formation (Kiss *et al.*, 2001; Bonfanti, 2006).

The expression of PSA is also developmentally regulated, but interestingly conserves its expression during adult state in certain brain regions where neuronal plasticity is persistent throughout life (subventricular zone, the olfactory bulb, the hippocampus; Theodosis *et al.*, 1999).

I.1.1.2.1- Synthesis of PSA-NCAM

Unfortunately there is not much evidence about the cellular mechanisms involved in the PSA-NCAM formation and the regulation of its levels during development or in adult state that could help to better understand how PSA-NCAM is modulated. Currently it is known that the addition of PSA to NCAM is a glycosylation mediated by two Golgi polysialyltransferases, ST8SiaIV -also called PST- and ST8SiaII -called STX- where either of them is sufficient to complete the synthesis of PSA chains (Eckhardt, 1995). ST8SiaII and IV show different enzymatic activity and biological function, and are expressed differentially in tissue-specific and cell-specific manners (reviewed in Angata & Fukuda, 2003). Both enzymes are expressed during developmental stages, and downregulated in the adult with different decline rates. Whereas ST8SiaII expression diminishes dramatically after birth, ST8SiaIV gradually declines but still is continuously expressed at lower levels in various tissues in adulthood (Bonfanti, 2006). Interestingly both transcripts are presents in the adult brain areas that undergo continuous synaptic rearrangement and continuously induce PSA-NCAM (Angata & Fukuda, 2003). As suggested by Bonfanti (2006) the expression of PSA-NCAM on the cell membrane is probably mediated by two main mechanisms: the transcription and/or the activity of ST8SiaII/IV regulating PSA-NCAM synthesis, and the transport of already assembled PSA-NCAM to the cell surface. Even though it has been suggested that the activity of both enzymes is Ca2+ dependent (Brusés & Rutishauser, 1998), and probably related to synaptic transmission through N-methyl-D-aspartate (NMDA)-evoked increase in the intracellular Ca2+ (Bouzioukh *et al.*, 2001), transcriptional pathways of either ST8SiaII or IV still remain diffuse (Rutishauser, 2008). Nevertheless, the transcription factor Pax3 has been considered as a potential candidate controlling the expression of the polysialyltransferases (Mayanil *et al.*, 2000, 2001). Overexpression of rodent Pax3 into a medulloblastoma cell lines *in vitro* induces an increase in ST8SiaII expression and a consequent increase in PSA-NCAM, which functionally alters the adhesive properties of Pax3 transfected cells (Mayanil *et al*., 2000). Pax3 is a member of the Paired Box (Pax) family of transcription factors involved in key roles in the formation of tissues and organs during embryogenesis (Lagha *et al.*, 2010). In particular, Pax3 is as an upstream regulator of myogenesis, controlling the behaviour of progenitor cells and their entry into the program of skeletal muscle differentiation (Buckingham & Relaix, 2007).

Moreover, Pax3 has a key role in neural crest cell migration and neural crest development. Pax3 is expressed early in development (~E8.5) in neural crest cells (NCCs), and continues to be expressed during their migration until NCCs begin to differentiate (Goulding *et al.*, 1991).

Pax3 is thought to have a main role, in conjunction with its transcriptional products, in the specification of progenitor cell position along the anterior-posterior and the dorso-ventral axis of the developing spinal cord and brain (Chalepakis *et al.*, 1993). Deficient Pax3 expression, as revealed by the homozygous null *Pax3* in *splotch* mutant mouse, leads to early embryonic death due to defects in both neural tube closure and neural crest development (Auerbach, 1954; reviewed in Monsoro-Burq, 2015), these embryos show a severe reduction of neural crest derivatives, including pigment cells, sympathetic ganglia, spinal ganglia, cardiac outflow tract, and enteric nervous system (Serbedzija & McMahon, 1997). In contrast, heterozygous Pax3 mutant mice only display a melanocyte development phenotype with pigmentation abnormalities (Auerbach, 1954). In humans, Pax3 gene mutation is responsible for Waardenburg's syndrome type 1 and 3, causing deafness and pigmentary disturbances (Tassabehji *et al.*, 1992; Read & Newton, 1997).

The main neural tube defects in Pax3-mutant mice have been related to inappropriate cell-ECM interactions within the neural tube or between the neural tube and somites (also expressing Pax3 during development; Serbedzija & McMahon, 1997). Among the possible explanations involved in the cell-ECM interaction failure, is the downregulation of PSA-NCAM in Pax3 mutant embryos (Glogarová & Buckiová, 2004), as PSA-NCAM has an important role in the cellular adhesion process. Also this study indirectly correlate the suggested link between Pax3 and the levels of PSA added to NCAM. Also, the chondroitin sulphate proteoglycan versican (ECM protein) has been related to defects in the Pax3 expression that could be mediating the negative adhesive effects in Pax3 mutant embryos (Henderson *et al.*, 1997).

On the other hand, it has been suggested that Pax3 is essential for neural crest survival during emigration, allowing NCCs to increase survival potential and resistance to apoptosis (Monsoro-Burq, 2015), and this role could be related to inhibitory effects rather than inducing neural tube-specific gene expression (Pani *et al.*, 2002).

I.1.2- Adult axonal re-growth

Axonal growth capacity normally decreases as the central nervous system matures; thus growth in the adult is secondary to a pathological state. Studies in developmental neurobiology as well as in cancer biology (cell growth deregulation), adult peripheral nervous system (PNS), and in lower animal models, have helped the identification of candidate molecules involved in adult post lesion plasticity mechanisms. Different approaches of gain and loss of function for these candidate molecules have revealed that adult CNS neurons possess intrinsically the machinery to adapt to injury, and can also respond to additional non-developmental stimuli (e.g. genetic and pharmacological signals) to increase axonal outgrowth.

From the first experiments until today, it has been difficult to identify a unified definition to describe post lesion-induced axonal re-growth after injury: terminal sprouting, collateral growth or axon regeneration. Inconsistent use of these terms lead to a misinterpretation about the underlying mechanisms regulating different forms of axon re-growth, which could be completely different. By asking the question *from where* the new axon growth arises, axon regeneration has been defined as any form of axon growth from the injured neuron, and collateral growth as the axonal growth emerging from remaining intact neurons (**Figure 1.3**; see Tuszynski & Steward, 2012; Geoffroy & Zheng, 2014).

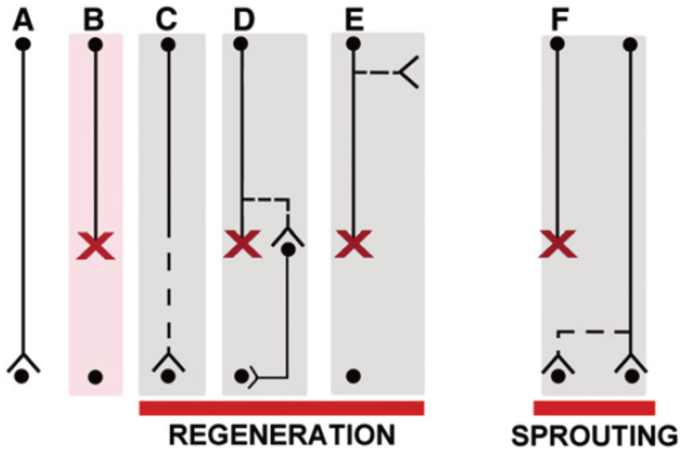

Figure 1.3: Axon regeneration and collateral sprouting concepts. (A) Intact axons. (B) Injured axons. (C-E) Regenerative axon growth, which involve the canonical axon regeneration with new growth arising from the tip of the transected axon (C), also the axon growth occurs from a region close to the injured tip (D) or either from a site remote from the injury (E). These last two forms of axon regeneration (D-E) have been called as well "regenerative sprouting" when deeper anatomical distinction is needed (Bareyre *et al.*, 2004). (F) Collateral sprouting involves the formation of a newly growing fibre from intact axons nearby to the injured axon. From Tuszynski & Steward, 2012.

I.1.2.1- Inherent Axon Regeneration potential

In the adult PNS there is a high plastic potential that spontaneously shifts to a regenerative state following lesion. Axotomy induces a retrograde signal activating the soma, which activates a cell survival pathway, and several re-growth associated genes (Mar *et al.*, 2014) .
The importance of the neuron's intrinsic plasticity and its potentiation by external stimuli in order to boost axon re-growth after damage was first demonstrated in the conditioning model of PNS sensory neurons (Chen & Zheng, 2014).
Each primary sensory neuron in dorsal root ganglia (DRG) has two major branches emerging from a unipolar axon, one projecting to the PNS and the other to the CNS. Each branch responds differently following axotomy in the adult state: the peripheral axon branch has the ability to regenerate back toward its proper target, but the CNS axon branch fails to regenerate and only shows abortive sprouting. However, if a previous injury has been performed on the peripheral axon (conditioning lesion), the plastic response can switch to a regenerative state after a second injury in either the central or peripheral branch (Richardson & Verge, 1986). The conditioning lesion model shows that DRG neurons have the competence to potentiate their intrinsic post lesion plasticity, which requires the activation of a growth-signalling pathway in the soma. This activated transcriptional program is complex and involves the expression of hundreds of genes and their upstream regulators (regeneration associated genes; RAGs; see Abe & Cavalli, 2008; Chandran *et al.*, 2016; Van Niekerk *et al.*, 2016). Among the activated pathways, there is a recapitulation of developmental growth-associated proteins such as GAP-43, CAP23 and SPRR1A. Also there is an upregulation of transcription factors including ATF-3, c-Jun, Sox11, and Smad1, as well as an increase in levels of arginase 1 (reviewed in Liu *et al.*, 2011). Another important change after conditioning lesion is the transient increase of cyclic adenosine monophosphate nucleotide (cAMP) levels, which support *in vivo* data where elevation of cAMP induces plastic re-growth of mature spinal axons following spinal cord injury (Qiu *et al.*, 2002). This cAMP-induced re-growth has been related to the ability of overcome myelin-related inhibitory environment, by the activation of downstream modulators such as protein kinase A (PKA; Cai *et al.*, 1999, 2001), cAMP response element-binding protein (CREB; Gao *et al.*, 2004), and arginase 1 (Cai *et al.*, 2002).

I.1.2.2- Trophic responses

As indicated above (Section **I.1.1.1**), neurotrophic factors have a well-known role mediating axonal growth and circuit formation throughout development (Reichardt, 2006). Therefore

they have been considered good molecular candidates to stimulate post-lesion plastic mechanisms and axonal re-growth. However the addition of exogenous neurotrophic factors into different CNS injury models has provided mixed results. In the injured corticospinal tract (CST), addition of exogenous NT-3, induces axon re-growth from damaged axons (Schnell et al 1994), whereas NGF, BDNF (Schnell *et al.*, 1994; Lu *et al.*, 2001) and insulin-like growth factor-1 (IGF-1) ((Hollis *et al.*, 2009) did not. In an optic nerve injury model, intravitreal injection of exogenous BDNF also fails to promote axonal regeneration, however the addition of ciliary neurotrophic factor (CNTF) into the damaged optic nerve can induce regeneration (described below; Smith *et al.*, 2009). The varied plastic response to exogenous neurotrophic factors may reflect an altered responsiveness of mature neurons to these factors (Liu *et al.*, 2011).

In the spinal cord injury (SCI) model, the administration of neurotrophic factors by different means, such trophic-factor expressing fibroblasts grafted into the damaged area, have been shown to activate re-growth in different neuronal populations (**Table 1.1**; Hollis & Tuszynski, 2011). However in most cases treatment with neurotrophic factors induced only moderate regenerative sprouting with limited functional recovery. A possible explanation is that the induced growth response in adult systems cannot directly overcome myelin-derived inhibition. The combination of these observations reveals that neurotrophic factors are generally ineffective as a treatment after SCI (Hannila & Filbin, 2008).

Table 1.1: Main neurotrophins potentiating plastic re-growth in a neuron type dependent manner after spinal cord injury. 5-HT$^+$, serotonergic raphaespinal tract; TH$^+$, tyrosine hydroxylase expressing coerulospinal tract. Modified from Hollis & Tuszynski, 2011.

Growth Factor	Central axon populations affected	References
NGF	ChAT$^+$ motor CGRP$^+$ sensory TH$^+$ coerulospinal	Tuszynski *et al.*, 1994, 1996; Grill *et al.*, 1997.
BDNF	5-HT$^+$ raphaespinal TH$^+$ coerulospinal NF200$^+$ proprioceptive sensory Rubrospinal	Bregman *et al.*, 1997; Kobayashi *et al.*, 1997; Liu *et al.*, 1999; Kwon *et al.*, 2002; Lu *et al.*, 2005.
NT-3	5-HT$^+$ raphaespinal NF200$^+$ proprioceptive sensory Corticospinal motor (axon plasticity only)	Bradbury *et al.*, 1999; Tuszynski *et al.*, 2003; Lu *et al.*, 2004; Taylor *et al.*, 2006.
NT-4	5-HT$^+$ raphaespinal TH$^+$ coerulospinal	Bregman *et al.*, 1997.

I.1.2.3- Anti-regeneration molecules

The early suggestion that intrinsic growth competence for CNS axons is usually diminished as neurons mature has been correlated to the decline in the expression of growth-related genes (Smith & Skene, 1997). But in addition, a second mechanism mediated by inhibitory regulators of growth signalling pathways has been suggested to contribute to restriction of axonal growth in adult neurons.

I.1.2.3.1- PTEN

Phosphatase and tensin homologue (PTEN) is a tumor suppressor enzyme that dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP_3) to repress the pro-proliferative PI3K-AKT pathway. The elimination of PTEN gene following optic nerve or CST lesion in the adult induces regeneration of injured axons and collateral sprouting (**Figure 1.4**; Park *et al.*, 2008; Liu *et al.*, 2010), and also improves the survival of retinal ganglion cells (RGCs) (Park *et al.*, 2008). Unfortunately, the recovery of visual function after axon regeneration induced by PTEN deletion still is under debate, as two different studies have shown contradictory data (Lima *et al.*, 2012; Luo *et al.*, 2013).

The main downstream effector by which PTEN-deletion induces axon re-growth is thought to be the mammalian target of rapamycin (mTOR) (Park *et al.*, 2008, 2010). The pharmacological inhibition of mTOR directly interrupts the post lesion growth response mediated by PTEN-inactivation (Park *et al.*, 2008). However whether the mTOR signalling pathways involved in axonal re-growth also regulate cell survival is unknown (Chen & Zheng, 2014).

A second PTEN target is the PI3K-AKT pathway, which in turn modulates the glycogen synthase kinase 3 (GSK-3) to activate the transcription factor SMAD 1 (Saijilafu *et al.*, 2013) and regulate post-lesion axon re-growth in sensory neurons.

Taken together these studies show that PTEN deletion can activate axon re-growth through different mechanisms. It remains to be evaluated whether these mechanisms are neuron type dependant and to what extent they promote functionally-beneficial neural circuit repair.

I.1.2.3.2- KLFs

Recently, a subset of transcription factors from the Kruppel-like factors (KLFs) family have been suggested to regulate axon growth in a development-dependent manner (Liu *et al.*,

2011). Among the KLF subsets, KLF4 inhibits axon outgrowth in cultured embryonic hippocampal neurons and RGCs (Moore *et al.*, 2009). KLF4 has high expression in mature RGCs (Moore *et al.*, 2009) so that its deletion after injury increases both neurite outgrowth in cultured RGCs and optic nerve regeneration in adult mice (Moore *et al.*, 2009). In contrast KLF6 and KLF7 expression declines with maturation, and their overexpression in cortical neurons enhances neurite outgrowth *in vitro* (Chen & Zheng, 2014). Moreover KLF7 overexpression in adult CST neurons induces re-growth of their axons after injury (Blackmore *et al.*, 2012).

Additional studies are needed in order to understand the role of the different types of KLFs on plasticity-related axon growth mechanisms in both developing and mature neurons, and to identify key potential transcriptional targets activated in post lesion neuronal plasticity. A gene expression analysis performed in zebrafish RGCs after optic nerve axotomy, a model showing robust post-injury re-growth, identified upregulation of KL6a and KLF7a which was necessary for RGCs axon re-growth (Veldman *et al.*, 2007). In addition, this expression analysis showed that not all the upregulated genes associated with regenerative growth are expressed during RGC developmental stages, meaning that post lesion plasticity in adult neurons is not a direct recapitulation of developmental growth pathways (Liu *et al.*, 2011).

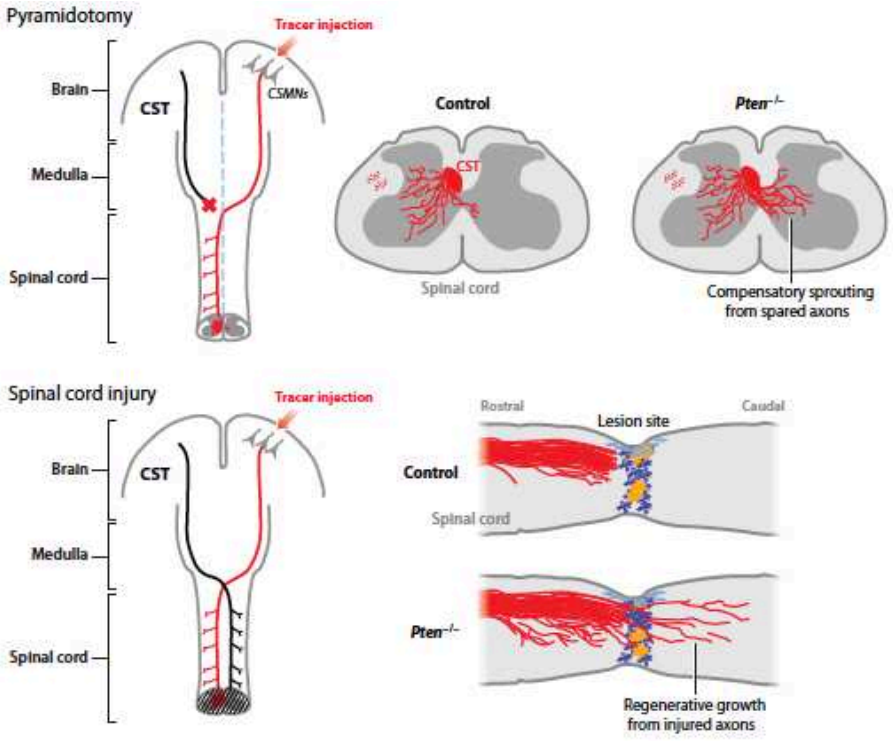

Figure 1.4: Intrinsic and PTEN-deletion mediated CST axon re-growth. Following unilateral transection of CST by pyramidotomy, induces the intrinsic collateral sprouting from intact CST. The PTEN deletion in this tract, promotes the two types of axon re-growth after injury by activating mTOR and PI3K/GSK-3 pathway. Modified from Liu *et al.*, 2011.

I.1.2.3.3- SOCS3

Suppressor of cytokine signalling 3 (SOCS3) is a negative regulator of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, which has been shown to promote plastic responses in CNS sensory axons (Qiu *et al.*, 2005). Using the conditioning lesion model, pharmacological blockade of JAK following the first injury to the peripheral nerve impaired the subsequent re-growth of central axonal branches (Qiu *et al.*, 2005). Similarly, Smith and co-workers (2009) deleted SOCS3 from axotomised RGCs, and found significant optic nerve regeneration. This SOCS3-deletion induced plasticity was dependent on the cytokine receptor gp130, which is also an upstream regulator of the JAK-STAT pathway. An important ligand of gp130-ligand is CNTF, which potentiates the re-growth of optic nerve axons lacking SOCS3 (Smith *et al.*, 2009). In vitro studies confirmed neurite outgrowth- promoting effect of CNTF on cultured mature RGCs, and found that this plastic response is mediated by the JAK-STAT pathway, but also by the activation of the PI3K-AKT signalling (Müller *et al.*, 2009).

Despite the ability of exogenous CNTF to induce post lesion plasticity in injured neurons, its use in mouse optic nerve injury does not promote circuit repair. Short-term CNTF treatment induces limited sprouting from injured RGC axons (Müller *et al.*, 2007), while Adeno-associated virus (AAV)-mediated CNTF overexpression strongly stimulates outgrowth either from damaged or intact axons (Pernet *et al.*, 2013). Unfortunately, these newly growing axons can only form aberrant paths.

I.1.2.4- Extrinsic factors

In addition to intrinsic neuronal responses involved in axon regeneration, the extracellular environment also plays a significant role. Key studies by Aguayo and collaborators in early 80's demonstrated that some injured CNS neurons have the intrinsic capacity to regrow into grafted permissive substrates (e.g. peripheral nerve environment; Aguayo *et al.*, 1981; David & Aguayo, 1981; Geoffroy & Zheng, 2014), revealing the inhibitory nature of the CNS environment. Further studies have confirmed that the composition of the extracellular matrix in the mature CNS changes dramatically after the differentiation of oligodendrocytes and the myelination of axons and contributes to the switch from axonal growth and elongation to growth-arrest and synaptogenesis. First, adult CNS-myelin expresses growth-inhibitory proteins (Liu *et al.*, 2006). Second, lesions cause local cellular breakdown and inflammation, which generate the glial scar, a well-known barrier for re growing axons (Yiu & He, 2006).

These environmental conditions directly affect the degree of intrinsic plastic response in the adult CNS.

I.1.2.4.1- Myelin-derived inhibitors

Three main myelin-derived proteins have been recognized: myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte myelin glycoprotein (OMgp), where the last two proteins are exclusively expressed by oligodendrocytes, and absent from the PNS. Myelin-derived proteins signal through multiple neuronal receptors (e.g. NgR and PirB), and co-receptors (p75NTR or TROY, and LINGO-1) to activate signalling cascades which converge on downstream effectors Ras homolog gene family member A (RhoA) and Rho-associated kinase (ROCK), that in turn induce growth cone collapse and the inhibition of intrinsic axon outgrowth (see Geoffroy & Zheng, 2014; Schwab & Strittmatter, 2014).

Pharmacological and genetic loss of function approaches, for these myelin-derived proteins and their main receptors, have found a variety of results related to axon re-growth and circuit repair after injury. Recent *in vivo* studies performing SCI to single or triple knockout mice for myelin proteins (Nogo/MAG/OMgp) conclude that removal of these myelin-derived proteins has a clear and reproducible effect permitting axon collateral sprouting (mainly OMgp and Nogo), but only a limited effect on axonal regeneration (Cafferty *et al.*, 2010; Lee *et al.*, 2010). Moreover, in none of the mutant conditions was functional recovery observed (Lee *et al.*, 2010). In contrast, other groups have found, after section of the CST in adult rats, that the neutralization of NogoA with antibodies promotes axon re-growth with functional recovery (Freund *et al.*, 2009).

I.1.2.4.2- Glial scar and CSPGs

Shortly after injury to the CNS, reactive astrocytes become hypertrophic and, in combination with other glial and fibroblast cells, form a scar at the site of the lesion. This glial scar represents a physical barrier for re-growing axons, and contains growth-inhibitory chondroitin sulphate proteoglycans (CSPG; Bradbury *et al.*, 2002). CSPGs are secreted by astrocytes and are ECM proteins heavily glycosylated by glycosaminoglycan (GAG), which is thought to mediate the inhibitory growth effect of CSPGs (McKeon *et al.*, 1995; Zuo *et al.*, 1998). Interestingly, the bacterial enzyme chondroitinase ABC (ChABC) catalyses the degradation of GAGs from CSPG, and is used in different conditions to block the inhibitory effects of CSPG and make ECM substrates more permissive to growth (Liu *et al.*, 2006). After CNS

injury, the addition of ChABC promotes post lesion axonal re-growth (Moon *et al.*, 2001; Bradbury *et al.*, 2002; Yick *et al.*, 2003; Chau *et al.*, 2004), and significant functional recovery in different types of SCI (Bradbury *et al.*, 2002; Caggiano *et al.*, 2005; Houle *et al.*, 2006). Unfortunately, the mechanisms underlying this functional recovery remain unclear. In a dorsal column lesion SCI model, Barritt and co-workers (2006) showed that post-lesion ChABC treatment promotes robust sprouting from both injured CST axons and intact serotonergic projections. These sprouting axons were able to extend below the level of the lesion and reach the spinal grey matter, suggesting that ChABC-induced recovery might be mediated by axon regeneration and/or collateral sprouting. The importance of these studies on ChABC-treatment is that they confirm the capacity for adult CNS neurons to mount an intrinsic plastic response post-lesion, which, in the absence of environmental inhibitors, may provide some degree of compensation both anatomically and functionally.

However, the glial scar is not an entirely negative post-injury reaction. It has its own function, being part of the tissue repair mechanism aiming to reduce inflammation and confine the damaged tissue (Silver & Miller, 2004). Thus, any treatment to promote post-injury axon growth must be cautious before disrupting or totally removing this scar, but rather aim to balance between the mechanism of wound repair and clearing a path for axon growth (Chen & Zheng, 2014). In addition, as it matures the protein components of the glial scar change, and astrocytes toward the scar centre express the permissive molecule PSA-NCAM, and its expression is correlated temporarily and spatially with the presence of axon sprouts (Dusart *et al.*, 1999; Camand *et al.*, 2004). Moreover, this more growth-permissive component of the glial scar can be manipulated. Two different groups using the SCI model in rodents have shown that overexpression of PSA-NCAM in the formed glial scar allows a substantial portion of sprouting CST axons to cross through the lesion site (El Maarouf *et al.*, 2006; Zhang *et al.*, 2007). Suggesting that the induced expression of PSA-NCAM into the glial scar provides a favourable environment for post lesion spontaneous regrowing axons.

I.1.2.5- Summary

In summary, the above descriptions indicate that mature CNS circuits conserve some degree of structural plasticity, which can be extrinsically stimulated in order to induce post-lesion neural circuit repair. This stimulation involves key steps: activation of presynaptic re-growth (e.g. RAGs, trophic factors) and/or manipulation of the molecular environment at the lesion

site (guidance cues, target-derived signals, myelin-related proteins). However, despite the potentiation of axon re-growth observed, proper functional recovery remains to be improved. Further studies need to be done to understand how to modulate appropriately a neuron's intrinsic post-injury plastic potential, and which other mechanism could be missing in the final axon-target reinnervation.

By looking at the normal process of circuit development, it has been classically accepted that following axon growth and elongation the pathfinding step, specially when invading target regions, it is highly mediated by post synaptic-derived signals (Sperry, 1963; Goodman & Shatz, 1993; Sotelo & Chédotal, 2005). Whether target neurons play a role in the post lesion plasticity mechanism has been difficult to evaluate in CNS injury models, mainly because of physiological and technical difficulties, to manipulate each synaptic components.

Interestingly, by using the olivocerebellar pathway it has been already observed that denervated target cells locally mediate their afferent reinnervation in adult states (Rossi *et al.*, 2007). In addition, to its high post lesion plasticity, it has a well-described topography, which is important to manipulate and evaluate synaptic partners role in the mechanism underlying plastic responses.

II- Rodent Olivocerebellar pathway as a model to study reinnervation

The olivocerebellar pathway (OCP) is one of the highest conserved tracts in the vertebrate brain (Llinás, 2014). This path originates from the inferior olivary nucleus (ION) and projects into the cerebellum to the contralateral cerebellar cortex and the deep cerebellar nuclei (DCN).

II.1- Cerebellum

The cerebellum is one of the regions of the central nervous system where the organization and intrinsic circuitry pattern is known in considerable detail. The rodent cerebellum is a highly foliated structure located dorsal to the brainstem (**Figure 1.5**).

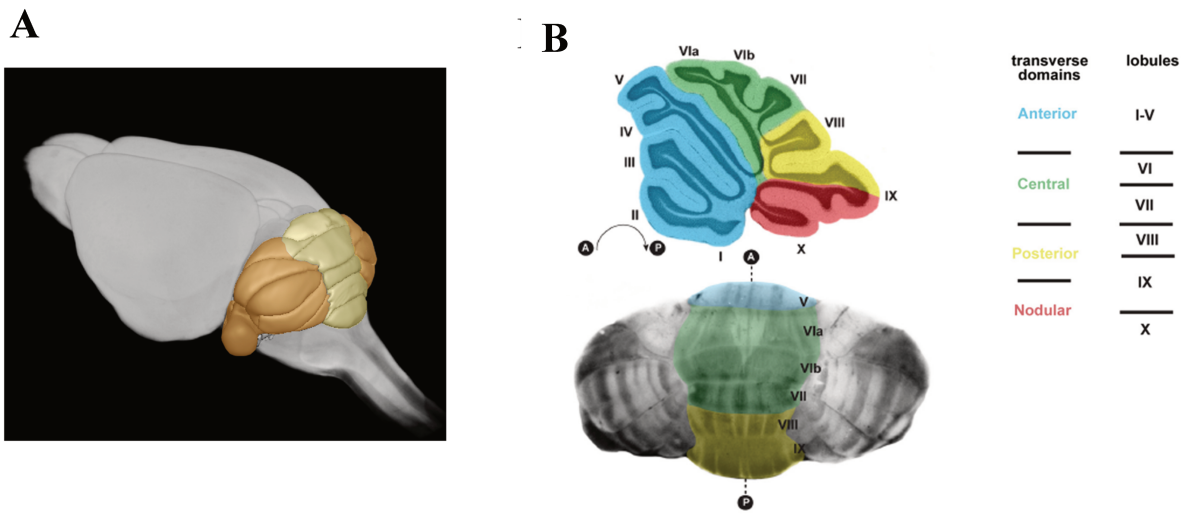

Figure 1.5: Morphology of adult mouse cerebellum. (A) Dorsolateral view of the cerebellum positioned on the mouse brain using magnetic resonance images. The vermis (yellow) surrounded by the cerebellar hemispheres (orange) are highlighted. (B) At the top, a sagittal section cut through the highly foliated cerebellar vermis (dorsal view at the bottom). Ten lobules can be recognized in this region, which can be further subdivided along the rostro-caudal axis in four transverse domains (Ozol *et al.*, 1999). Color-coded according to the panel. Modified from (A), Ullmann *et al.*, 2012; (B), Reeber *et al.*, 2013.

It is connected to the brainstem and other parts of nervous system by three pairs of cerebellar peduncles, the superior (almost entirely efferent), the middle and the inferior (mainly afferent; Ruigrok *et al.*, 2015). On a macro scale the cerebellum has two symmetric hemispheres united in the midline by the central longitudinal vermis. Following the anteroposterior axis two deep fissures divide the cerebellum into three main lobes, the anterior and posterior lobes (by the

primary fissure) and the flocculonodar lobe (separated from the posterior lobe by the posterolateral fissure). These lobes are further subdivided by shallow horizontal fissures forming in total 10 lobules, numbered from I to X according Larsell`s roman terminology for rodents (**Figure 1.5 B**; Larsell, 1952; Reeber *et al.*, 2013).

Within the cerebellum, the cerebellar cortex forms the superficial grey matter, which is subdivided into three layers, and an internal white matter formed of nerve fibres and 4 pairs of deep cerebellar nuclei. The cerebellum receives 3 types of extrinsic afferents, climbing fibres (CFs), mossy fibres and monoaminergic fibres (Ramon y Cajal, 1911; Palay & Chan-Palay, 1974; Ito, 2006), and its output arises from the DCN (Ruigrok *et al.*, 2015).

II.1.1- Cerebellar cortex

The cerebellar cortex is a simple and uniform structure containing seven main neuronal cell types which are distributed within three layers: the granular layer, Purkinje cell layer and the molecular layer (**Figure 1.6**).

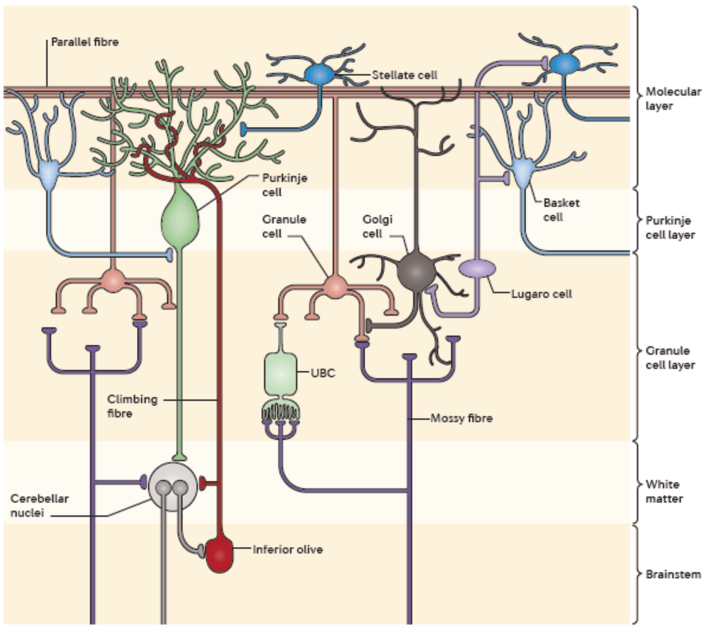

Figure 1.6: Cerebellar cytoarchitecture. Schematic of the simplified cerebellar cortex, which consist of the granule cell layer, Purkinje cell layer and the molecular layer. The basic cytoarchitecture is built of the Purkinje cells, granule cells, Golgi cells, Lugaro cells, unipolar brush cells (UBCs), stellate cells and basket cell interneurons. The two main cerebellar afferents are the climbing and the mossy fibres; the former fibres innervate the Purkinje cell and subsets of neurons in the cerebellar nuclei. Modified from Cerminara *et al*., 2015.

The granular layer lies in the inner part of the cortex and contains a high density of small granule cells (GCs; soma diameter 5 to 8 μm); these glutamatergic neurons relay the input from mossy fibres (described below) to Purkinje cells. In the granule cell layer three different types of interneurons are present, two inhibitory (GABAergic), Golgi and Lugaro cells, and the excitatory (glutamatergic) unipolar brush cell (Mugnaini *et al.,* 2011; Cerminara *et al.*,

2015). The Golgi cell receives inputs from both GCs and mossy fibres, and projects back to the mossy fibre-GC synapses as an inhibitory feedback loop.

The middle Purkinje cell layer is formed by a monolayer of PC somata interposed between the inner granular layer and the superficial molecular layer. Given that PCs are the sole output of the cerebellar cortex, they are of key importance to cerebellar cortical information processing (Apps & Garwicz, 2005). From the PC soma one or two main dendrites emerge to give rise a highly arborized dendritic tree (up to multiple tertiary dendrites) entirely contained in the molecular layer. Oriented parasagittally and perpendicular to the long axis of the lobule it spans 300-400 μm in width, and only 15-20 μm deep (transversal plane; Palay & Chan-Palay, 1974). Each PC receives up to 200,000 synaptic contacts from granule cell axons - parallel fibres- onto long-necked spines (Ramnani, 2006), which are restricted to the distal dendrites (Leto *et al.*, 2015). It also receives input from a single CF (Sugihara, 2006), which makes numerous synapses on short, stubby spines that are restricted to the primary dendrites and the apical part of the soma (Sotelo & Rossi, 2013).

The PC axon arises from the basal portion of the soma and becomes myelinated immediately after its initial segment. It traverses the granular layer to innervate, with GABAergic synapses, DCN neurons. Within the granular layer the PC axon projects several collaterals that terminate on other PCs, Golgi cells, Lugaro cells and basket cells (Ruigrok *et al.*, 2015) in plexuses which lie just above and below the PC somata (Palay & Chan-Palay 1974).

Purkinje cells form a heterogeneous population of neurons according of their expression of certain marker proteins, which are restricted in clusters throughout the cerebellar cortex and divides the cerebellum into "compartments" (Herrup & Kuemerle, 1997; Apps & Hawkes, 2009). Histological techniques have revealed stripe (or band)-shaped expression patterns of several marker proteins along the rostrocaudal axis of the cerebellum, including 5'-nucleotidase (Scott, 1963), acetylcholinesterase (Marani & Voogd, 1977), Zebrin I and II (Hawkes *et al.*, 1985; Herrup & Kuemerle, 1997), excitatory amino acid transporter 4 (EAAT4; (Dehnes *et al.*, 1998), integrin β1 (Murase & Hayashi, 1996), and early B-cell factor 2 (EBF2; Croci *et al.*, 2006). Among these markers the most studied is Zebrin II, which corresponds to the glycolytic enzyme aldolase C (Lang *et al.*, 2016). Its expression is restricted to parasagittal compartments of PCs alternated with PCs that do not express Zebrin II, therefore forming Zebrin-positive and Zebrin-negative stripes (nomenclature introduced by Hawkes & Leclerc, 1987) (**Figure 1.5B and 1.7**). Moreover, within zebrin bands, subsets of PCs can express other molecular markers along the transverse plane, subdividing the

cerebellar cortex into even finer stripes. For example heat shock protein 25 and human natural killer antigen 1 are expressed within different zebrin+ stripes (Armstrong *et al.*, 2000; Marzban *et al.*, 2004). This further subdivision reveals the complexity of PC heterogeneity, and depending on the labeling of different molecular markers is how boundaries between PCs subpopulations are defined.

This banding arises from the settlement of PC subpopulations in the cerebellar plate early during development. Shortly after PCs differentiate in the ventricular zone (VZ) they migrate towards defined positions forming specific subpopulations that disperse after birth to form the stripe-shaped compartments (Fujita *et al.*, 2012; Arancillo *et al.*, 2015).

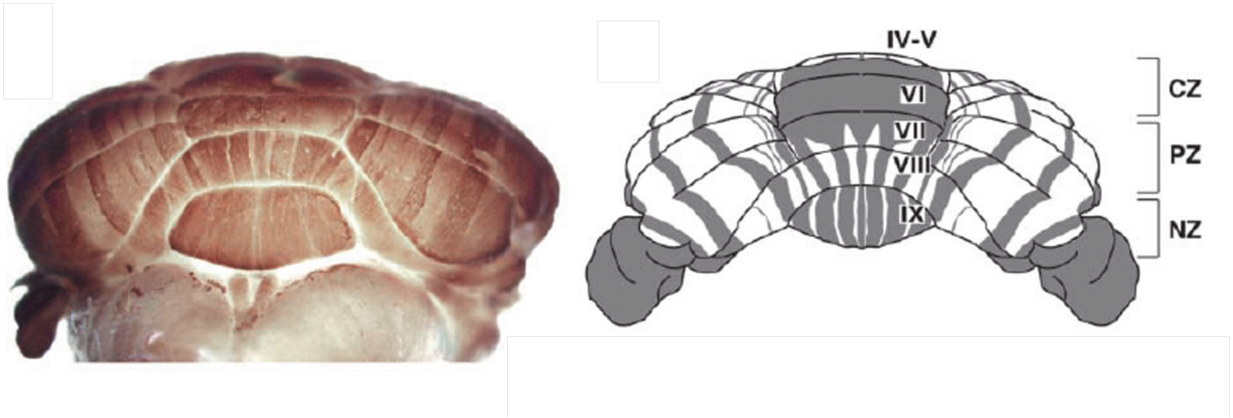

Figure 1.7: Parasagittal zones in the cerebellar cortex. Left panel, distribution of Purkinje cells in the adult mouse cerebellum showing alternated immunoreaction to Zebrin II (seen from the posterior view). Right panel, schematic representation of the Zebrin II-positive stripes (grey) and negative-stripes (white). Cerebellar vermis lobules (from IV to IX) and transverse zones (central zone: CZ; posterior zone: PZ; nodular zone; NZ) are represented. Modified from Sillitoe & Hawkes, 2002.

The molecular layer is the outermost layer of the cerebellar cortex and contains the major components of the cerebellar cortical circuit. Within the molecular layer there is a small population of two GABAergic interneurons, basket and stellate cells, but it mainly consists of neuronal processes: Purkinje cell dendrites, granule cell axons and CF terminals (described below). Purkinje cells extend their dendritic trees through this layer. GCs axons ascend vertically into the molecular layer where they bifurcate (T shape) and run horizontally parallel to the long axis of the lobules as parallel fibres (PFs). As they run perpendicular to the PC dendrites they form *en passant* synapses with approximately 300 PCs (Eccles *et al.*, 1967), and the inhibitory interneurons. Golgi cell dendrites are also projected from the granular layer into the molecular layer where they receive PF synapses.

II.1.2- Deep cerebellar nuclei

The deep cerebellar nuclei are the sole output of the cerebellum to the rest of the central nervous system. They are located in the deep cerebellar white matter and are composed of 4 pairs: medial cerebellar (fastigial) nucleus, anterior and posterior interposed nuclei and the lateral cerebellar (dentate nucleus; Ruigrok, 2013). These nuclei present a non-homogeneous population with inhibitory (GABAergic) neurons that project to inferior olivary neurons (ION), excitatory (glutamatergic) neurons projecting mainly to the thalamus, red nucleus and other brainstem nuclei, and a special population of glycinergic neurons, co-expressing GABA, that are believed to represent inhibitory interneurons (Chen & Hillman, 1993; Sultan *et al.*, 2003). DCN neurons receive mainly PC axons, but also collaterals from the mossy fibres and inferior olivary axons.

II.1.3- Cerebellar afferents

The cerebellum receives two major types of extracerebellar afferents, the mossy fibres and the olivocerebellar (climbing fibre) axons, and a third monoaminergic afferent, which is a fine network of serotonergic, noradrenergic, acetyl cholinergic fibres, derived from raphe nuclei reticular formation and locus coeruleus (Ito, 2006). These fibres run through granule and molecular layers to contact PCs, and are considered as modulatory system (Ruigrok *et al.*, 2015). On the other hand, both mossy and climbing fibres bring sensorimotor information, which is integrated by the PCs in the cerebellar cortex. As these two inputs interact through the PC, it is postulated that the cerebellar cortex and specifically the PCs are the region where cerebellar learning/decisions takes place.

II.1.3.1- Mossy Fibres

The mossy fibres (MFs) are derived from multiple sources in the brainstem and spinal cord (Ruigrok, 2011) but their terminals all share similar morphological features. After traversing the cerebellar peduncles these myelinated axons reach the deep cerebellar white matter and emit collaterals to the DCN and the granular layer. In the granular layer MF terminals form excitatory (mostly glutamatergic) synapses with dendrites of granule cells and with Golgi cells, this specialized synaptic complex in its glial sheath is called a glomerulus. In this manner, the mossy fibres input to PCs indirectly, being relayed by GCs and their PF axons in

the molecular layer. The activation of the MF-GC-PF pathway generates the discharge of an action potential in the PCs called "simple spikes" (Llinás, 2014), which occurs at a high frequency (Miall *et al.*, 1998).

II.1.3.2- Climbing Fibres

The second major cerebellar afferent are the CFs, which arise exclusively from inferior olivary neurons (Desclin, 1974) and are described in detail in the following section.

II.2- Inferior olivary nucleus

In mice, inferior olivary nucleus (ION) neurons are grouped in a pair of symmetric nuclei located each side of the midline in the ventrocaudal medulla (Azizi, 2007). Each nucleus is composed of three main sub divisions: the principal olive (PO) and the medial and dorsal accessory olives (MAO and DAO respectively Voogd, 2014), plus several smaller sub nuclei such as the dorsal cap of Kooy, ventrolateral outgrowth, beta-nucleus and dorso medial cell column (**Figure 1.8 A**; Ruigrok *et al.*, 2015). In general, each olivary subnucleus projects to one or more discrete longitudinal zones in the cerebellar cortex (**Figure 1.8 B**; Azizi & Woodward, 1987; Ruigrok & Voogd, 2000).

Inferior olivary neurons possess distinctive morphological features that make them unique. They can be classified into two types of neurons according to their dendritic pattern; the main type possesses highly branched dendrites, which curl back towards the soma, while the second type presents a rather simple dendritic arborisation with long main dendrites radiating long distances from the soma (De Gruijl *et al.*, 2013). In mice both type of neurons are present in all the subnuclei. Additionally, ultrastructural analysis of olivary dendrites reveals the presence of dendritic spines, which are electronically coupled between inferior olive neurons through gap junctions formed by Connexin36 (Cx36; De Zeeuw *et al.*, 2003; Llinás, 2014). Dendritic terminals from different olivary neurons are grouped together within a glial sheath forming an inferior olivary glomerulus (De Zeeuw *et al.*, 2011). These glomeruli are present throughout the inferior olive and receive both excitatory and inhibitory afferents (reviewed in De Zeeuw *et al.*, 1998; Llinás, 2014). The gap junction-mediated electronic coupling of IO neurons in the glomerulus is thought to synchronize oscillations among neighbouring olivary neurons (Devor & Yarom, 2002; Schweighofer *et al.*, 2013) leading to the synchronous activity in connected Purkinje cells (Lang *et al.*, 1999). However this

function needs to be revisited since recent work from De Zeeuw's group did not find changes in PC synchrony (Kistler *et al.*, 2002) in the absence of functional gap junctions (connexin36 null-mutant mice). Using computational modelling they suggest that olivary gap junctions may integrate synaptic input rather than synchronize the neuronal output (Kistler & De Zeeuw, 2005).

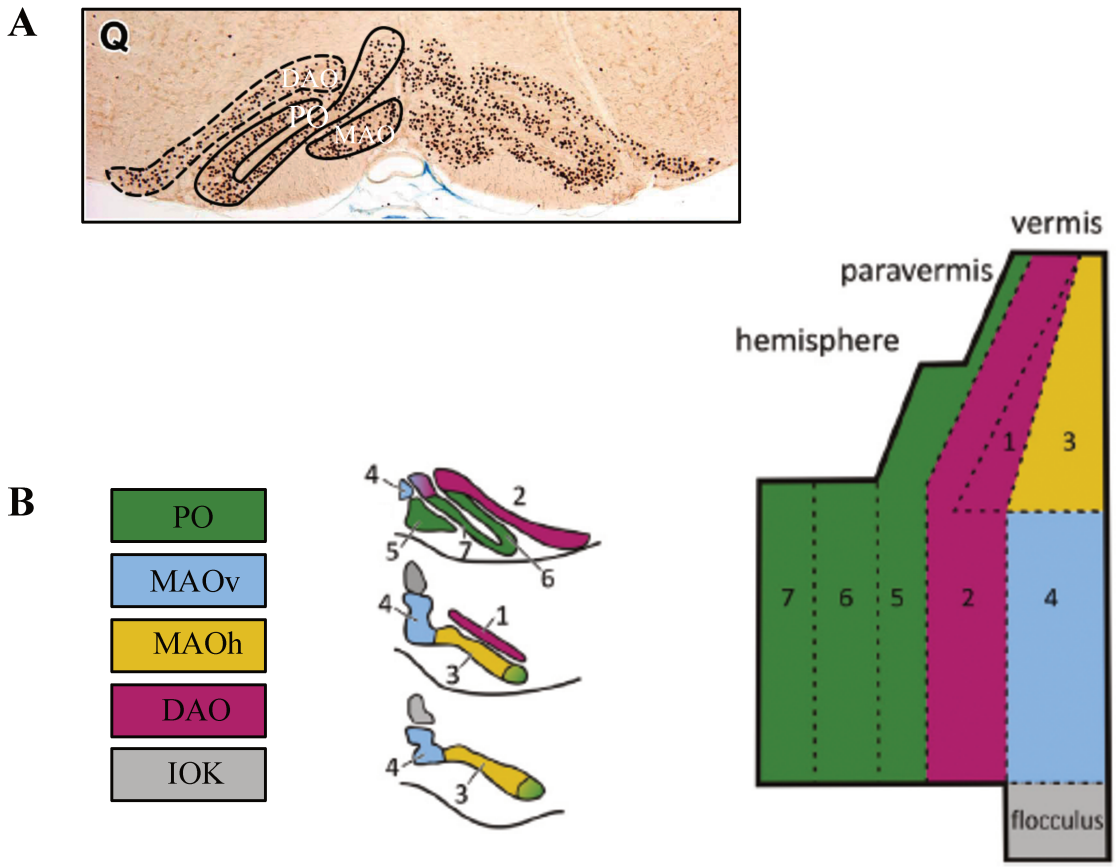

Figure 1.8: Olivary subdivisions and olivocerebellar organization. (A) Photomicrograph of FoxP2 staining in coronal sections of a 14 months old rat brainstem showing the major subnuclear division of the inferior olivary nucleus: dorsal accessory olive, DAO; principal olive, PO and the medial accessory olive, MAO. Note that the Dorsal Cap of Kooy (IOK) is not present in this section. (B) Simplified diagram of the topographic organization of the olivocerebellar pathway as described originally by Azizi & Woodward (1987) and complemented by Ruigrok *et al.*, (2015). MAOv, MAO vertical; MAOh, MAO horizontal. Modified from (A) Fujita & Sugihara, 2012; (B) Ruigrok *et al.*, 2015.

II.2.1- Afferents to the ION

The ION receives excitatory and inhibitory inputs from a variety of afferents. Excitatory afferents bring sensory and motor signals from several sources such as trigeminal nuclei,

dorsal column nuclei, pretectal complex, and red nuclei, as well as a collection of nuclei from the mesodiencephalic junction (De Zeeuw & Ruigrok, 1994; De Zeeuw *et al.*, 2011). In addition, GABAergic terminals from the DCN contact olivary neurons, which modulate the efficiency of electronic coupling (Sotelo *et al.*, 1986; Medina *et al.*, 2002), and thus secondarily synchronous activity of Purkinje cells (Lang, 2002) as a direct negative feedback loop (see below).

II.3- Inferior Olive output, the OCP

The total outflow of each inferior olive projects to the contralateral hemicerebellum as the olivocerebellar path. Already in the late 19th century, Ramón y Cajal (1890) defined projections "innervating", i.e. terminating upon, Purkinje cells in the cerebellar cortex, as climbing fibres. Nowadays we know that the only source of cerebellar CFs are inferior olivary (IO) neurons (Desclin, 1974; Sotelo & Chédotal, 2005). Their axons cross the midline passing through the opposite nucleus, ascend the lateral edge of the medulla to enter the cerebellum through the inferior cerebellar peduncle (Sotelo & Chédotal, 2005; De Gruijl *et al.*, 2013) .
Axons ramify in the deep white matter into thick branches and thin collaterals (Sugihara *et al.*, 1999). In adult rodents a single olivocerebellar (OC) axon produces about seven thick branches, each ascending through the granular layer to terminate as CFs on single Purkinje cells in a one-to-one relationship (Sugihara *et al.*, 1999, 2001). Thin olivary axon collaterals project mainly to the cerebellar nuclei (van der Want *et al.*, 1989), and also to the granular layer where they terminate on Golgi interneurons (Scheibel & Scheibel, 1954; Shinoda *et al.*, 2000), although proper synaptic contacts between them have still not been conclusively shown (Galliano *et al.*, 2013). This well-defined organization at cellular level builds up a precise connectivity between the inferior olive, cerebellar cortex and DCN. Olivary axons send collaterals to contact neurons in the deep cerebellar nuclei, which receive inhibitory synapses from Purkinje cells in the sagittal zone innervated by the same cluster of olivary neurons (Ruigrok & Voogd, 1990). A subset of these contacted DCN neurons, mainly GABAergic (Uusisaari *et al.*, 2007), in turn project back to the initial cluster of olivary neurons, forming a closed olivo-Purkinje-nuclear-olivary loop (De Zeeuw *et al.*, 1998; Sotelo & Chédotal, 2005).

Each CF forms around 150-300 synaptic contacts along the proximal dendritic branches of the innervated PC (Shinoda & Sugihara, 2013). As a result, the activation of a CF generates a

strong all-or-none excitatory response in the PC soma (Sugihara, 2006). The "complex spike" response (CS; Davie *et al.*, 2008) occurs relatively infrequently (~1 Hz) in contrast to the sustained high frequency of the simple spikes (10-200 Hz; Warnaar *et al.*, 2015). The CSs are the result of somatically generated Na^+ currents followed by dendritic Ca^{2+} spikes (Weber *et al.*, 2003) that propagate and cause Ca^{2+} influx from proximal to most distal portion of the dendritic tree, including the terminal spines where PF synapses are located (Najafi & Medina, 2013). Through the dendritic Ca^{2+} conductance, CFs exert a strong effect on PF-PC synapses by decreasing their synaptic strength (Tanaka *et al.*, 2007; reviewed in Gao *et al.*, 2012). This CF-dependant long-term depression (LTD) of PF-PC synapses has been described for more than 30 years (Ito & Kano, 1982). Nowadays it is also known that CFs can trigger long-term modulation in molecular layer interneuron synapses (Kano *et al.*, 1992; Mittmann & Ha, 2007), and in the CF synapse itself (Bosman *et al.*, 2008). The exact functional significance of this synaptic modification remains unknown, but they are thought to operate synergistically at a defined time and within a defined cerebellar area to create optimal PC output for behaviour (Gao *et al.*, 2012).

II.3.1- Topography of the OCP

In order to modulate specific PC activity for a given behaviour the OCP is organized in longitudinal zones through the cerebellar cortex forming a precise topographic network, which has gradually been revealed over the last forty-years (Voogd, 1967; Andersson & Oscarsson, 1978; Azizi & Woodward, 1987; Buisseret-Delmas & Angaut, 1993; Sugihara *et al.*, 2001; Apps & Hawkes, 2009). In the rodent OCP, particular sub-nuclei within the inferior olive project their axons to cerebellar Purkinje cells specifically distributed in a narrow longitudinal zone (200-300 μm wide in rats) in the same or different cerebellar lobules (**Figure 1.9**; Sugihara *et al.*, 2001; Sugihara & Shinoda, 2007). Nearby sub-nuclei of olivary neurons do not necessarily project to adjacent narrow longitudinal zones, thus resulting in a characteristic discontinuous topographic projection with sharp boundaries (**Figure 1.10 A-B**). Electrophysiological mapping from parasagittal bands has shown that the topographic organization of narrow longitudinal band is a functional domain (Fukuda *et al.*, 2001). In this small functional unit, called microzone (Oscarsson, 1979), PCs have similar responsiveness in their complex spike activity that is thought to reflect the synchronized activity of neighboring IO neurons through their electronic coupling (Fujita & Sugihara, 2013). The longitudinal

microzone organization is thought to be present throughout the cerebellum and to represent the basic operational unit of the cerebellar cortex (Apps & Hawkes, 2009).

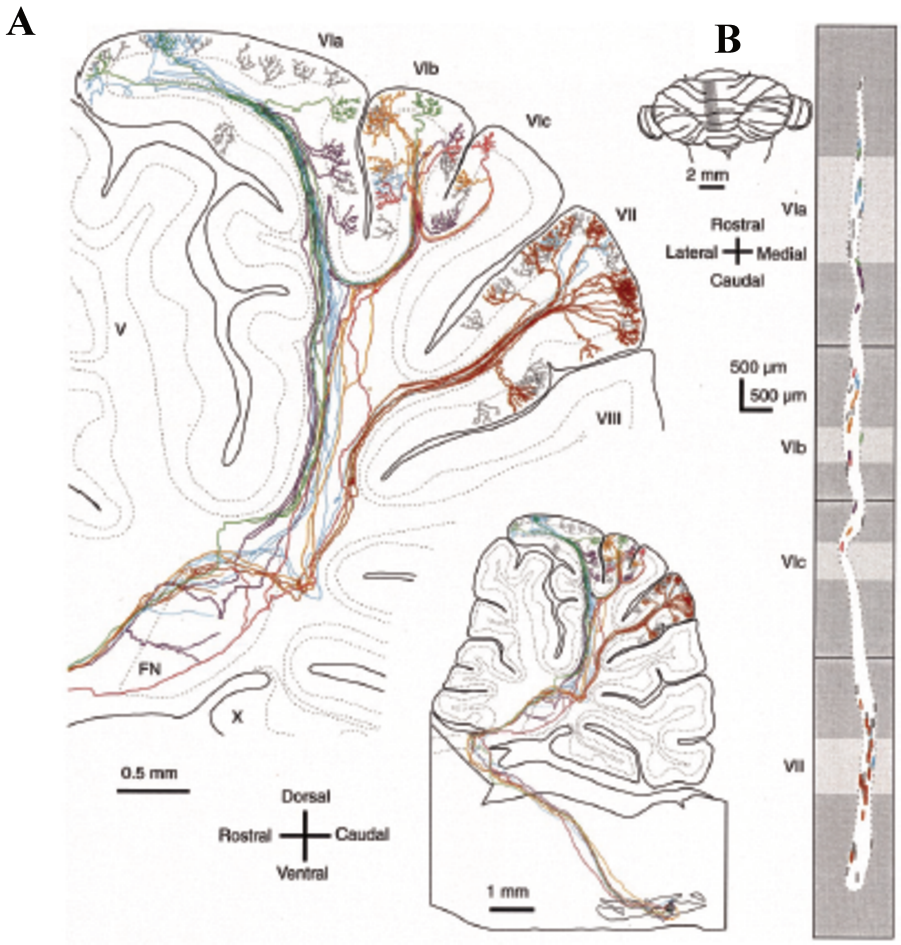

Figure 1.9: Longitudinal organization into microzones of olivocerebellar pathway. (A) Anatomical reconstruction of six olivary axons (represented in different colours) originated from the same sub olivary nuclei. Their terminal climbing fibres project to the cerebellar cortex in a narrow longitudinal band of around 200 µm of width, which define an anatomic microzone. (B) Unfolded view of the innervated cerebellar vermis. Extracted from Sugihara *et al.*, 2001.

Additionally, the OCP topography is closely correlated with the longitudinal compartmentalisation of heterogeneous PCs in the cerebellar cortex. Detailed mapping using both zebrin II- expression pattern as a template and different axonal tracers have shown that CFs projecting to the narrow longitudinal zones are aligned within the zebrin II stripes (**Figure 1.10 A-B**; Voogd *et al.*, 2003; Sugihara & Shinoda, 2004). This correlation between topography and molecular heterogeneity of PCs has been suggested to be depended on the target PCs, acting as a scaffold that instruct the organization of climbing fibres during development to generate longitudinal zones (Apps & Hawkes, 2009; "matching hypothesis"

detailed in Section **II.3.3.2**).

Furthermore, Sugihara and Shinoda (2007), have demonstrated that the PC compartmental organization applies to both olivo-PC and cortico-nuclear projections. Axons from PCs restricted to either zebrin II positive or negative-stripes in the cerebellar cortex terminate in compartmentalized subregions of the DCN separated from each other as zebrin II-positive or negative groups. Strikingly, collateral projections from small subsets of OC axons terminate within a DCN sub-region, which overlaps with the corticonuclear projection from PCs zones that in turn receive climbing fibres from the same subset of OC axons (**Figure 1.10 C**). This interconnected olivo-cortico-nuclear topography between defined nuclear compartments form a functional unit in the cerebellum (Sugihara & Shinoda, 2007) and probably represent the morphological correlate to the functional unit of the "corticonuclear microcomplex" proposed by Ito (1984).

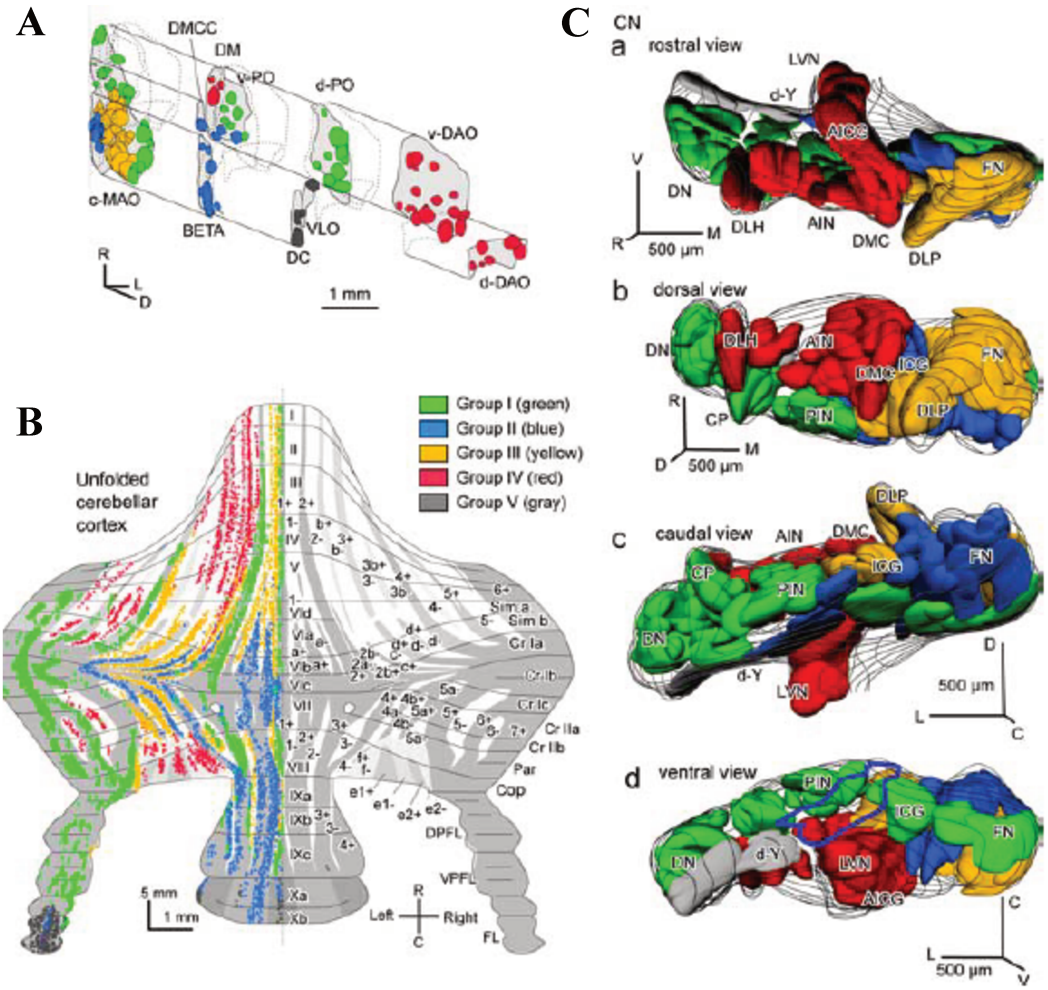

Figure 1.10: Compartmentalization of olivo-cortico, olivo-nuclear and cortico-nuclear topography. Five groups of individual subnuclei of the inferior olive (A; in colours) project to zebrin II positive or negative stripes in the cerebellar cortex (B; grey colour) and to defined areas of the deep cerebellar nuclei (C). Zebrin-positive Purkinje cell axons projects to deep cerebellar nuclei areas innervated by collaterals of the same climbing fibres from which they are contacted. Extracted from Sugihara & Shinoda, 2007.

II.3.2- Function of the OCP

Knowledge about the structural organization and electrophysiological properties of the OCP have supported two main hypotheses regarding the function of this tract. In general, 1) the motor timing hypothesis (Llinás, 2011) suggests that groups of olivary neurons fire rhythmically and synchronously with a heterochronic conduction velocity, thus the signal reaches at the same time the target PCs irrespective of their position (i.e. PCs are synchronously activated; Llinás, 2014), providing accurate timing of signals for proper motor coordination (De Zeeuw *et al.*, 1998). 2) The motor learning hypothesis proposes that the olivary neurons transmit error signals (ascending sensory information about inaccurate motor performance) to Purkinje cells leading to a change of the parallel fibre-induced activity, which in turn could correct the motor error (reviewed in De Zeeuw *et al.*, 1998; Llinás, 2011; Schweighofer *et al.*, 2013).

Currently, both hypotheses are still a matter of debate but given that they do not exclude each other (Sotelo & Chédotal, 2005) there is an increasing support of the idea that the OCP is involved in both motor learning and motor control function of the cerebellum (Lang *et al.*, 2016).

II.3.3- Development of the OCP

II.3.3.1- Neurogenesis

During brain development, the first neurons of the OCP to be produced are the inferior olive, followed by the DCN and later on the PCs (and cerebellar interneurons). The rodent inferior olive neurons are generated from the most caudal part of the germinal neuroepithelium adjacent to the roof plate of the fourth ventricle called the rhombic lip (Sotelo & Chédotal, 2005; Hidalgo-Sánchez *et al.*, 2012). In the rat, they differentiate on embryonic day 12 and 13 (E12 and E13; Bourrat & Sotelo, 1988, 1991), and migrate ventrocaudally through a specific route along the lateral edges of the brainstem in a sub marginal stream (**Figure 1.11**; Sotelo & Chédotal, 2005). In common with other precerebellar neurons, olivary neurons actively extend their axon during the migration process (Bourrat & Sotelo, 1988). Between E16 and E17 the somata of inferior olivary neurons arrive at their final destination adjacent to the floor plate. Although the cell bodies do not cross the midline, their axons do (already by E15; Sotelo & Chedotal, 2005), and by E16-E17 they have entered the contralateral cerebellar plate through the inferior peduncle (Wassef *et al*., 1992). The restriction of olivary neurons to the

ipsilateral side of the floor plate with regard their origin, is controlled by chemo-repellent cues released in this region (Bloch-Gallego *et al.*, 1999; Causeret *et al.*, 2002; Marillat *et al.*, 2004; Renier *et al.*, 2010) .

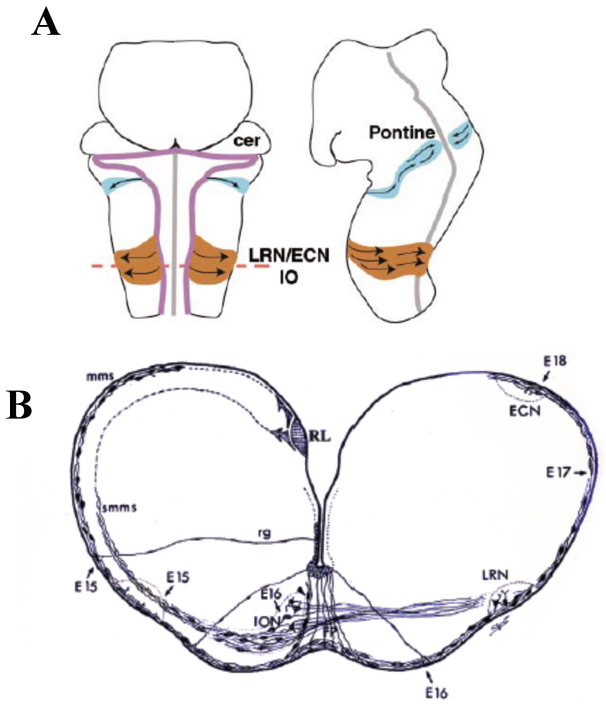

Figure 1.11: Schematic representation of migratory pathways of precerebellar neurons. (A) Dorsal (left drawing) and lateral (right drawing) view of rat developmental hindbrain. Precerebellar neurons migrate from rhombic lips ventrally and tangentially to radial glia toward the floor plate in specific migratory route. (B) Transverse section view showing the deeper pathway of inferior olivary neurons in the submarginal stream compared to superficial migration of the other precerebellar neurons. Extracted from (A) Marillat *et al.*, 2004; (B) Sotelo & Chedotal, 2005.

On the other hand, cerebellar neurons derive from two distinct germinal neuroepithelia, the rostral rhombic lip -originates glutamatergic neurons- and the ventricular zone –produces GABAergic neurons-. The rostral half of the rhombic lip generates a secondary germinal zone called the external granular layer (EGL), which contains the precursors of the GCs (GCPs; Ruigrok *et al.*, 2015). The GCPs proliferate, and then from E13 (mouse) migrate tangentially over the surface of the cerebellar primordium. Soon after birth, GCPs begin to produce postmitotic GCs that radially migrate ventrally through the PC plate to the internal granule cell layer. The EGL continues to generate new GCs and reaches a proliferation peak at P8 in mice (Butts *et al.*, 2013), which ceases completely by P15, and their migration ends by P20 (reviewed in Sotelo, 2004).

The ventricular zone is the origin of all the cerebellar GABAergic neurons (Hoshino *et al.*, 2005). The DCN are born from E11 to E13 and are the first neurons that migrate into the cerebellar plate by E13 (Elsen *et al.*, 2013) moving in a rostro-medial direction along a subpial stream to reach the depth of the cerebellar primordium. PCs are produced slightly after the DCN by E11-E13 (Sotelo, 2004). They migrate dorsally toward the pial surface of the cerebellar anlage between E14 and E17 (Sotelo & Rossi, 2013). During this “ascent”,

which is thought to be mediated by radial glial processes (Altman & Bayer, 1997), PCs leave their axon terminals in the DCN establishing synaptic contacts before birth (Eisenman *et al.*, 1991). Once PCs terminate their migration close to the pial surface of the cerebellar plate they aggregate in clusters expressing specific molecular markers (Armstrong & Hawkes, 2000), such as En-1, En-2, PLCβ4 and EphA4 (Millen *et al.*, 1995; Fujita *et al.*, 2012). This already heterogenic PC subpopulations form evident embryonic compartmentalization in the mouse cerebellar primordium by E17.5 (Millen *et al.*, 1995; Fujita *et al.*, 2012). During the first postnatal week these defined clusters disperse along the rostro-caudal axis to form the characteristic mono-cellular alignment arranged in parasagittal zones; a process mediated by reelin signalling (Apps & Hawkes, 2009).

II.3.3.2- Olivocerebellar map formation: Matching hypothesis

Shortly after forming clusters, target PCs are innervated by precerebellar afferents (Armstrong & Hawkes, 2000). Olivocerebellar axons reach the rat cerebellum at around E17 and already contact PCs by the end of E19 (Chedotal & Sotelo, 1992; Morara *et al.*, 2001). By P0-P5, CFs topography strongly overlaps with the nascent PC stripes (Reeber *et al.*, 2013). This early termination of CFs over restricted PC subpopulations (clearly revealed during adult state) suggests an active role for PC in defining the pattern of the olivocerebellar map.

Sotelo and colleagues have postulated that the development of the olivocerebellar topography is directed by positional guides cues (chemospecific interactions) between biochemically heterogeneous subpopulation of IO neurons and PCs (Sotelo, 2004; Sotelo & Chédotal, 2005). This statement called the "matching hypothesis" has different supporting evidence. Firstly, cortical compartmentation of molecularly heterogeneous PCs is a cell-autonomous process and does not depend on the afferent olivocerebellar fibres (Wassef *et al.*, 1990; Oberdick *et al.*, 1993). Second, IO neurons already present molecular expression domains before targeting their cerebellar territory in its stripe-like formation (Chedotal & Sotelo, 1992); and third, the topographic organisation between IO neurons and PCs compartments during development is congruent with the adult olivocerebellar map (Wassef *et al.*, 1992). Even though the identification of genes controlling the matching process has been elusive until now, potential candidates have been suggested based in their expression pattern in the OCP. For example, members of adhesion molecules families such as BEN, type-II classic cadherin and δ-protocadherin, are expressed in longitudinal domains of PC subpopulations, and in subnuclei of the inferior olive (see Sotelo, 2004; Reeber *et al.*, 2013). Additionally, the system of Eph

receptors/Ephrin ligands has been suggested as candidate molecules involved in the organization of olivocerebellar topography (Sotelo & Chédotal, 2005). EphA receptor and Ephrin A are compartmentalized in the OCP (EphA3, EphA4, ephrin-A2 and ephrin A5 are expressed in subset of PCs, and EphA2 and A4 in the inferior olive), and their altered expression disrupts the targeting of olivocerebellar projections (ephrin-A2; Nishida *et al.*, 2002). On the other hand activity dependent processes are related with the refining of the OCP and not in the formation of the olivocerebellar topography (Apps & Hawkes, 2009). Moreover, Rekling and co-workers (2012) have recently found in postnatal mouse brain slices that clusters of inferior olive spontaneously generate synchronous Ca^{2+} transients. Further studies need to be done in order to elucidate a role of neuronal activity in the development of the olivocerebellar compartmentation.

II.3.4- Postnatal organization and remodelling of CFs- PCs interaction

Once the olivocerebellar axons establish the primitive zonal map, both synaptic partners (CFs and PCs) undergo postnatal morphological changes until they reach the functional mature circuit.

II.3.4.1- PCs differentiation

From the end of the migration phase (E18-E19) until the day of birth (P0) the target PCs show a bipolar and elongated shape recognized as "simple-fusiform cells" phase (**Figure 1.12**; McKay & Turner, 2005). At this phase CFs make the first transient contacts with PCs (Chedotal & Sotelo, 1992; Morara *et al.*, 2001). At P0 PCs are arranged in a plate 6 to 12 cells deep (Altman & Bayer, 1985), and show a branched apical dendrite ("complex-fusiform" phase). From P2 to P4, PCs become non-polarized by pruning their long dendritic processes ("regressive-atrophic dendrites" phase), and continuing to grow perisomatic protrusions that emerge in all directions ("stellate cells with disoriented dendrites" phase). During this phase PCs begin to line up in a monolayer that is almost complete by P5. Around P6 a large apical dendrite begins to develop, while lateral and basal perisomatic processes are rapidly withdrawn. To compensate the loss of lateral processes numerous spine-like pseudopodia emerge from the soma and contact with immature CFs arbors (Chedotal & Sotelo, 1992). Despite the irregular shape of PCs at this stage, they remain polarized with a short apical extension, and from P6 to P10 is the period where the final polarity of these neurons is built. By P10 PCs show again a polarized morphology with the apical dendritic tree vertically

oriented into the molecular layer and absorption of the fine perisomatic processes. At P15 the elaborated and complex dendritic tree reaches the adult width, and the PC soma is synaptically mature (Leto *et al.*, 2015). From now on the dendritic growth plane follows a vertical orientation, significantly extending its height until P30 (Sotelo, 2004)

Intrinsic and extrinsic factors have been suggested to modulate the development of the PC dendritic tree. The initial growth and shaping of the PC dendrites seems to be regulated by a cell-autonomous mechanism, as purified cultures of PCs have shown *in vitro* to develop dendrites and even pruning-like process in absence of afferents or other cerebellar neurons (Baptista *et al.*, 1994). Moreover in mutant mice devoid of GCs (staggerer and weaver) differentiation of Purkinje cells proceeds normally until P7 (Sotelo & Dusart, 2009). During late phase of PC development different extrinsic factors have been identified, which shape their dendrites and dendritic tree. Among them, synaptic interactions with afferent (mainly PFs), trophic factors (NT-3, NT-4, BNDF), thyroid hormone and sex hormones (progesterone and estrogen) are main regulators of the orientation and growth of mature PC dendritic trees (see Kapfhammer, 2004; Sotelo & Dusart, 2009). Synaptic interaction with CFs is thought to be important in the sculpture of the ultimate dendritic arbors (Sotelo, 2004) .

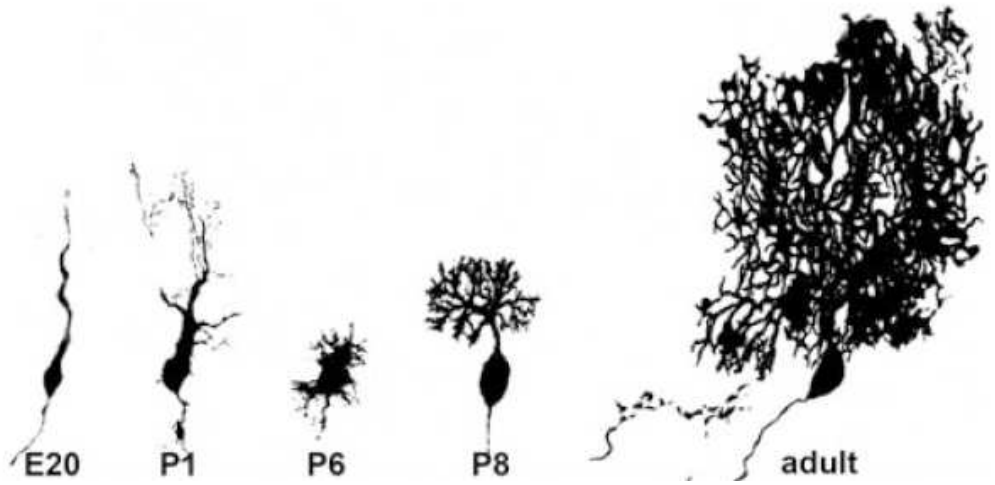

Figure 1.12: Morphological development of the rat cerebellar Purkinje cell. Before birth, early changes to the dendrites initiate progressive changes from a simple spindle shape until reach the characteristic multi-branched adult state, is reached around second to third postnatal week.

II.3.4.2- CF refinement

Simultaneously to developmental PC differentiation, the immature PC is being innervated by multiple CFs. The process of CF-PC synaptic maturation involves extensive remodelling of

the afferents before reaching the mature one-to-one relationship. This postnatal remodelling of CFs undergoes four major successive stages, i.e. the “creeper” stage (Chedotal & Sotelo, 1993), the “pericellular nest" stage, the “capuchon” stage, and the “dendritic” stage (Ramon y Cajal, 1911). Once reaching immature PCs around P0, very thin CFs creep over PC perikarya and extend unbranched terminal arbours around their somatic processes (“creeper” stage; Sugihara, 2006; **Figure 1.13)**. By P3 these creeper-type CFs form a few synaptic contacts on PCs, which are already functional, and express the type 2 vesicular glutamate transporter (VGLUT2; Watanabe & Kano, 2011). At this stage PCs are innervated by multiple CFs, each of which induce excitatory postsynaptic currents (EPSCs) with a longer duration and smaller synaptic amplitude compared to mature PCs (Hashimoto & Kano, 2003).

Before forming the “nest” stage CFs massively retract their terminal arbours (Sugihara, 2006). Initially, each olivocerebellar axon presents around 100 creeper-type CFs, but by P5 only about 10 develop into a nest-like structure around PC somata aligned in a monolayer (“pericellular nest” stage; **Figure 1.13**). The pericellular nest comprises several CFs multi-innervating each target PC, on average 4 CFs per PC (Crepel *et al.*, 1976; Mariani & Changeux, 1981) although electrophysiologically up to 6CFs/PC can be identified (Uesaka *et al.*, 2014).

During the “capuchon” stage (~P9) CFs are progressively displaced onto the main stem of the developing PC dendritic tree; reorganizing the CF innervation-site from perisomatic to peridendritic position (Watanabe & Kano, 2011). At this stage there is a major reduction in the number of CFs innervating each PC, with only about 50% of PCs remaining multi-innervated (mainly by 2 CFs; Kano *et al.*, 1995).

After P12, the perisomatic CF synapses virtually disappear and the CFs climb along the developing PC dendrites (“dendritic” stage; Sugihara, 2005). Because of the vertical growth of the PC dendrites into the developing molecular layer, the height of CF terminal arbors increases strikingly (**Figure 1.13**; Sugihara, 2006). At this stage the last important regression of CF multi-innervation of PCs takes place to finally reach the mature one-to-one relationship (Crepel *et al.*, 1976; Hashimoto & Kano, 2003).

The CF-PC synapse refinement during development is crucial for the proper function of the OCP. Behavioural motor deficits due to the persistent multiple innervation of mature PCs by CFs have been shown in a variety of transgenic/mutant mice with altered elimination of supernumerary CFs. Three of these animal models have revealed an important role for GCs (*weaver* and *reeler* mutant mice; Crepel *et al.*, 1976; Mariani *et al.*, 1977; Puro & Woodward, 1977) and PF-PCs synapses (*staggerer* mutant mice; Mariani & Changeux, 1980; Mariani,

1982) in the refinement of CFs synapses on PCs. Moreover several molecules regulating postsynaptic pathways have been identified to mediate the CF-PCs synapses elimination, including glutamate receptor GluRδ2 (Kashiwabuchi *et al.*, 1995), metabotropic glutamate receptor mGluR1 (Kano *et al.*, 1997), PLCβ4 (Kano *et al.*, 1998), PKCγ (Kano *et al.*, 1995), Sema3A-7A (Uesaka *et al.*, 2014) and most recently presynaptic downstream effector such as C1ql1 (Kakegawa *et al.*, 2015; Sigoillot *et al.*, 2015) (for molecular details see, Watanabe & Kano, 2011).

In combination to this molecular blueprint, PC electrical activity seems to have a role in the elimination of redundant CFs. During development N-methyl-D-aspartate (NMDA)-receptor is transiently expressed in PCs, and poorly expressed in GCs. Rabacchi and co-workers (1992) have shown that pharmacologically blocking NMDA-receptor in the rat cerebellum *in vivo* impairs the regression of multiple CFs synapsing on PCs. Even tough this observation was confirmed by Kakizawa and collaborators (2000), further experiments are need to identify properly the neuron-type with blocked NMDA-receptor.

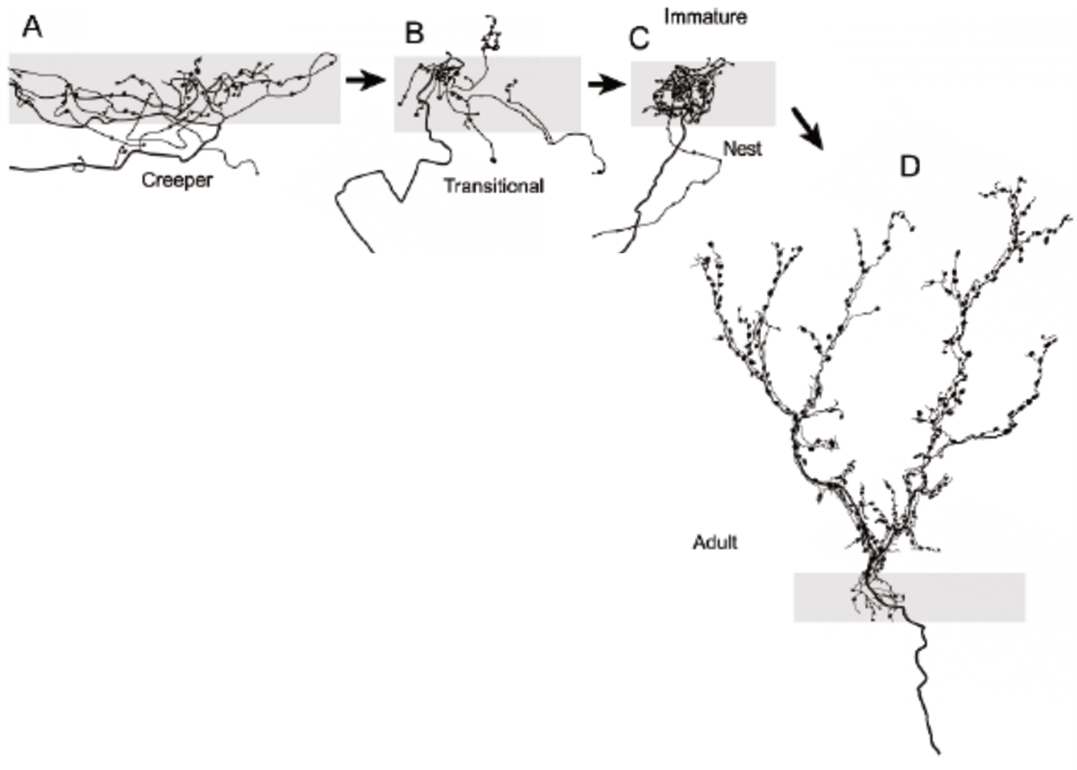

Figure 1.13: Diagrams of developmental remodelling of climbing fibre morphology. Drawings of climbing fibres from P5 (A) until adult state (D) show the dramatic rearrangement of their structure before reaching the final adult state. Grey zones represent the Purkinje cell layer. Modified from Sugihara, 2006.

III- Structural plasticity in the mature rodent OCP following lesion

As discussed in section I, in the mammalian central nervous system the intrinsic response of afferent neurons following injury is critical to determine the extent of circuit repair. It has been established that this response is neuron-type and developmental state-dependant; and in some cases, it can be modulated extrinsically with growth-related molecules (e.g. neurotrophic factors). Despite this knowledge important questions remain to be answered: To which extent these determinants can influence each other in order to boost the intrinsic plastic response? Are they activating the same or different molecular mechanisms?

The OCP is a good model to understand these questions. PCs denervation by removing their CF afferents, generates a well-established system where the CF plasticity can be studied at different developmental stages and conditions, and where detailed morphology and synaptic interaction between CF-PCs allows the recognition of proper target re-innervation.

III.1- Developmental plasticity of the OCP

In newborn rats, unilateral transection of the inferior cerebellar peduncle (pedunculotomy; Px; **Figure 1.14A**) leads to the rapid degeneration of CF terminals within the corresponding hemicerebellum (Zagrebelsky *et al.*, 1997) and the later degeneration of the axotomized inferior olivary neurons (Angaut *et al.*, 1985; Sherrard & Bower, 1986; Zagrebelsky *et al.*, 1997). In response to denervated PCs, the uninjured contralateral OCP rapidly activates a plastic mechanism by sprouting fine terminal branches, which cross the midline and reach the denervated hemicerebellum already at 18 hours post lesion (**Figure 1.14B**; Zagrebelsky *et al.*, 1997). The number of these collaterals increases during the next day, in addition with the emergence from the uninjured tract of transcommissural axons growing through the cerebellar white matter that elongate into the deafferented hemicerebellum between 24-48 hours post lesion (**Figure 1.14B**; Zagrebelsky *et al.*, 1997). The origin of these axons has been related to newly generated afferents from intact contralateral inferior olivary neurons (Sherrard *et al.*, 1986; Sugihara *et al.*, 2003) but further studies remain to validate this observation. Both transcommissural collaterals and axons produce terminal CF plexuses into the denervated cortex, which after 4 days post injury shown a pericellular nest like-stage around denervated PCs (Zagrebelsky *et al.*, 1997). The re-innervating CF arbours distribute normally along the proximal dendrites of PCs in the molecular layer (**Figure 1.15**; Zagrebelsky *et al.*, 1997;

Sugihara *et al.*, 2003), and as shown by electrophysiological studies, the new interaction between both synaptic partners displays normal physiological function (Sugihara *et al.*, 2003). This developmental CF reinnervation to PCs conserves normal steps of circuit maturation where multiple CFs reinnervate a single denervated PC followed by the regression of the supernumerary terminals to finally reach the mature one-to-one CF-PC relationship (Lohof *et al.*, 2005).

Interestingly, the reinnervating transcommissural CFs are organized into longitudinal zones in the deafferented-reinnervated cerebellar cortex similar in width to microzones observed in the intact cerebellum (250-500 µm; Sugihara *et al.*, 2003). In addition, they align within zebrin II stripes (Zagrebelsky *et al.*, 1997), and form an almost mirror image of the topographical projection in the intact hemicerebellum (Angaut *et al.*, 1982; Sherrard *et al.*, 1986).

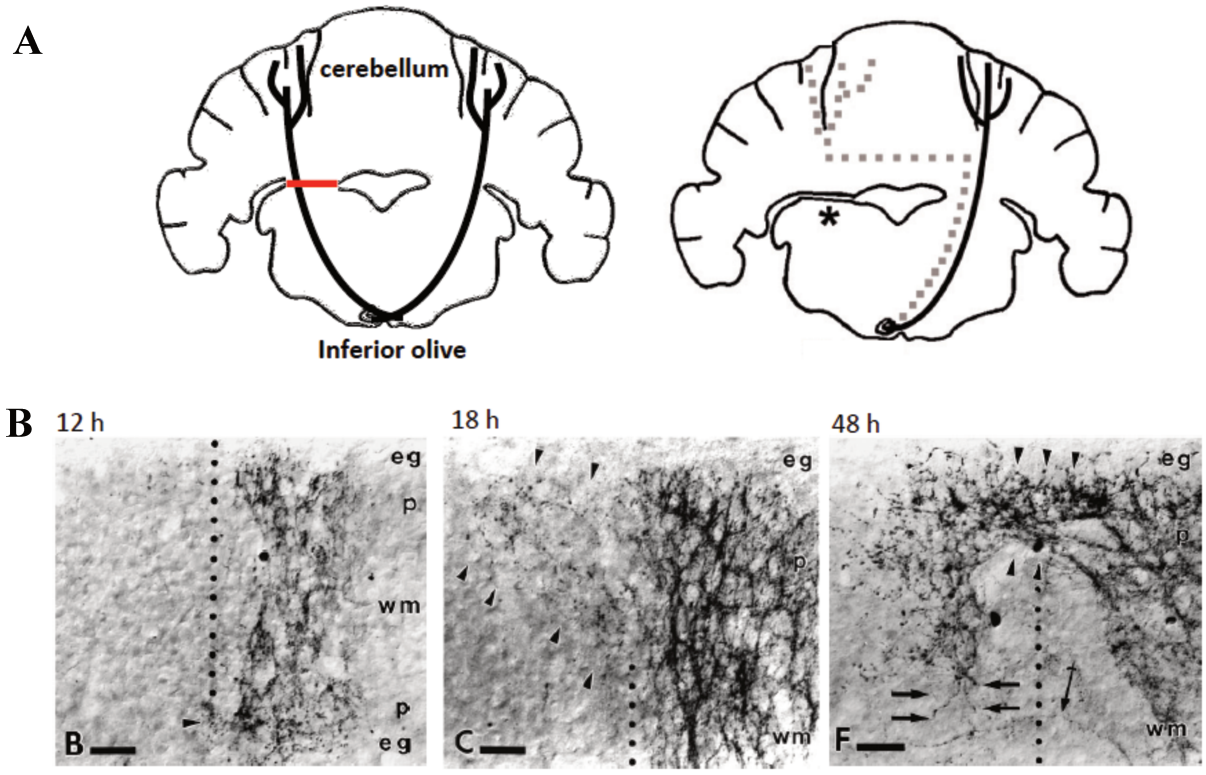

Figure 1.14: Developmental post lesion plasticity of the olivocerebellar pathway. (A) Schematic diagrams illustrating the normal olivocerebellar projection (black lines). After unilateral transection of the left olivocerebellar tract (left schema, red line) compensatory reinnervation by transcommissural projections (right schema, grey dashed line) arise from the intact tract. (B) Early steps of developmental OCP plasticity following pedunculotomy as shown in A. Calcitonin gene related-peptide (CGRP)-immunolabelled climbing fibres rapidly disappear from the left hemicerebellum12 hours after Px, compared to the adjacent intact right olivocerebellar tract (separated by the midline dotted lines). At 18 hours post injury terminal CF sprouts (arrowheads) reach the denervated hemicerebellum, and by 48 hours post lesion an increased number of these sprouting branches from the intact olivocerebellar tract (arrowheads) are accompanied by transcommissural axons that grow through the white matter (thin arrow) and invade the deafferented hemicerebellum (wide arrow). eg, external granular layer; p, Purkinje cell layer; wm, white matter. Scale bars 530 µm. Modified from (A), Willson *et al.*, 2008; (B), Zagrebelsky *et al.*, 1997.

On the other hand, reinnervating transcommissural olivocerebellar axons also extend branches to the DCN in both intact and denervated cerebellar sides; although less dense in the denervated hemicerebellum they conserve normal terminal arbors in areas consistent with the normal olivo-cortico-nuclear circuit (Zagrebelsky *et al.*, 1997; Sugihara *et al.*, 2003).

The topographical organization of the re-innervating OCP into the deafferented hemicerebellum and the appropriate DCN, strongly suggests that newly formed CFs are responding to positional target-related cues expressed by both denervated PCs and deep cerebellar neurons. However, exactly which molecules direct the reorganization of the OCP topography following Px is still unclear, and whether they are involved in the instructive developmental growth program or are related to a different re-growth mechanism.

Even though in the deafferented hemicerebellum not all the PCs are contacted by new CFs (around 80% of PCs are reinnervated in vermal cortex and only half of them in more lateral regions; Sugihara *et al.*, 2003) the CF reinnervation to PCs restore both motor and cognitive functions (Dixon *et al.*, 2005; Willson *et al.*, 2007). Thus, developmental plasticity of the OCP following a physical lesion, translates into the generation of a trans-commissural compensatory path that is capable of extending through immature white matter and projecting to appropriate target neurons.

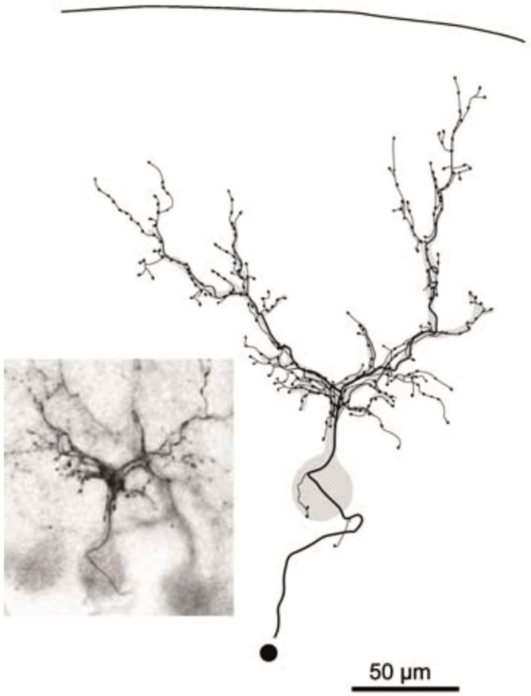

Figure 1.15: Reinnervating climbing fibre arbour. Camera lucida drawing and photomicrograph (box) showing a CF reinnervated PC in a normal one-to-one relationship; following 14 days post pedunculotomy at P3. From Sugihara *et al*., 2003.

III.2- Plasticity in the mature OCP

In contrast to its developmental plasticity, the mature OCP does not spontaneously form a compensatory path following pedunculotomy (Sherrard *et al.*, 1986). However it does show a high degree of local CF terminal plasticity in the presence of deafferented PCs, as was revealed following a subtotal neurotoxic lesion to the ION in adult rats (Benedetti *et al.*, 1983). The intraperitoneal administration of 3-acetylpyridine (3-AP) in adult rats induces a selective degeneration of the majority of IO neurons and their terminal CFs (Desclin & Escubi, 1974; Llinás *et al.*, 1975). The surviving olivary neurons synapse normally on PCs, and are capable of reinnervating neighbouring denervated PCs (Benedetti *et al.*, 1983). Morphological studies by Rossi and co-workers (1989,1991a,b) have shown that a few days after 3-AP treatment several thick branches emerge from intact CF arbors and elongate through the molecular layer until they contact deafferented PCs (**Figure 1.16**). The reinnervating CF branch develops a new terminal arbour, which covers the proximal dendrites of the dennervated PC, where it forms several varicosities (Aoki & Sugihara, 2012) that are functional synapses (Benedetti *et al.*, 1983). Like developmental plasticity, this re-innervation is also restricted within parasagittal zebrin II stripes (Zagrebelsky *et al.*, 1996), suggesting that structural plasticity of mature CFs also responds to target-derived signals (both denervation and positional cues).

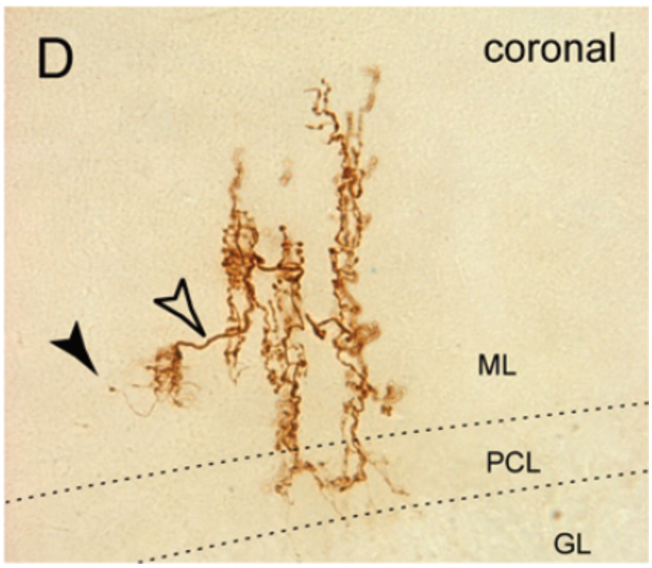

Figure 1.16: Climbing fibre branching after neurotoxic lesion. Photomicrograph of BDA-labelled CF in 3-AP-treated rat cerebellum showing reinnervated neighboured PCs by transverse expansion of large CF (open arrowhead), also the presence of thinner transverse branches is identified (filled arrowhead). ML, molecular layer; PCL or P, Purkinje cell layer, GL, granular layer. Modified from Aoki & Sugihara, 2012.

Another approach to evaluate whether the CF plasticity is activated by non-innervated PCs rather than factors originated from the lesion itself (inflammation, cellular debris, etc.), has been done by grafting embryonic cerebellar tissue into an intact adult host cerebellum, and

observing how intact CFs behave. Rossi and co-workers (1992, 1994) also showed that mature CFs extend collaterals onto the grafted cerebellar tissue where they innervate immature PCs. The newly formed CF-PC synapses conserve the morphology and the physiology of normal CF inputs (Tempia *et al.*, 1996). According to this evidence, the intrinsic plasticity of mature CFs seems to be regulated by target Purkinje cells and not by factors associated with injury (Strata & Rossi, 1998).

III.2.1- Modulated plasticity of the mature OCP.

As revealed by either the neurotoxic lesion or grafted PCs, mature CFs dynamically respond to adjacent non-innervated PCs by collateral sprouting and appropriate target selection. However this mature OC re-innervation does not occur after unilateral axotomy of olivary neurons. As described above, Px in newborn rats during the first postnatal week leads to trans-commisural CF reinnervation of deafferented PCs, but this intrinsic developmental plasticity diminishes gradually until ends by P10. This critical period can be further extended by the addition into the denervated hemicerebellum of certain growth factors such as, insulin like growth factor-1 (IGF-1; Sherrard & Bower, 1997, 2003), neurotrophin-3 (NT-3; Sherrard & Bower, 2001), and notably, brain derived neurotrophic factor (BDNF; Sherrard & Bower, 2001; Dixon & Sherrard, 2006). As shown by Sherrard and Bower (2001), 24 hours post-pedunculotomy at P11, intracerebellar injections of BDNF into the denervated hemicerebellum induces the trans-commisural growth of CFs to re-innervate deafferented PCs (**Figure 1.17 A**). The reinnervating CFs are not confined to the BDNF injection sites but distribute throughout the hemivermis and to some extension into the hemispheres, where they show a topographic organization in parasagittal stripes following a partial mirror image of the intact hemicerebellum (Dixon & Sherrard, 2006). In addition the newly formed CFs contact normally the proximal dendrites of denervated PCs (**Figure 1.17 B**; Dixon & Sherrard, 2006) forming functional synapses (Letellier *et al.*, 2007); which, unlike developmental synaptogenesis, PCs are only re-innervated by a single CF (mono-innervation). Interestingly the BDNF-induced mature OCP plasticity partially compensates motor and spatial learning (Willson *et al.*, 2008).

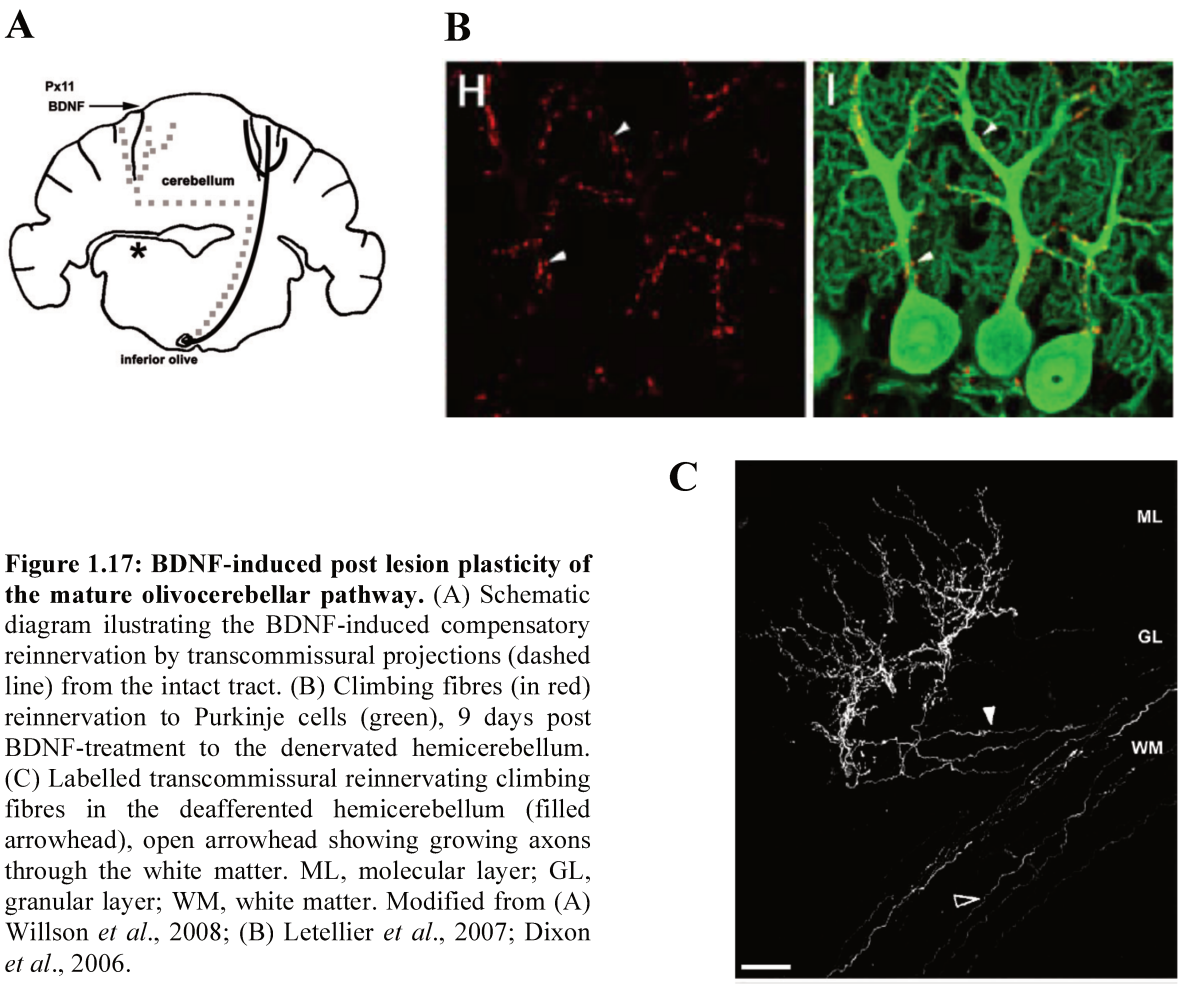

Figure 1.17: BDNF-induced post lesion plasticity of the mature olivocerebellar pathway. (A) Schematic diagram ilustrating the BDNF-induced compensatory reinnervation by transcommissural projections (dashed line) from the intact tract. (B) Climbing fibres (in red) reinnervation to Purkinje cells (green), 9 days post BDNF-treatment to the denervated hemicerebellum. (C) Labelled transcommissural reinnervating climbing fibres in the deafferented hemicerebellum (filled arrowhead), open arrowhead showing growing axons through the white matter. ML, molecular layer; GL, granular layer; WM, white matter. Modified from (A) Willson *et al*., 2008; (B) Letellier *et al*., 2007; Dixon *et al*., 2006.

In consequence, the intrinsic plasticity of the mature OCP seems to have a more "local" response to non-innervated PCs than BDNF-induced reinnervation. After either the 3-AP treatment or the grafted immature cerebellum, CFs only innervate adjacent deafferented PCs, by forming collaterals already within the denervated target region and growing them through the molecular layer where repulsive cues (e.g. myelin-derived proteins) are absent. In contrast, in BDNF-induced plasticity CFs grow collaterals over longer distances, and cross the deep cerebellar white matter (an inhibitory environment for newly growing axons) before reaching the denervated PCs (**Figure 1.17 C**). This raises several questions: How does the extrinsic BDNF stimulate mature CFs to induce reinnervation to PCs following Px? What are the molecular mechanisms controlling the BDNF-induced re-innervation?

In a transcommissural compensatory path that is morphologically similar to the contralateral sprouting developed during developmental plasticity does the BDNF-induced reinnervation recapitulate the mechanisms of the developmental reinnervation?

As discussed in (section **I.1.1.1**), BDNF shows a variety of physiological roles that could be acting in neural circuits after damage. The BDNF-TrkB receptor-signalling pathway is well known to activate pre-synaptic molecular cascade in order to directly induce axonal growth and collaterisation (Dinocourt *et al.*, 2006; reviewed in Park & Poo, 2013). In the inferior olivary neurons TrkB is expressed with high levels from P0 to P5 (Riva-Depaty *et al.*, 1998; Sherrard *et al.*, 2009), the time frame when developmental CF-PC synapses are being formed (Mariani & Changeux, 1981) and developmental plasticity can be activated (Angaut *et al.*, 1982, 1985; Sherrard *et al.*, 1986; Sugihara *et al.*, 2003).

Trk receptors can be anterogradely transported to axonal terminal portions (Bhattacharyya *et al.*, 2002; Gomes *et al.*, 2006) to respond to target-derived neurotrophins. Interestingly, expression of TrkB in the CF terminals has been identified during developmental stages (Sherrard *et al.*, 2009).

Moreover, in the cerebellum Trk receptors are also expressed by the major neuronal populations, especially PCs and GCs (Ohira & Hayashi, 2003) , and even though TrkB signalling is involved in PF-PC (Shimada et al., 1998), MF-GC (Rabacchi *et al.*, 1999), and GABAergic connectivity (Seil, 1999; Rico *et al.*, 2002) its activation seems to be unrelated to circuit formation, with the exception of the CF-PC synapses, where TrkB activation could be modulating synaptogenesis (Bosman *et al.*, 2006; Sherrard *et al.*, 2009). On the other hand, BDNF does have a role in PCs differentiation (Schwartz *et al.*, 1997; Shimada *et al.*, 1998), and GCs survival (Schwartz *et al.*, 1997). The BDNF-dependent PC survival still remains unclear, with contrasting evidence showing dependence (Larkfors *et al.*, 1996; Morrison & Mason, 1998) or independence on BDNF (Mount *et al.*, 1994) .

Additionally, BDNF shows a chemotrophic property turning axonal growth cones *in vitro* (Song *et al.*, 1997), and *in vivo* (Tucker *et al.*, 2001). But given that secreted neurotrophins readily bind to cell surfaces and extracellular matrix, it is suggested that this effect is local rather than as a long-range guidance cue (Park & Poo, 2013).

III.3- Growth intrinsic mechanisms in the inferior olivary neurons

The remarkable structural plasticity shown by mature IONs has been largely related to high constitutive expression of several growth-associated proteins during the adult state (Strata & Rossi, 1998; Carulli *et al.*, 2004), including GAP-43 (Kruger *et al.*, 1993; Wehrlé *et al.*,

2001), PSA-NCAM (Fernandez *et al.*, 1999; Quartu *et al.*, 2010), early growth response 1factor (EGR-1; also known as transcription factor KROX-24; Herdegen *et al.*, 1995; Buffo *et al.*, 1998), and the protein kinase C myristoylated alanine-rich C kinase substrate (MARCKS; Mcnamara & Lenox, 1997). From all these markers related to growth/regenerative processes in the IO neurons, GAP-43 has been the most studied.

GAP-43 is associated with a direct role in maintaining CFs structure in normal conditions, and promoting the formation of CF collaterals sprouting following lesion of the ION (Grasselli *et al.*, 2011; Allegra Mascaro *et al.*, 2013). After downregulation of GAP-43 in rodent ION neurons, CFs decrease their length, branching and number of synaptic boutons (Grasselli *et al.*, 2011). Moreover after neurotoxic lesion with 3-AP, CFs lacking GAP-43 are unable to sprout collaterals to neighbouring denervated PCs. In support to this evidence, Allegra-Mascaro and co-workers (2013) performed *in vivo* single axon lesion by laser axotomy, and found that CFs silenced for GAP-43 cannot generate reactive sprouts, suggesting a role for this protein in the initiation of post-injury axonal outgrowth. These loss of function approaches, suggest a direct role for GAP-43 in the mechanism of the intrinsic OCP plasticity.

Notably, even though the overexpression of GAP-43 induces axonal sprouting in different neural systems (Aigner *et al.*, 1995; Buffo *et al.*, 1998), an upregulation of GAP-43 related to sprouting is not clear after IO neurons injury (see review Carulli *et al.*, 2004). As a matter of fact, neither IO neurons nor CFs show changes in GAP-43 mRNA/protein expression levels following neurotoxic lesion with 3-AP (Carulli *et al.*, 2004), suggesting that the already high constitutive GAP-43 expression seems to be enough to start the sprouting response, and probably does not need a cell body-mediated upregulation (Smith & Skene, 1997).

IV- Summary and Aims

Within the CNS, the olivocerebellar pathway is generally considered to be a plastic neural circuit, with intrinsic potential to reinnervate denervated Purkinje cells globally via transcommissural collaterals following unilateral pedunculotomy during development (Zagrebelsky *et al.*, 1997; Sugihara *et al.*, 2003; Dixon *et al.*, 2005; Willson *et al.*, 2007) or focally from transverse branches after partial inferior olivary lesion (Rossi *et al.,* 1991a&b;

Dhar *et al.,* 2016). Despite the decrease with maturation of post-lesion transcommissural plasticity, treatment with exogenous BDNF into the denervated hemicerebellum extends the potential for transcommissural plasticity into mature, even adult, states and induces functional CF reinnervation to PCs (Sherrard & Bower, 2001; Dixon & Sherrard, 2006; Willson *et al.*, 2008).

IV.1- General aim

The general aim of this thesis project is to understand the mechanisms activated by BDNF that are involved in the plastic reinnervation response of mature OCP following injury. To address this aim, the *ex vivo* model of the OCP will be used to lesion the OCP.

More specifically, the overall aim has three components:

(1) Addition of BDNF into the denervated hemicerebellum induces CF reinnervation to PCs. But it is not known whether BDNF has a direct role on the denervated hemicerebellum or acts preferentially on remaining afferent CFs and subsequently their ION cells of origin. **The afferent/target-related responses to BDNF treatment into the denervated hemicerebellum will be differentiated** using morphological and molecular studies.

(2) Different intracellular signalling pathways have been related to potential neuronal plasticity, including that mediated by BDNF. **The molecular effectors of BDNF-induced CF-PC reinnervation in the mature OCP will be identified** by gene expression studies of candidate molecules and their modulation by pharmacological or genetic approaches. The timecourse of gene expression, in the cerebellum and inferior olive, will be correlated with known plastic-events (e.g. axonal growth) to assess their role **in each synaptic partner.**

(3) Axotomy to early post-natal OCP activates an intrinsic plastic response and reinnervation that declines with maturation; however it can be extended to mature states after the addition of exogenous BDNF into denervated hemicerebellum. **To assess whether the BDNF-activated plastic window in the mature OCP share molecular mechanism with developmental plasticity**, the lesion to OCP will be reproduced at equivalent postnatal day 3. Following injury, gene expression analysis and manipulation of candidate molecules established in the aim 2 will be performed.

The results of the studies addressing the above aims will be presented as 2 articles.

CHAPTER 2

Results – Article 1

Chapter 2 - Article 1

Pax3 mediates BDNF-induced post-lesion axon collateral outgrowth: reinnervation within the olivocerebellar path *in vitro*

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Running title: Pax3 induces axon elongation

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ABSTRACT

The adult mammalian central nervous system (CNS) has limited capacity for repair after injury due to intrinsic neuronal properties and inhibitory extracellular molecules, which prevent effective circuit reformation. Addition of neurotrophic factors, such as brain derived neurotrophic factor (BDNF), can result in reorganisation of neuronal connections that promote functional recovery. In the olivocerebellar pathway (OCP), climbing fiber (CF) terminals of inferior olivary nucleus (ION) axons innervate cerebellar Purkinje cells (PCs) with a precisely-organised topography. Following unilateral transection of the OCP, BDNF treatment to the denervated hemi-cerebellum induces CF-PC reinnervation, with appropriate topography that provides functional recovery. How BDNF induces this effect is unclear. Using an *ex vivo* model of the mouse OCP, we show that addition of exogenous BDNF into the denervated hemi-cerebellum increases the neuroplasticity biomarker, polysialic acid-neural cell adhesion molecule (PSA-NCAM), in both cerebellar and ION regions, and that PSA-NCAM mediates the amount of BDNF-induced reinnervation. Moreover olivary overexpression of the PSA-NCAM synthetic enzyme, ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase (Sia2), allows CF reinnervation of denervated PCs. The transcription factor Pax3, which is upregulated by BDNF, regulates Sia2, providing a link for BDNF to PSA-NCAM. BDNF treatment of the deafferented hemi-cerebellum increases Pax3 expression in the reinnervating ION. Pax3 overexpression in the ION induces significant PC re-innervation and potentiates the effect of exogenous BDNF, while olivary Pax3 knockdown almost abolishes the reinnervation of BDNF. Our results show a novel role for Pax3: its expression is essential in the afferent region (ION) to permit olivocerebellar reinnervation and potentially underlying BDNF-induced plasticity. We propose that BDNF induces Pax3 expression in olivary neurons and causes an increase of PSA-NCAM on their axons, which may increase their motility and therefore promote reinnervation of target PCs.

Keywords Reinnervation; olivocerebellar path; climbing fibre; collateral sprouting; Pax3; PSA-NCAM; sialtransferase

INTRODUCTION

Understanding the mechanisms for the maintenance and repair of neuronal connections in the central nervous system is a fundamental question in neurobiology. The adult mammalian central nervous system (CNS) has limited capacity for repair after injury due to intrinsic neuronal properties (Neumann & Woolf, 1999) and inhibitory extracellular molecules (Silver & Miller, 2004), which prevent effective circuit reformation.

However, some remaining uninjured axons can develop collaterals in grey matter that reinnervate denervated neurons (spinal cord = SC, Bareyre *et al.,* 2004; hippocampus, Deller & Frotscher, 1997; cerebellum, Rossi *et al.,* 1991). Thus augmenting this spontaneous plastic response of mature neurons could include stimulating a neuron's growth capacity, increasing growth-permissive molecules or decreasing molecules that inhibit axonal outgrowth. Neurotrophins, in particular brain derived neurotrophic factor (BDNF), have been shown to increase axon collateral sprouting (SC, Zhou & Shine, 2003; Vavrek *et al.,* 2006; cerebellum, Sherrard & Bower, 2001; Dixon & Sherrard, 2006). In addition reducing extracellular growth-inhibition, by myelin protein blockade (Freund *et al.,* 2009) or chondroitinase removal of chondroitin-suphate proteoglycans (Chau *et al.,* 2004), and increasing growth-permissive molecules such as polysialated neural cell adhesion molecule (PSA-NCAM; pharmacologically, Mehanna *et al.,* 2010; genetically, Zhang *et al.,* 2007 EJN), promote axonal sprouting and collateral outgrowth. Unfortunately, at present all of these methods require administration of the molecule directly into the injured neural tissue (Egleton & Davis, 2005 ; Freund *et al.,* 2007 ; Price *et al.,* 2007 ; Mehanna *et al.,* 2010), and are thus not practical for clinical use. It is therefore important to understand the mechanisms activated by these molecules in order to identify more practical therapeutic strategies.

The olivocerebellar path provides a useful model of post-lesion repair, as its anatomy is very well described and its behavioural functions can be readily tested (Willson *et al.,* 2008, Dixon & Sherrard 2006). Olivocerebellar axons enter the cerebellum via the contralateral inferior peduncle and terminate on Purkinje cells (PCs) as climbing fibres (CFs) organised in narrow parasagittal microzones (Sugihara *et al.,* 2001). This path regulates motor learning (Apps a Lee, 2002), the procedural aspects of navigation (Rondi-Reig & Burguière, 2005), and spatial memory (Meignin *et al.,* 1999). Following unilateral transection of the olivocerebellar path (pedunculotomy, Px), the contralateral (axotomised) inferior olive degenerates. Intracerebellar injection of BDNF, which is involved in olivocerebellar development (Bosman

et al., 2006; Sherrard *et al.,* 2009), induces remaining intact olivocerebellar axons to sprout transcommissural collaterals that reinnervate PCs of the denervated hemicerebellum and partly recreate the olivo-cortico-nuclear circuit (Sherrard & Bower 2001; Dixon & Sherrard 2006; Willson *et al.,* 2008). The resulting olivocerebellar repair improves performance of complex motor and cognitive tasks (Dixon *et al.* 2005; Willson *et al.,* 2008).

In the current study, we have used the olivocerebellar path *ex vivo* (Chedotal *et al.,* 1997; Letellier *et al.,* 2009) as a highly reproducible model with which to investigate mechanisms underlying BDNF-induced olivocerebellar reinnervation. We began by studying molecules downstream of BDNF signalling which are also expressed within the olivocerebellar system, in particular during the developmental period of active axonal growth and synaptogenesis. We observed that BDNF induces rapid sprouting of CF horizontal branches and that these elongate to reinnervate denervated PCs in association with increased PSA-NCAM expression, which is both necessary and sufficient for this repair. We then investigated the transcription factor Pax3, as a link between BDNF and PSA- NCAM (Kioussi & Gruss, 1994; Mayanil *et al.,* 2000) and identify that its expression is upregulated in the inferior olivary nucleus during BDNF-induced collateral growth, and that, like PSA-NCAM, it is both necessary and sufficient for this repair. This is the first demonstration that Pax3 is involved in axonal outgrowth in the mammalian CNS.

MATERIALS AND METHODS

Organotypic cultures

Pregnant Swiss mice were purchased from Janvier (Le Genest-St-Isle, France). Animal housing and all procedures were performed under the guidelines established by *le comité national d'éthique pour les sciences de la vie et de la santé* which are in accordance with the European Communities Council Directive (2010/63/EU).

Hindbrain explant cultures were performed as described previously (Chedotal, Bloch-Gallego, & Sotelo, 1997; Letellier *et al.,* 2009) using Swiss mice at E14. Briefly, the embryos' heads were put into ice-cold Gey's balanced salt solution (Eurobio, Courtaboeuf, France) with 5 mg/ml glucose, and brains were quickly dissected out. The hindbrain (including the cerebellar anlage and the inferior olive nucleus), was isolated and the meninges removed. Explants were transferred onto 30 mm Millipore culture membranes (pore size 0.4 μm;

Millicell CM, Millipore, Bedford, MA) and placed in six-well plates with 1ml/well of medium containing 50% basal medium with Earle's salts (Invitrogen, Carlsbad, CA), 25% Hank's Balance Salt Solution (Invitrogen), 25% horse serum (Invitrogen), 1 mM L-glutamine, and 5 mg/ml glucose. Explants were cultured at 35°C in a humidified atmosphere with 5% CO2. The day of dissection was 0 days *in vitro* (0 DIV). The medium was replaced every 2–3 days.

Cerebellar denervation and explant injection

To denervate cerebellar tissue and induce olivo-cerebellar reinnervation, the cerebellar plates were removed from their explant brainstem at 21 DIV (equivalent to P15), when the olivocerebellar path is relatively mature, and corresponding to an age at which post-lesion repair in vivo requires BDNF. The co-cultured cerebellar plate (graft) was placed with the proximal surface apposed to the "host" cerebellar tissue. In this configuration, reinnervating olivary axons must grow through white matter and pass neurons of the deep cerebellar nuclei, which is similar to in vivo post-pedunculotomy repair (Sugihara *et al.,* 2003). Twenty-four hours after co-culture some cerebellar plates were treated with 1µl (4µM) recombinant *human brain derived neurotrophic factor* (hBDNF; Alomone, 0.1% BSA in H_2O), which induces climbing fibre reinnervation in mature animals in vivo (Dixon & Sherrard, 2006; Sherrard & Bower, 2001).

To investigate mechanisms associated with BDNF-induced CF-PC reinnervation, co-culture explants also received other treatments: (1) 350 IU of neuraminidase EndoN (AbCys, France) injected onto the graft (denervated) hemicerebellum on 23 and 28 DIV to digest PSA from PSA-NCAM; (2) lentiviral mediated gene transfer to induce gene over-expression in either the graft cerebellar plate (1µl) or inferior olive (4x50nl in each olivary region); (3) inferior olivary injection of lentivirus containing pooled siPax3/hPax3/Pax3i/GFP lentivirus (LVsiPax3; Gentaur, Paris France) that knocks-down Pax3 expression by 70% (according to the manufacturer).

Recombinant lentiviral vector production

Recombinant plasmid vectors encoding for *sia2, sia2-3'UTR, sia4, sia4-3'UTR, pax3 or enhanced green fluorescent protein (GFP)* under the control of the PGK promoter were used to prepare stocks of lentiviral particles as previously described (Zennou *et al.,* 2001). All constructs were FLAG-tagged to allow for the identification of transduced cells using a FLAG antibody. Briefly, HEK 293T cells were transiently cotransfected with the p8.91 encapsidation plasmid (Zufferey *et al.,* 1997), the pHCMV-G (Vesicular Stomatitis Virus pseudotype) envelope plasmid and the pFlap recombinant vectors containing the transgene. The supernatants were collected 48 hours after transfection, treated with DNAseI (Roche Diagnostics) and filtered before ultracentrifugation. The viral pellet was then resuspended in PBS, aliquoted and stored at -80°C until use. The amount of p24 capsid protein was determined by the HIV-1 p24 ELISA antigen assay (Beckman Coulter, Fullerton, CA). Virus from different productions averaged 175 ng/µl of p24 antigen.

Immunohistochemistry

To identify the presence or absence of CF reinnervation, virally-transfected neurons, and different cell populations in the cerebellum or inferior olivary nucleus, we used double or triple fluorescent immunolabelling. Explants were fixed for 4 hours with 4% PFA in 0.1 M PBS at various post-lesion survival times. Some explants were cryo-protected in 10% sucrose in 0.1 M PBS, embeded in 7.5% gelatine with 10% sucrose added and subsequently frozen in isopentane, and transverse cryostat sections (10 µm thick) were cut. Explants or cryosections were incubated overnight at 4°C with different combinations of primary antibodies. Immunolabelling was visualized with FITC-, AMCA-, or Cy3-conjugated species-specific donkey secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, sections or explants were mounted in Mowiol (Calbiochem, La Jolla, CA) or CFM3 (Citifluor Ltd, UK), which increases optical resolution (Fouquet *et al,* 2015), for the study of CF transverse branches, and examined using either standard epifluorescence microscopy (Leica DM6000) or a confocal microscope (6000, Leica SP5, Vienna, Austria).

Primary antibodies used were as follows: mouse or rabbit anti-Calbindin D28K (CaBP, 1:5000; Swant, Bellinzona, Switzerland); goat anti-Parvalbumin (PV, 1: 5000; Swant); guinea-pig or mouse anti-vesicular glutamate transporter-2 (VGLUT2, 1:2000; Chemicon, Temecula, CA); rabbit anti-GFP (1:100; Milipore,Temecula,CA); rabbit anti-Forkhead box protein P2 (FOXP2, 1:300; Abcam) or goat anti-FOXP2 (1:2000; Abcam); mouse anti-

Flag(1:2000; Sigma, St Louis, MO); mouse anti-Pax3 (1:250; R&D systems, Minneapolis, MN); rabbit anti-cFos (1:500; Santa Cruz Biotechnology, Santa Cruz, CA).

The amount of CF reinnervation was measured by the number of CaBP-positive PCs (soma and primary dendrites) colocalised with VGLUT2 per field of view (grid) and expressed as percentage PCs per field. This quantification was made systematically on z-stacks taken in rows through the cerebellar graft with increasing distance from the host-graft interface. Data from rows 1 and 2 were defined as a proximal zone, and those from rows 3-5 were defined as a distal zone.

Protein analysis: Western Blot and ELISA

Cerebellar plates or inferior olivary tissue were taken from explants during development from 9 - 35 DIV and at 1, 2, 4, or 12 days post-lesion. Tissue was homogenized in 500 µl of lysis buffer (pH; 120 mM NaCl; 50 mM TRIS, 1 mM EDTA; 25 mM NaF; 1 mM Na_3VO_4; 0.5% SDS), sonicated and centrifuged (14 000 rpm, 30 min) and the supernatants were analyzed by ELISA or western blot.

PSA-NCAM concentration was measured using an ELISA kit (PSA-NCAM ELISA ABC0027B) Eurobio France) according to manufacturer's instructions. Samples were run in duplicate.

Pax3 protein was measured by western blot; 30 µg of total protein was separated by SDS-PAGE, transferred onto PVDF membranes and probed with anti-Pax3 (1:250; R&D systems, Minneapolis, MN) and then anti-β-actin (1:1000; Abcam). Bound antibody was visualised using HRP-conjugated anti-mouse secondary antibody (Amersham Biosciences) and ECL Advance for chemiluminescence (2 minutes exposure time; Amersham Biosciences). Bands were identified by size (Pax3: 53 kDa; βactin: 42 kDa), intensities were measured (ImageJ, Gelplot macro) and normalised against the amount of β-actin in each well.

qRT-PCR

To evaluate changes in gene expression during the process of reinnervation, RNA was extracted from either the cerebellar plate or the inferior olivary region of lesioned or control explants. Tissue from 6 cerebellar plates and inferior olive regions were pooled. Total RNA was extracted using Trizol (Life Technologies) according to manufacturer's instructions

(Chomczynski & Sacchi, 1987) and RNA concentration was measured by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) before being stored at -80°C.

200ng of total RNA was reverse transcribed in 20µl using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was amplified on a LightCycler® 480 (Roche Applied Bioscience, USA) in 10 µl reaction volume using SYBR Green I Master Mix (annealing temperature 58 °C, 50 cycles). Housekeeper primers were TUB5 and ARBP.

TUB5: Forward GCTAAGTTCTGGGAGGTGATAAGCG,
Reverse CCAGACTGACCGAAAACGAAGTTG;

ARBP: Forward TGCCAGCTCAGAACACTGGTCTA,
Reverse GGGAGATGTTCAGCATGTTCAGCA;

BDNF: Forward TCACTGGCTGACACTTTTGAGCA,
Reverse CGCCGAACCCTCATAGACATGTTT;

Pax3: Forward AGCAAACCCAAGCAGGTGACA,
Reverse AGGATGCGGCTGATAGAACTCACT;

ST8SiaII: Forward AGCACAATGAACGTGTCCCAGAA,
Reverse GAGCCAGGTTGCACCTTATGACA;

ST8SiaIV: Forward TTCCGGCATTCTGCTAGACAGTG,
Reverse CGAAAGCCTCCAAATGCTCTTTGC.

All samples were amplified in triplicate and the mean was used to calculate gene expression in each tissue sample. Raw data were pre-processed with Lightcycler 480 software (Roche Applied Bioscience, USA) according to Pfaffl's equation. Target gene expression was normalised to the harmonic mean of 2 housekeeper genes.

Electrophysiology

Whole-cell patch-clamp recordings from PCs were performed as previously described for acute cerebellar slices (Llano *et al.,* 1991). Patch pipettes were filled with a solution containing: 120 mM Cs-D-Gluconate, 13 mM biocytin, 10 mM HEPES, 10 mM BAPTA, 3 mM TEACl, 2 mM Na2ATP, 2 mM MgATP, 0.2 mM NaGTP, pH 7.3, 290–300 mM mOsm. Explants were continuously perfused with a bath solution containing: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, 25 mM glucose, and bubbled with 95% O2 and 5% CO2. Picrotoxin (100 µM) was added to block inhibitory currents. CF-EPSCs were elicited by stimulation with a saline-filled glass pipette in the area

surrounding the PC. We distinguished CF-EPSCs from currents mediated by parallel fibers (PF-EPSCs) by their all-or-none character and by the demonstration of paired-pulse depression. To determine the number of CFs innervating a PC, we counted the number of discrete CF-EPSC steps that appeared when the intensity of stimulation was gradually increased or when the position of the stimulation electrode was changed.

Following electrophysiological recordings, explants were fixed in 4% paraformaldehyde (PFA) in PB 0.1 M and processed for immunohistochemistry; the recorded (biocytin-filled) PCs were visualized after incubation with AF488-conjugated avidin (Invitrogen, Molecular Probes).

Statistics

Transformed (square-root) data revealed homogeneity of variance and inter-group comparisons were analysed using analysis of variance (ANOVA) and either Bonferroni's or Dunnets T3 (if normality was not attained) post hoc tests. The number of CF branches was assessed using the χ^2 test. All values were stated as mean±SEM and $\alpha = 0.05$.

RESULTS

We used our in vitro model of the olivocerebellar pathway (Chédotal *et al.,* 1997; Letellier *et al.,* 2009) to elucidate mechanisms underlying appropriately-targeted post-lesion reinnervation that is induced by BDNF.

In vitro BDNF increases terminal sprouting from intact CFs to reinnervate denervated PCs

We first confirmed that, as *in vivo*, BDNF injection into the target cerebellum (Sherrard & Bower 2001; Dixon & Sherrard 2006; Willson *et al.,* 2008) could induce olivocerebellar reinnervation in the maturing pathway. Cerebellar plates were denervated at 21 DIV, the equivalent of postnatal day 15 (P15), apposed to an intact explant and treated with BDNF or vehicle (Fig 2.1A). Consistent with *in vivo* studies (Willson *et al.,* 2008), BDNF induces the growth of VGLUT2-labelled terminals into the denervated cerebellar plate, which localise around the PC somata and primary dendrites (Fig 2.1B). In all explant co-cultures a very small number of VGLUT2-positive terminals were observed around PCs adjacent to the host hemicerebellum, consistent with previous studies (Letellier *et al.,* 2009). However,

quantification of reinnervated PCs at different distances from the host-graft junction showed that significant reinnervation occurred only in the presence of BDNF and that reinnervation density decreased further from the junction ($F_{3,49} = 99.3$, $p<0.0001$; Fig 2.1C). This pattern is similar to that observed following in vivo pedunculotomy (Willson *et al.,* 2008).

Given that the half-life of exogenous BDNF in brain parenchyma is very short (about 1 hour; Soderquist *et al.,* 2009), we examined CF terminals in the intact host cerebellar plate, to identify the initial effects of BDNF that may explain their subsequent growth to reinnervate PCs in the graft. LV-GFP was injected into the ION regions at 7DIV to label CFs. In the intact explant, GFP-filled CFs surrounded target PC soma and major dendrites (Fig 2.2A) and project a few short thin branches typical of horizontal branches *in vivo* (Sugihara *et al.,* 1999; Nishiyama *et al.,* 2007). In the lesioned-co-culture cerebella, 24h after BDNF treatment these horizontal branches were longer ($F_{2,52} = 19.93$, $p<0.0001$) and more numerous (ANOVA, $F_{2,57} = 12.7$, $p<0.0001$; Fig 2.2B); specifically, after BDNF treatment there were more branches per CF than in either vehicle-treated lesioned explants ($p<0.001$) or intact tissue ($p<0.0001$). In addition, there were more CFs with branches >40 μm in BDNF-treated tissue than either in vehicle-treated tissue or intact (non-lesioned) controls ($\chi^2 = 29.32$, 2df, $p<0.0001$).

As that there was also some CF sprouting in vehicle treated explants, we evaluated how far the BDNF activation may have spread using expression of the immediate early gene, c-fos. Explants were lesioned and cocultured at 21DIV and treated with BDNF 24h later. Post BDNF treatment, some explants were fixed at 1.5h and others taken for RNA extraction and qPCR at 1h, 6h, 24h, and 48h. Immunohistochemistry revealed c-fos labelling only in the BDNF-treated graft cerebellar hemisphere (Fig 2.2C) and not in the adjacent host, nor in vehicle treated tissue. qPCR analysis confirmed this, showing an acute 4-fold increase over vehicle treatment for cerebellar *cfos* at +1h after BDNF ($p<0.001$), which gradually returned to baseline by +24h. These data suggest that CF horizontal branch sprouting is primarily due to the presence of denervated PCs and not to a direct effect of the BDNF.

BDNF-induced olivocerebellar reinnervation requires PSA-NCAM

Although BDNF significantly increased innate CF sprouting in as little as 24h, its very short half-life, combined with strong cerebellar expression of truncated TrkB receptors (tTrkB) that

binds free BDNF (Sherrard *et al.,* 2009; Bosman *et al.,* 2006), raise questions about *how* BDNF induced sufficient CF outgrowth to reach and reinnervate PCs in the grafted hemicerebellum. Because neural circuit reformation is thought to recapitulate normal developmental processes (Harel & Strittmatter, 2006), we looked at molecules associated with olivocerebellar development, and post-lesion axonal outgrowth. Polysialic acid (PSA)-NCAM is one such molecule, being highly expressed in early development and decreasing with maturation (Rutishauser, 2008), and is involved in early OC maturation (Avella *et al,* 2006) and post-lesion plasticity in the cerebellum (Dusart *et al.,* 1999; Zhang *et al.,* 2007).

We measured PSA-NCAM, in the cerebellar plate and inferior olivary region (Figure 2.3), during reinnervation after co-culture. As *in vivo* (Rutishauser, 2008), in non-lesioned explants PSA-NCAM concentration decreased during maturation (after P15) in both the cerebellar plate and the inferior olive region (cerebellum: ANOVA $F_{5,21} = 18.7$; inferior olive: ANOVA $F_{5,29} = 26.3$, $p < 0.0001$ for both; Fig 2.3A,B). However, following denervation and co-culture this decrease was attenuated in both regions (Fig 2.3A,B), so that there was significantly more PSA in inferior olive tissue of lesioned BDNF-treated versus intact explants from 4 days post-lesion (ANOVA $F_{8,65} = 7.1$, $p<0.001$; Fig 2.3B). This increase in PSA after co-culture and BDNF treatment, and to a lesser extent with just co-culture (although not statistically significant), is consistent with the differential degree of CF horizontal branch sprouting in vehicle vs. BDNF treated explants (above). Interestingly, an isolated cerebellar plate treated with BDNF (Fig 2.3A) did not maintain its PSA-NCAM levels, suggesting that the olivary axons carry this adhesion molecule.

To identify whether increased PSA-NCAM is related to olivocerebellar reinnervation, we altered cerebellar PSA-NCAM concentration and re-evaluated CF reinnervation. First, we treated the grafted hemicerebellum of BDNF-treated or vehicle-treated co-cultures with Endo-N (see Methods) to remove PSA from NCAM. Not only does Endo-N reduce PSA-NCAM in lesioned-BDNF-treated tissue (ANOVA $F_{7,50} = 3.1$, $p<0.01$; Fig 2.3A,B) but it also significantly reduces BDNF-dependant reinnervation ($F_{5,59} = 58.49$, $p<0.01$; Fig 2.3C). Second, we used LV-Sia2 transfection of the denervated cerebellar plate to overexpress *sia2*, an enzyme that adds PSA to NCAM (Rutishauser, 2008) and increases PSA-NCAM (Canger & Rutishauser, 2004). The percentage of reinnervated PCs after LV-Sia2 is the same as that induced by BDNF, and greater than vehicle ($F_{2,27} = 77,11$, $p<0.0001$; Fig 2.3C). Interestingly, immunohistochemistry also revealed that PCs were not transfected (not shown) to

overexpress *sia2*, which suggests that CF reinnervation only requires PSA-NCAM generally in the cerebellar environment rather than specifically on its target PCs.

Taken together these data indicate that BDNF induces CF reinnervation by increasing PSA-NCAM in the olivocerebellar environment, and that a general increase of growth-permissiveness within the cerebellar extracellular milieu is sufficient to allow inferior olivary axon ingrowth without interfering with subsequent target selection (CFs synapse onto PCs and not other neurons) or having to be specific to the synaptic partners (PCs were not transfected to overexpress *sia2*).

Afferent axon PSA-NCAM permits olivocerebellar reinnervation.

Our PSA ELISA data also showed that denervation, co-culture and cerebellar BDNF significantly increased PSA-NCAM in the inferior olivary region (Fig 2.3B above), suggesting that retrograde BDNF signalling also acts on olivary PSA-NCAM to promote reinnervation. We examined whether an increase of PSA-NCAM by the afferent olivary axons was sufficient to produce PC reinnervation post-lesion, by overexpressing *sia2* or *Sia4* (LV-Sia2, LV-Sia4) in the reinnervating inferior olive, 24 hours after lesion and co-culture (Fig 2.4A). After 10 days to allow CF outgrowth, we compared PC reinnervation after LV-Sia2 or LV-Sia4 ± BDNF, LV-GFP+BDNF and LV-GFP (control) treatments.

LV-Sia2 infection +/- BDNF in the inferior olive resulted in significantly greater CF-PC reinnervation than either BDNF or vehicle treatment (LV-GFP +/- BDNF) for both proximal and distal zones ($F_{3,30} = 119.3$, $p<0.0001$). Also, the effects of LV-Sia2 and BDNF were not additive (LV-Sia2+BDNF = LV-Sia2+Vehicle; Figure 2.4B). In contrast, the effect of LV-Sia4 was less pronounced, inducing similar reinnervation to BDNF (Fig 2.4B; $F_{5,60} = 92,58$, $p<0.0001$) and less than LV-Sia2 ($p<0.001$). However, the percentage of PC reinnervation increased when LV-Sia4 and BDNF were combined (LV-Sia4+BDNF > LV-Sia4+Veh; $p<0.01$) to equal that induced by LV-Sia2 +/- BDNF. Since Sia4 is expressed preferentially in the mature nervous system, specifically in regions of synaptic plasticity (Uryu *et al,* 1999; Rutishauser, 2008), its induction of less reinnervation than Sia2 is consistent with different roles for these two enzymes in the developing/mature nervous system.

Given the large distance between the medullary inferior olive and sprouting cerebellar CF axon terminals, we tested whether targeting the upregulated mRNA to the axon terminal

would have greater effect. Inferior olivary injections of LV-Sia2-3'UTR or LV-Sia4-3'UTR in lesioned co-culture explants only had the same effect as the non-3'UTR vectors (data not shown).

Paired homeobox transcription factor Pax3 induces olivocerebellar reinnervation

We next sought to understand the signalling interactions between BDNF and the production of PSA-NCAM. Database searching revealed that the transcription factor *pax3* is expressed in the immature cerebellum (Schuller *et al.,* 2006), upregulated by BDNF in cerebellar neuronal culture (Kioussi & Gruss 1994) and in turn upregulates the expression of ST8Sia2 (Mayanil *et al.,* 2000) and ST8Sia4 (Lagha *et al.,* 2010). We evaluated the expression of *pax3* in the cerebellar graft and inferior olive from 1 - 48 hours after the application of BDNF at 22DIV (equivalent P16) following denervation at 21DIV (equivalent P15). Expression of *pax3* mRNA transiently increased in the inferior olive 24h post BDNF ($p<0.05$) and this was followed by increased Pax3 protein at 48h ($p<0.01$; Fig 2.5A,B). There were no changes in Pax3 mRNA or protein in the cerebellum, nor in vehicle-treated controls. Taken together these results indicate that increased olivary Pax3 is not simply the result of the lesion but occurs in response to the cerebellar BDNF application and at the time when olivary neurons are actively growing axonal branches.

To test whether the transient upregulation of its gene expression is involved in olivocerebellar reinnervation, we altered olivary *pax3* expression and observed dramatic changes in olivocerebellar reinnervation ($F_{11,105} = 82,72$, $p<0.0001$). First, we increased inferior olivary *pax3* expression in lesioned co-cultures by transfecting olivary neurons with LV-Pax3, 24h after BDNF/vehicle injections. We found that LV-Pax3 induced CF-PC reinnervation ($F_{7,77} = 99,61$, $p<0.0001$) equal to that induced by BDNF, but which was also additive with BDNF in both proximal and distal zones (LV-Pax3 < LV-Pax3 BDNF, $p<0.01$ for both; Fig 2.5C). Conversely LV-siPax3, which reduces basal *pax3* expression by 70% (manufacturers data), significantly reduced BDNF-induced reinnervation ($F_{5,47} = 58,53$, $p<0.0001$; Fig 2.5C) down to the percentage in vehicle treated explants.

Secondly, as induction of axonal outgrowth and olivocerebellar reinnervation is a new function for Pax3, we verified that the VGLUT2-labelled terminals were CFs. Whole-cell PC recordings in the grafted cerebellar plates of olivary *pax3*-overexpressing explants identified some PCs showing CF synaptic currents (CF-EPSCs), with classic all-or-none activity and

paired-pulse depression. Other PCs had no CF-EPSCs. Subsequent histological analysis revealed that CF-EPSC-positive PCs colocalised with VGLUT2, whereas those without such responses generally did not (Fig 2.5D).

Thirdly, as Pax3 regulates *sia2* (Mayanil *et al.,* 2000), we tested whether it acts, at least in part, by providing a growth permissive environment. As after BDNF treatment, 4 days after lesion and co-culture, there was increased cerebellar PSA-NCAM in explants transfected by olivary LV-Pax3 ($F_{11, 117} = 6{,}416$, $p<0.0001$; Fig 2.5E).

Taken together, these data show a novel function for Pax3, that of inducing axonal outgrowth and neo-synaptogenesis in the olivocerebellar pathway; and that this function is in the afferent pre-synaptic component, since Pax3 expression is required in the reinnervating inferior olive and not in the cerebellar PCs.

DISCUSSION:

In this study we aimed to understand some of the signalling pathways that are activated during post-lesion BDNF-induced axon collateral growth, using our *ex vivo* model of the olivocerebellar path. We demonstrate that BDNF induces rapid sprouting of thin horizontal CF branches and upregulation of PSA-NCAM and the transcription factor Pax3. Both PSA-NCAM and Pax3 are necessary for CF-PC reinnervation to take place and, individually, are able to induce it. However, PSA-NCAM appears to function as a permissive cellular environment, whereas Pax3 acts only as a pre-synaptic agent to stimulate olivary axonal growth.

BDNF induces rapid sprouting of CF terminal horizontal branches

It has been previously described that cerebellar Purkinje cells respond to CF denervation by producing an unknown signal that induces sprouting of nearby climbing fibre terminals, which grow through the molecular layer to reinnervate adjacent deafferented PCs (Rossi *et al.,* 1991; Tempia *et al.,* 1996; Dhar *et al.,* 2016). It is also known that the addition of BDNF to the denervated hemicerebellum promotes olivocerebellar axon collateral growth through the cerebellar white matter to promote CF-PC reinnervation (Dixon & Sherrard 2006; Willson *et al.,* 2008). Here we show for the first time the source of these collaterals may be the horizontal branches of CF arbors. Thin horizontal CF terminal branches have been previously

described (Rossi *et al.,* 1991; Sugihara *et al.,* 1999; Nishiyama *et al.,* 2007), however their function has not been identified, as they do not form synaptic terminals (Nishiyama *et al.,* 2007). In agreement with previous studies (Rossi *et al.,* 2001; Dhar *et al.,* 2016), we observed that these CF horizontal branches sprout in the presence of denervated PCs. However despite prolonged extension through the molecular layer (Rossi *et al,* 2001; Dhar *et al,* 2016), the capacity for these collaterals to form new terminal arbors decreases with increasing distance (Dhar *et al.,* 2016). This has been interpreted as showing a limit to the plastic capacity of ION neurons (Dhar *et al.,* 2016), which may explain why spontaneously generated CF collaterals are unable to enter the graft hemicerebellum and reinnervate PCs (in vitro, this study; in vivo, Dixon & Sherrard 2006; Willson *et al.,* 2008). Given that reinnervating transcommissural CF collaterals generated post-lesion during development can support 15-fold more terminal arbors (up to 89/CF were identified; Sugihara *et a.l,* 2003), it is also likely that the limited number of reinnervating arbors generated in the adult (Rossi *et al.,* 1991; Dhar *et al.,* 2016) reflects axon collaterals having to grow through an environment that is only partially-permissive. In contrast, we now show that when BDNF is added, these CF horizontal branch sprouts are longer, more numerous and can extend long collaterals that innervate deafferented PCs in the graft tissue. BDNF thus augments an inherent plastic capacity of a pre-existing CF structure, but whether this is direct growth-promotion within the CF terminals, modulation of the extracellular environment or both, cannot be concluded.

BDNF upregulates PSA-NCAM, which is necessary and sufficient for CF-PC reinnervation

The cellular environment is fundamental to the facilitation/blockade of axonal outgrowth (Silver & Miller, 2004; Freund *et al.,* 2009; Chau *et al.,* 2004), including olivocerebellar axons (Bravin *et al.,* 1997). We first examined the extracellular matrix molecule PSA-NCAM, because it regulates CF maturation (Avella *et al.,* 2006) and promotes axonal growth (Zhang *et al.,* 2007; Mehanna *et al.,* 2010). We observed increased PSA-NCAM in both cerebellar and inferior olivary tissue 2-4 days following lesion and BDNF treatment, i.e. at the time when reinnervating CF collaterals are growing into the denervated hemicerebellum. The relevance of PSA-NCAM upregulation to CF reinnervation is confirmed by modulating PSA-NCAM levels and finding concomitant changes in the amount of CF-PC reinnervation in lesioned explants: EndoN lysis of cerebellar PSA-NCAM diminished reinnervation after BDNF treatment; and lentiviral-driven expression of the synthetic sialtransferase enzymes (sia2 and sia4), in either the cerebellum or inferior olive, induced reinnervation as great or

greater than that induced by BDNF (Figs 2.3C and Fig 2.4B). PSA-NCAM expression on the growing olivocerebellar axons seems to be the most important factor: we observed greater BDNF-induced upregulation of PSA-NCAM in the ION than in the cerebellum, and very low PSA-NCAM expression in isolated hemicerebellar anlage (i.e. without any extracerebellar afferents) even after BDNF treatment. Axonal PSA-NCAM has been demonstrated during hippocampal reinnervation and is entirely consistent with its role directly promoting axonal elongation (Bonfanti, 2006). These results suggest that at least one effect of BDNF in converting post-lesion plastic sprouting to effective reinnervation comes from increasing this permissive substrate in the tissue.

Pax3 induces olivocerebellar axonal outgrowth to reinnervate Purkinje cells

Although PSA-NCAM may regulate BDNF-TrkB interactions (Bonfanti, 2006), a direct link between BDNF and PSA-NCAM upregulation is not obvious. To try to understand the molecular events linking BDNF signalling to *sia2* and *sia4* expression, and therefore PSA-NCAM, we evaluated expression of *pax3*, which upregulates *sia2* and *sia4* (Mayanil *et al.,* 2000; Largha *et al.,* 2010), after lesion and BDNF treatment and showed a transient increase in the ION. Pax3 is strongly expressed in the developing hindbrain where it regulates regional developmental genes (Vennemann *et al.,* 2008). It is also upregulated by BDNF (Kioussi & Gruss 1994), through TrkB induction of Klf4 (Yin *et al.,* 2015) that binds to response elements in the *pax3* promoter (http://www.informatics.jax.org/marker/MGI:1342287) suggesting that this transcription factor may be directly induced by BDNF-TrkB signalling. Therefore we modified *pax3* expression in both the cerebellum and inferior olive to evaluate its potential role in olivocerebellar reinnervation. Over-expressing *pax3* in the cerebellum had almost no effect on CF reinnervation, so the percentage of reinnervated PCs after LVPax3 in the cerebellum was significantly less than after BDNF. In contrast, olivary *pax3* overexpression increased PSA-NCAM levels in the grafted cerebellar plate and provided post-lesion PC reinnervation by correctly functioning CFs at the same level to that induced by BDNF. Moreover, interfering with *pax3* expression by siRNA significantly reduced BDNF-induced post-lesion repair. Taken together these experiments show that the increase of olivary Pax3 after lesion/BDNF is *necessary* for CF axon collateral outgrowth and PC reinnervation (cf BDNF vs. Veh: Western Blot, Fig 2.5B; reinnervation score, BDNF vs. BDNF/siPax3, Fig 2.5C), and is in itself *sufficient* to induce olivary axon collateral growth (cf LV-Pax3 vs. LV-GFP, Fig 2.5C). A possible mechanism underlying the phenomenon is the increase of PSA-NCAM (Fig 2.5E) providing a more growth-permissive environment.

The involvement of Pax3 in neural circuit repair and reinnervation is a new function for this transcription factor. Potential links between Pax3 and axonal outgrowth are mixed, with potential involvement in *C. elegans* motor neuron regeneration (Nix *et al.,* 2014), but not in mouse DRG conditioning lesions (Vogelaar *et al.,* 2004), suggesting that any involvement may be system (or perhaps species) specific. In contrast to the sciatic nerve crush-DRG conditioning lesion, our data do reveal a positive effect of Pax3 in the mammalian CNS. But we also reveal a potential mechanism of action since Pax3 can only induce CF reinnervation via its olivary expression; i.e. its role is presynaptic, indicating that it "pushes" axonal outgrowth rather than "pulls" as a target-derived attractant. Moreover, preliminary *in silico* searches in Gene Ontology databases reveal that among the transcription factors containing Pax3 binding sequences in their promoter regions, there is significant enrichment of factors involved in axon extension, guidance and regeneration, as well as synaptogenesis, at least some of which are expressed in the cerebellum (Allen Brain Atlas). The fact that the effect of Pax3 can be further potentiated by cerebellar BDNF (cf LV-Pax3 vs. LV-Pax3 + BDNF) supports the hypothesis that, by directly promoting axonal outgrowth, Pax3 forms one mechanism by which BDNF induces olivocerebellar reinnervation; but that other signalling cascades activated by BDNF-TrkB (Liu & Snider, 2001) are also involved. While the mechanisms by which BDNF has these additional effects are not shown in this study, future studies are needed to examine them.

Conclusion

Our study thus provides clear links between BDNF, Pax3 expression, Sia2 upregulation and increases in PSA-NCAM in this model of lesion and repair of the olivocerebellar path. This is a novel role for Pax3, and future studies in other neuronal systems should demonstrate whether these mechanisms are can be generalized and may provide interesting therapeutic possibilities.

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FIGURE LEGENDS

Figure 2.1. BDNF administration allows post-lesion repair in an ex vivo model of the olivocerebellar path.

A: The E14 mouse hindbrain containing the cerebellum and inferior olivary nucleus is isolated and cultured in "open-book" configuration. Isolated (denervated) cerebellar plates are positioned next to the host cerebella to provide denervated target PCs for reinnervation by the intact olivocerebellar axons (grey arrows). Grafted cerebellar plates can be treated with BDNF or specific lentivirus to induce reinnervation (red dotted arrows).

B: Immunostaining for calbindin (CaBP, PCs) and VGLUT2 (CFs) allows quantification of CF reinnervation in the grafted cerebellar plate following BDNF of lentivirus application to the denervated tissue. CaBP/VGLUT2 co-labelled PCs are counted on image z-stacks taken in rows with increasing distance from the host (1 to 4). Bar = 25μm.

C: Reinnervation of PCs in the grafted cerebellar plate, following lesion at 21DIV (equivalent to P15), is significantly higher if BDNF is applied (Px15B) compared to vehicle-treated controls (Px15V; ANOVA $F_{3,49} = 99.3$, $p<0.0001$).

Between group post-hoc comparisons BDNF vs. vehicle: ***, $p<0.001$.

Figure 2.2. BDNF administration rapidly increases post-lesion CF collateral sprouting.

A: Horizontal branches projecting from GFP-expressing CFs which innervate PCs in the normal host hemicerebellar palate. In the intact explant (left panel), thin CF horizontal branches (white arrows) are rare. After lesion and coculture (middle panel) these branches appear longer and are also more numerous in the presence of BDNF (right panel).

B: 24 hours after lesion and coculture, horizontal CF branches were quantified. They are more numerous (left panel; ANOVA $F_{2,57} = 12.7$, $p<0.0001$) and longer (ANOVA $F_{2,52} =$

19.93, p<0.0001) in lesioned cocultures treated with BDNF. The number of branches longer than 40 mm was significantly higher after BDNF application (χ^2 = 29.32, 2df, p<0.0001).
Between group post-hoc comparisons BDNF vs. vehicle: ***, p<0.001.

C: Labelling for c-fos after BDNF or vehicle injections to the graft cerebellar plate. The schema (top left) shows which tissue was analysed: red squares indicate cerebellar tissue taken for qPCR and the black dotted line shows the orientation of frontal sections of the fixed explant. qPCR analysis (bottom left) shows a 4-fold increase of *cfos* expression at 1hr (p<0.001), which is still raised at +6h (p<0.01) but has returned to baseline at +24h. Immunolabelling (right) shows c-fos (red) after BDNF application to the graft (top panels) but none after vehicle treatment (lower 2 panels). In the BDNF-treated explant the c-fos is essentially in the graft cerebellum (right hand side) with almost none in the host (centre) or brainstem (left). The vertical dotted lines indicate the separations between the different areas of the explant

Figure 2.3. Olivocerebellar reinnervation is associated with increased PSA-NCAM.

A & B: PSA-NCAM decreases during maturation in the cerebellar plate (A) and inferior olivary region (B). Post lesion, BDNF administration partly counters this decrease, resulting in higher PSA-NCAM tissue concentration, which is significant in the inferior olive (ANOVA $F_{8,65}$ = 7.1, p<0.001). Notably, in isolated cerebellar plates treated with BDNF (CbmB) there is very much less BDNF. Additional post-BDNF treatment with EndoN to lyse PSA from NCAM (PxB+E) greatly reduces PSA-NCAM when compared to PxV or PxB (ANOVA $F_{7,50}$ = 3.1, p<0.01).

C: PC reinnervation in the grafted cerebellar plate induced by BDNF (PxB) is reduced when PSA-NCAM is lysed by the neuraminidase EndoN (PxB+E; ANOVA, $F_{5,59}$ = 58.49, p<0.01). Also, overexpression of *sia2* in the cerebellar plate to increase PSA-NCAM, promotes CF-PC reinnervation to a similar level as BDNF (Px+Sia2; $F_{2,27}$ = 77,11, p<0.0001).
Between group post-hoc comparisons BDNF/sia2 vs. vehicle: ***, p<0.001.
Between group post-hoc comparisons BDNF vs. BDNF+EndoN: ##, p<0.01.

Figure 2.4. Post-lesion reinnervation is increased by olivary overexpression of *sia2*.

A: Lentivirus injected (4x50 nl) into the ION of explants (left panel) is detected by GFP expression (right panel) or FLAG immunohistochemistry (not shown). The injection remains highly localised to the inferior olivary region. The white dotted line represents the junction between the host and graft hemicerebella. Bar = 1mm.

B: CF-PC reinnervation in proximal and distal regions of the grafted cerebellar plate, following olivary injection of LV-Sia2 or LV-Sia4 alone (red or pink bars) or with BDNF (blue border), LV-GFP with (blue bar) or without (black bar) BDNF. In both proximal and distal regions, *sia2* overexpression ± BDNF induced more reinnervation than BDNF or LV-GFP/Vehicle alone ($F_{3,30}$ = 119.3, p<0.0001); but the 2 treatments were not additive. *Sia4* overexpression alone was less effective ($F_{5,60}$ = 92,58, p<0.0001), equalling BDNF. However BDNF (PxB+Sia4) multiplied this effect of *sia4* increasing reinnervation (p<0.01) to that induced by *sia2*.

Between group post-hoc comparisons BDNF/sia2/sia4 vs. vehicle: *, p<0.05; ***, p<0.001.

Between group post-hoc comparisons Sia4 vs. Sia4+BDNF: ##, p<0.01.

Between group post-hoc comparisons Sia4 vs. Sia2: $, p<0.05.

Figure 2.5. Olivary *pax3* overexpression is involved in post-lesion CF-PC reinnervation.

A: Quantitative PCR reveals transient *pax3* upregulation in the inferior olive, but not the cerebellum, 24h after lesion and BDNF treatment (p<0.05).

B: Western blot analysis also revealed a transient increase in Pax3 protein 48h (2 days) after lesion and BDNF treatment (PxB vs PxV; p<0.01), which subsequently fell at 96h (p<0.05) to vehicle treated levels.

Between group post-hoc comparisons PxB vs. PxV: **, p<0.01.

Between group post-hoc comparisons PxB 2d vs. PxB 4d: #, p<0.05.

C: CF-PC reinnervation in proximal and distal regions of the grafted cerebellar plate, following olivary injection of LV-Pax3 alone (orange bar) or with BDNF (blue border), LV-GFP with (blue bar) or without (black bar) BDNF. In both proximal and distal regions, *pax3* overexpression induced reinnervation equivalent to BDNF but more than or LV-GFP/Vehicle alone ($F_{7,77}$ = 99,61, p<0.0001). The 2 treatments were additive, so that the effect of LV-Pax3 with BDNF was greater than any single treatment (LV-Pax3 < LV-Pax3 BDNF, p<0.01). When *pax3* was knocked-down by LVsiPax3, BDNF-induced reinnervation was decreased ($F_{5,47}$ = 58,53, p<0.0001) to the percentage in vehicle treated explants.

Between group post-hoc comparisons BDNF/Pax3/Pax3+BDNF vs. vehicle: ***, p<0.001.

Between group post-hoc comparisons Pax3 vs. Pax3+BDNF: ##, p<0.01.

Between group post-hoc comparisons siPax3 vs. BDNF/Pax3/Pax3+BDNF: $$$, p<0.0001.

NB: the statistical difference shown in the proximal zone also exist in the distal zone, but for clarity of the figure they have not been added to the graphic.

D: To ensure that the CaBP-VGLUT2 colocalisation did represent CF-PC synapses, we recorded CF induced EPSCs in PCs from graft explants following olivary LV-Pax3 transfection. Superimposed CF-EPSC traces (left) showing characteristic paired-pulse depression. The immunolabelled image (right) reveals a PC filled with biocytin during the recording and a few colocalised VGLUT2-positive terminals (white arrows).
Bar = 25μm.

E: As a potential mechanism underlying Pax3-induced reinnervation, we observed increased cerebellar PSA-NCAM from 4 days after lesion and olivary LV-Pax3 transfection when compared to intact or lesion vehicle/LV- GFP controls ($F_{11, 117} = 6{,}416$, $p<0.0001$).
Between group post-hoc comparisons LV-Pax3 vs. vehicle: **, $p<0.01$.

Figure 2.1

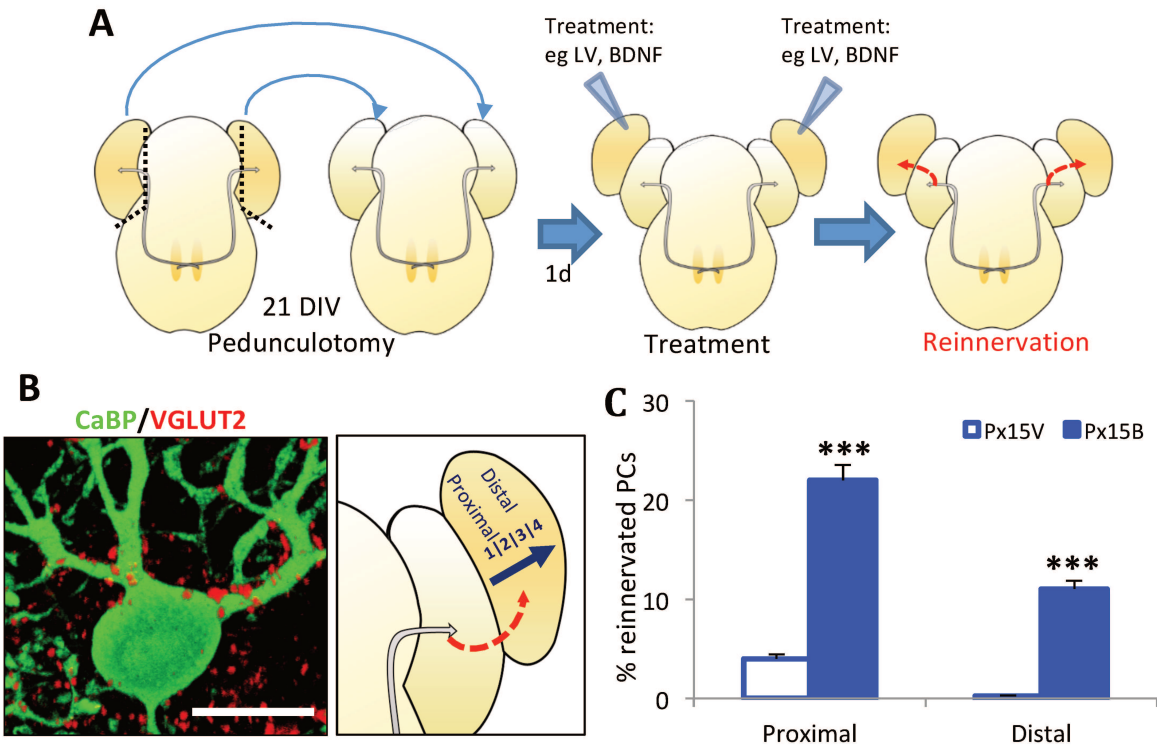

A
Treatment:
eg LV, BDNF
Treatment:
eg LV, BDNF
1d
21 DIV
Pedunculotomy
Treatment
Reinnervation
B
CaBP/VGLUT2
Distal
Proximal
1|2|3|4
C
30
20
10
0
% reinnervated PCs
Px15V
Px15B
***
***
Proximal
Distal

Figure 2.2

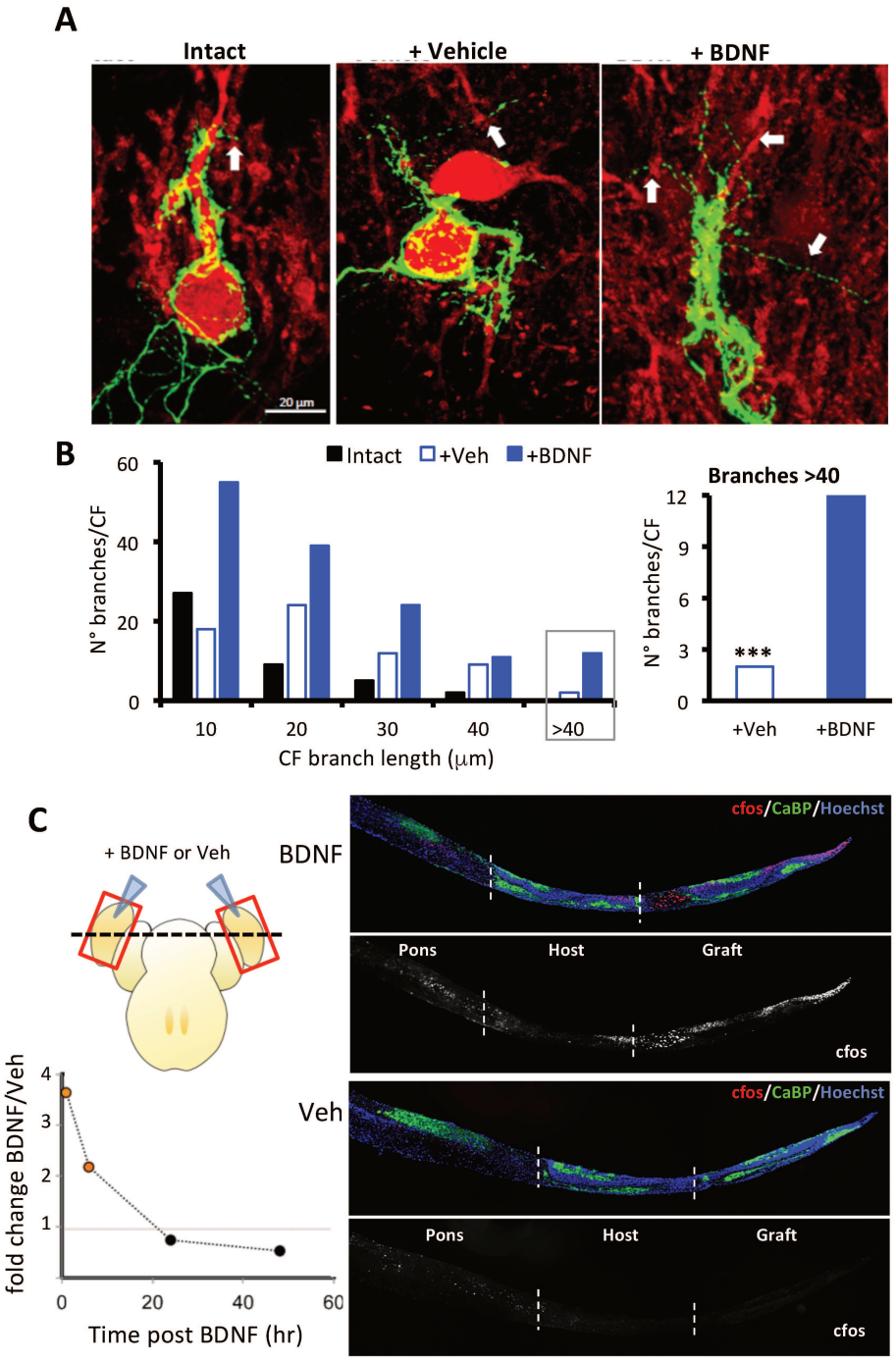

A
Intact
+ Vehicle
+ BDNF
20 µm
B
Intact
+Veh
+BDNF
N° branches/CF
60
40
20
0
10
20
30
40
>40
CF branch length (µm)
Branches >40
12
9
6
3
0
***
+Veh
+BDNF
C
+ BDNF or Veh
BDNF
cfos/CaBP/Hoechst
Pons
Host
Graft
cfos
Veh
fold change BDNF/Veh
4
3
2
1
0
20
40
60
Time post BDNF (hr)

Figure 2.3

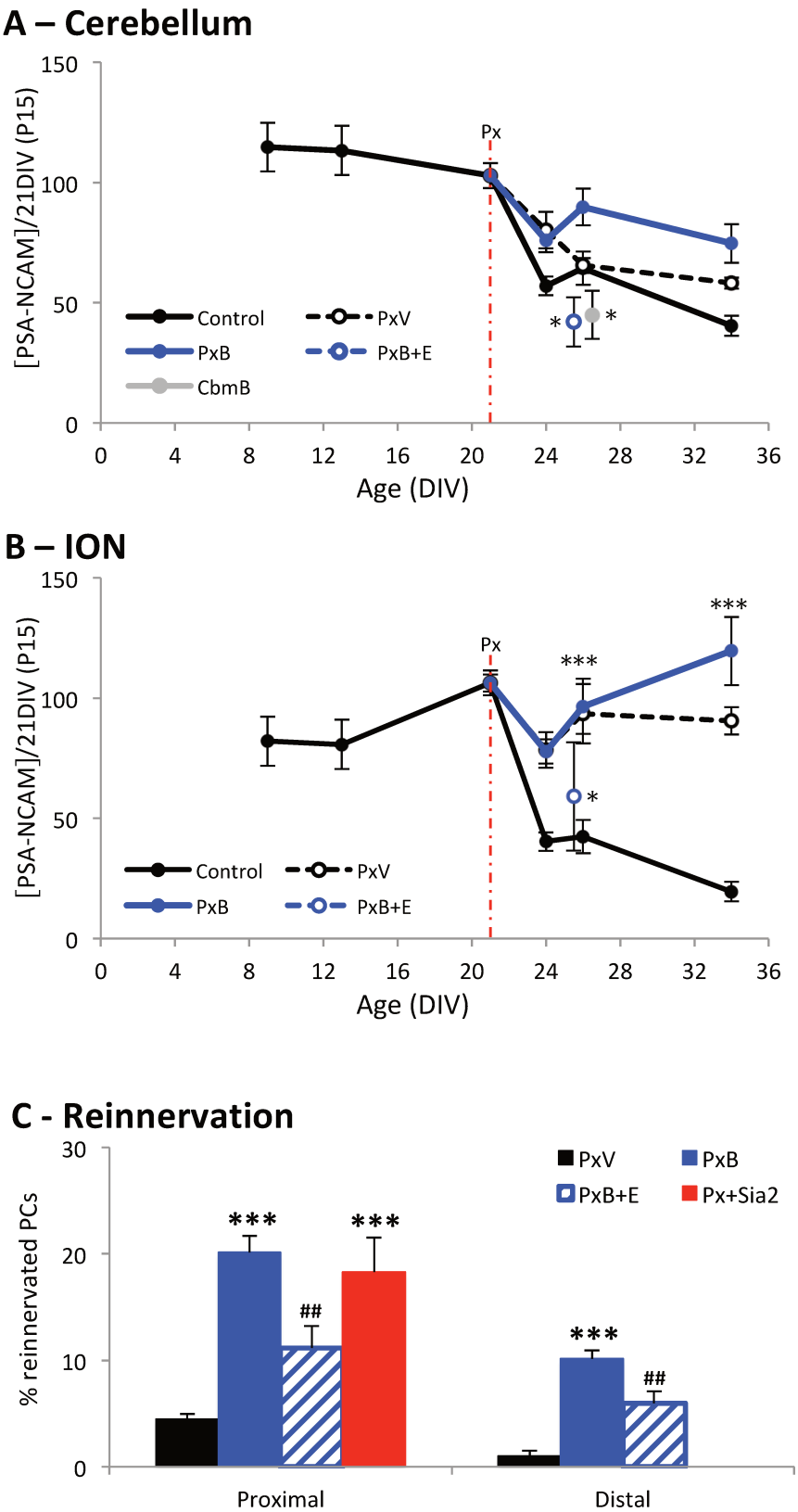

A – Cerebellum
[PSA-NCAM]/21DIV (P15)
150
100
50
0
Px
Control
PxV
PxB
PxB+E
CbmB
*
*
0
4
8
12
16
20
24
28
32
36
Age (DIV)
B – ION
[PSA-NCAM]/21DIV (P15)
150
100
50
0
Px
***
***
*
Control
PxV
PxB
PxB+E
0
4
8
12
16
20
24
28
32
36
Age (DIV)
C - Reinnervation
% reinnervated PCs
30
20
10
0
PxV
PxB
PxB+E
Px+Sia2
***
***
##
***
##
Proximal
Distal

Figure 2.4

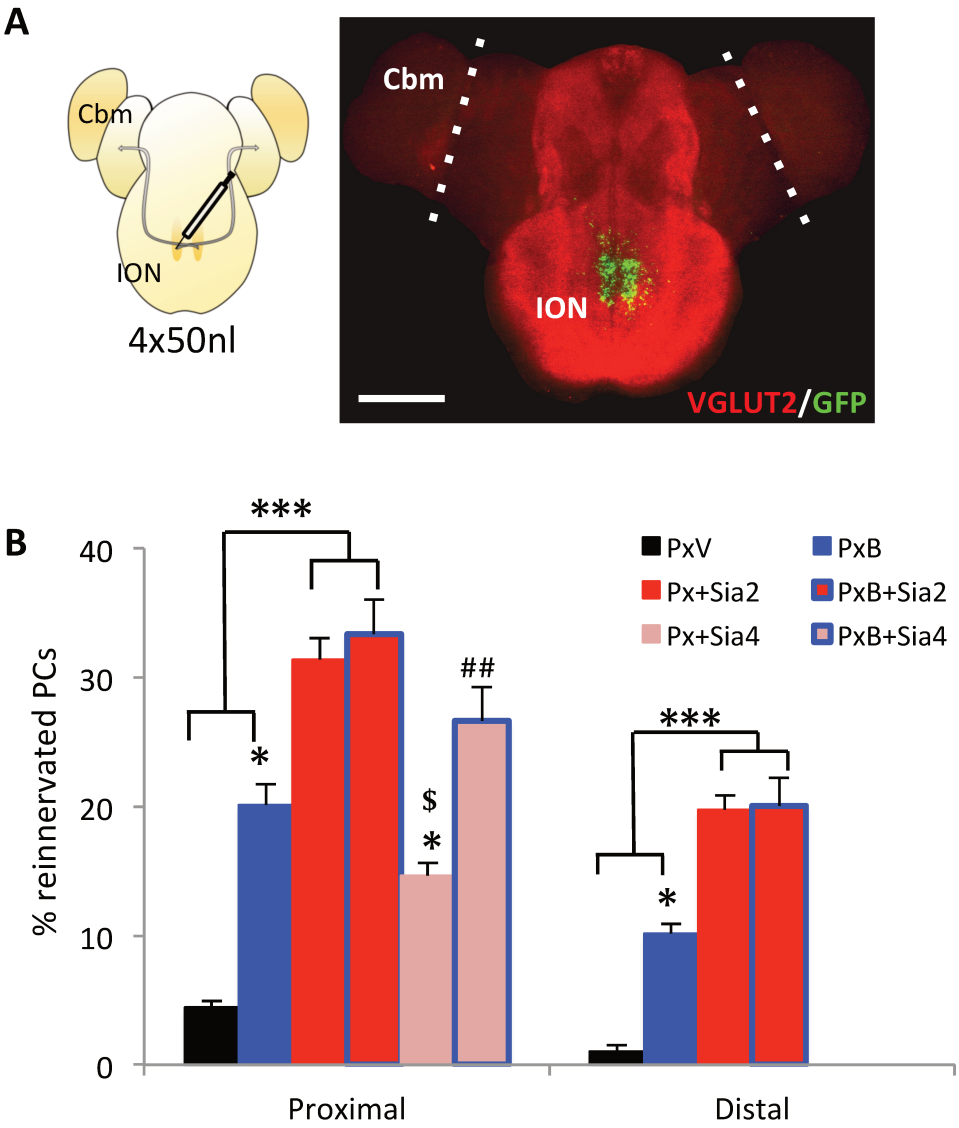
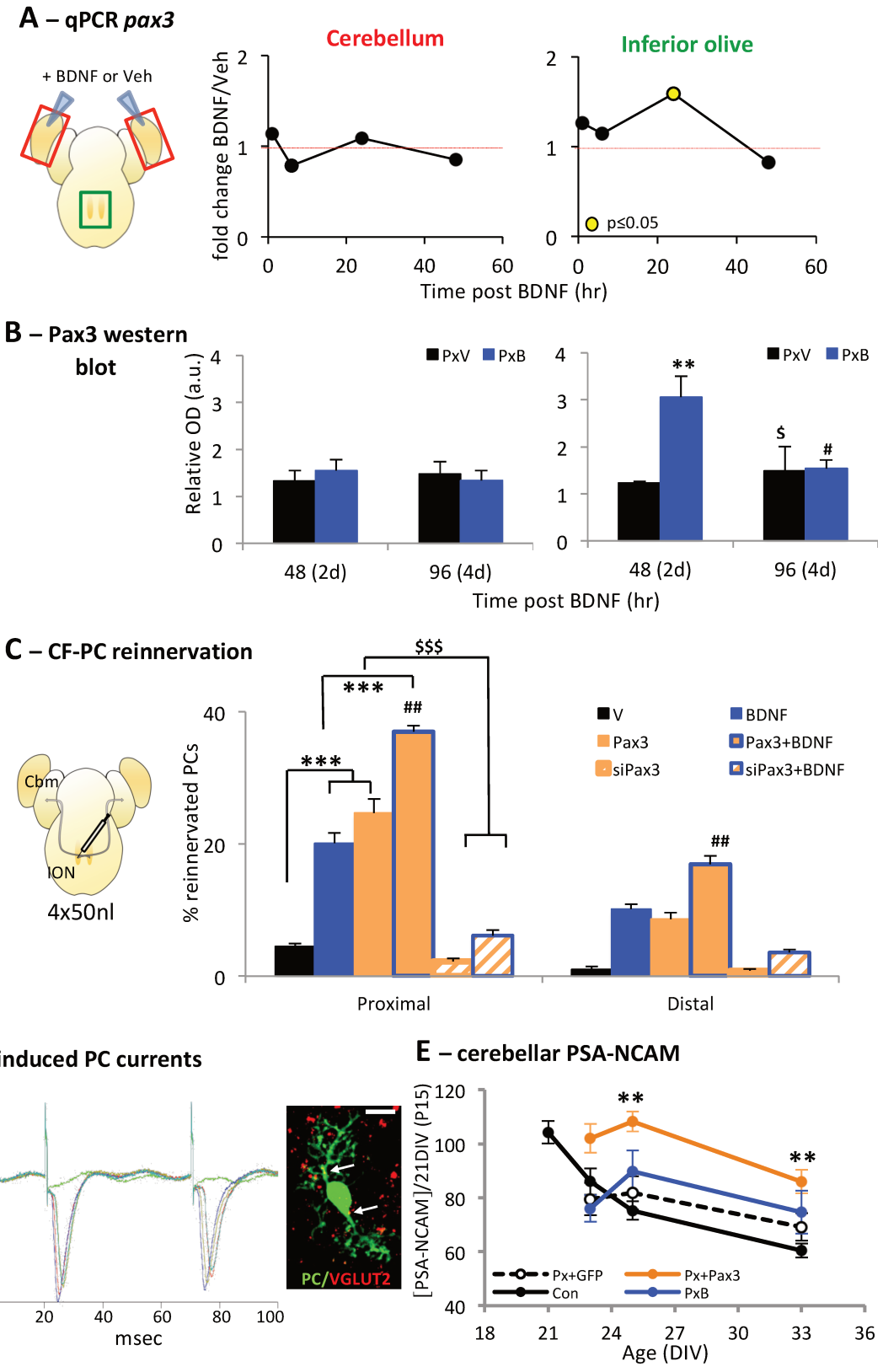

A
Cbm
ION
4x50nl
Cbm
ION
VGLUT2/GFP
B
% reinnervated PCs
40
30
20
10
0
***
*
##
$
*
***
*
PxV
PxB
Px+Sia2
PxB+Sia2
Px+Sia4
PxB+Sia4
Proximal
Distal

Figure 2.5


A – qPCR pax3
+ BDNF or Veh
Cerebellum
Inferior olive
fold change BDNF/Veh
p≤0.05
Time post BDNF (hr)
B – Pax3 western blot
PxV
PxB
Relative OD (a.u.)
48 (2d)
96 (4d)
Time post BDNF (hr)
C – CF-PC reinnervation
Cbm
ION
4x50nl
% reinnervated PCs
V
BDNF
Pax3
Pax3+BDNF
siPax3
siPax3+BDNF
Proximal
Distal
D – CF induced PC currents
Current (nA)
msec
PC/VGLUT2
E – cerebellar PSA-NCAM
[PSA-NCAM]/21DIV (P15)
Px+GFP
Px+Pax3
Con
PxB
Age (DIV)

CHAPTER 3

Results – Article 2

Chapter 3 – Article 2

The role of transcription factor Pax3 during developmental reinnervation of the Olivocerebellar pathway

In the previous chapter we described a potential signalling pathway by which BDNF injection in target tissue can induce axon collateral sprouting in surviving afferents to generate an appropriately targeted reinnervation; specifically, cerebellar injection of BDNF promotes sprouting and outgrowth of olivocerebellar CF axons, leading to reinnervation of Purkinje cells. We identified that the glycoprotein cell adhesion molecule, PSA-NCAM, is involved in this process, being both essential for the axonal outgrowth and able to induce the reinnervation by itself. The involvement of PSA-NCAM in neuronal plasticity is not surprising, as it is known to promote axonal growth and post-lesion collateral sprouting (Zhang *et al*., 2007; Mehanna *et al.,* 2010). However, the finding that Pax3 is also both necessary and sufficient to induce this reinnervation is new. Pax3 is known as a transcription factor involved in early embryonic development, particularly neural differentiation in the neural tube and hindbrain (Mansouri *et al.*, 1996), which includes the inferior olive. Pax3 regulates organogenesis and cellular differentiation within these regions (Mansouri *et al.*, 1996; Terzic & Saraga-Babic, 1999) and especially neural crest derivatives including Schwann cell precursors (Kioussi *et al.*, 1995; Blake & Ziman, 2013). Although Pax3 mRNA has been identified within axons (Ji & Jaffrey, 2014) its role in axonal sprouting and/or regeneration has not been previously identified.

It has often been stated that "regeneration recapitulates development" (Filbin, 2006; Harel & Strittmatter, 2006) and given the role of PSA-NCAM in olivocerebellar development (Avella *et al*., 2006) and the early developmental actions of Pax3, we wanted to know whether Pax3 is involved in olivocerebellar development and/or developmental plasticity. To address this question we examined the developmental profile of Pax3 expression, changes in this expression during spontaneous developmental post-lesion reinnervation, and the effect of reducing Pax3 expression on this process.

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The role of transcription factor Pax3 during developmental reinnervation of the Olivocerebellar pathway

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Running title: Pax3 is required for post-lesion reinnervation in the olivocerebellar path

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ABSTRACT

The adult central nervous system (CNS) displays poor functional recovery following lesion, due to inhibitory intrinsic and extrinsic factors. The addition of neurotrophic factors such as brain derived neurotrophic factor (BDNF) can result in reorganisation of neuronal connections through the growth of axon collaterals, which can promote functional recovery. This effect has been identified in the adult olivocerebellar pathway. We have observed (Results Chapter 2) roles for the cell-adhesion molecule, PSA-NCAM, and the transcription factor, Pax3, in BDNF-induced post-lesion repair in our *ex vivo* model.

In contrast to the adult, post-lesion plasticity involving axon collateral outgrowth and neural circuit reorganisation occurs spontaneously in the immature CNS. In the olivocerebellar path this reinnervation requires endogenous BDNF signalling (Sherrard *et al*., 2009). Thus the aim of this study is to identify mechanisms underlying developmental post-lesion olivocerebellar axonal outgrowth and cerebellar reinnervation, and a possible connection to those mechanisms underlying adult BDNF-induced reinnervation. We showed that a high concentration of PSA-NCAM is necessary for this reinnervation in the immature system. We also observed a 2-fold increase of Pax3 expression in the immature inferior olive 6 hours after olivocerebellar lesion. In order to determine whether this increase was necessary for reinnervation, experiments to knockdown Pax3 in the inferior olive were performed. Results showed a decrease in the number of reinnervated PCs following Pax3 knockdown compared to the controls. Understanding the role of Pax3 in developmental reinnervation of the olivocerebellar pathway and its relationship to the same process in the adult system may provide insight into mechanisms that might be utilised as potential treatments in neural circuit repair after injury.

Keywords: Reinnervation; olivocerebellar path; climbing fibre; collateral sprouting; Pax3; PSA-NCAM

INTRODUCTION

The central nervous system (CNS) has a high degree of precision in its connections, which is essential for the correct function and precise regulation of motor or cognitive behaviours (Williams, 2010). After an injury, any new connections that are formed must also be precise to permit functional recovery (Smith *et al.,* 2007). Unfortunately, the intrinsic properties of neurones and their cellular environment significantly limit the capacity of axons to regenerate and the nervous system to repair itself (Dulin *et al.*, 2015; Dixon & Sherrard, 2006; Filbin, 2006; Willson *et al.,* 2008). Furthermore, pathway complexity makes directing axons to their correct target challenging and further complicates repair following neural injury. Understanding the brain's wiring and the key molecules driving the process of innervation and post-injury reinnervation is of paramount importance in any attempt to increase the brain's capacity for repairing connectivity following injury.

In contrast to the adult, axon collateral sprouting through white matter tracts to re-form projections with appropriate afferent-target connections can occur in the injured neonatal CNS (corticospinal, Hicks & D'Amato, 1970; corticorubral, Naus *et al*., 1987; vision, Spear, 1995; cerebellum, Zagrebelsky *et al*., 1997). The new circuits compensate motor (SC, Weber & Stelzner, 1977; cerebellum, Dixon *et al*., 2005) and cognitive (Levine *et al*., 1987; Willson *et al*., 2007) deficits, in proportion to the specificity with which they recreate the original circuit (vision, Finlay *et al*., 1979; cerebellum, Gramsbergen & Ijkema-Paassen, 1982; Willson *et al*., 2007). This plasticity decreases progressively with maturation, thus understanding the mechanisms underlying developmental neuroplasticity may allow it to be recreate in the mature CNS in order to may improve recovery following injury.

The olivocerebellar pathway provides a good experimental model for these studies. The structure, topography, function and development of this pathway have been extensively described providing a template against post-lesion repair can be compared. Inferior olivary nuclear (ION) axons cross the midline, ascend through the medulla and enter the cerebellum through the inferior cerebellar peduncle to terminate on Purkinje cells (PCs) as climbing fibres (CFs) (Lohof, *et al.,* 2005; Sugihara, 2006); organised with precise parasagittal topography (Sugihara *et al*., 2001). This path regulates motor learning (Apps & Lee, 2002) and spatial cognition (Meignin *et al*., 1999; Rondi-Reig & Burguière, 2005). The olivocerebellar pathway also shows post-lesion repair, depending on its maturation. Following

unilateral lesion to the path (pedunculotomy, Px), new axons can arise from the unaffected ipsilateral inferior olive to reinnervate the denervated hemicerebellum. (Sherrard *et al,* 1986; Zagrebelsky *et al,* 1997; Sugihara *et al*., 2003; Lohof *et al.,* 2005) (Fig 3.1). During development this reinnervation occurs intrinsically (Sherrard *et al,* 1986; Zagrebelsky *et al,* 1997) and in the mature nervous system it can be induced by growth factors, such as Brain-Derived Neurotrophic Factor (BDNF; Dixon & Sherrard, 2006; Willson *et al,* 2008), a neurotrophin that facilitates axonal growth and regeneration (Butenschon *et al.*, 2016; Letellier *et al.*, 2007).

Identifying the mechanisms that BDNF triggers is vital in understanding how the olivocerebellar pathway (and potentially other BDNF-responsive pathways; Fawcett, 2006) responds to injury. The BDNF receptor, TrkB, closely interacts with a cell adhesion molecule, PSA-NCAM, which is associated with axonal growth and post-lesion regeneration (Mehanna *et al.,* 2010). PSA-NCAM consists of polysialic Acid (PSA), a carbohydrate, bound to the Neural Cell Adhesion Molecule (NCAM) on neurons (Guirado *et al.*, 2016). Studies indicate the involvement of PSA-NCAM in the regeneration of motor axons in the PNS (Franz *et al.,* 2005). Furthermore, several studies have illustrated that the sensitivity of the cells to BDNF depends on PSA-NCAM (Vutskits *et al.*, 2001; Quartu *et al.,* 2010), which introduces a potential molecular pathway of BDNF-induced reinnervation. Both PSA and NCAM are known to be upregulated following nerve injury in the peripheral nervous system in association with motor axonal regeneration, as well as correct target selection (Franz *et al.,* 2005). We have shown (Results: Chapter 2) that in our reinnervation system, cerebellar treatment with BDNF upregulates PSA-NCAM. This raises the question of what is the link between BDNF and PSA-NCAM, and whether other molecules contribute to the reinnervation processes.

Pax3 is a transcription factor known for its involvement in the formation of the neural tube and neural crest (Kioussi and Gruss, 1994). In addition, Mayanil *et al.* (2001) reported that overexpression of Pax3 can up-regulate expression of the PSA synthetic enzyme and thus increase PSA-NCAM (Mayanil *et al.* 2000). We have shown that BDNF, PSA-NCAM and Pax3 interact in important ways to promote post-lesion reinnervation in the mature olivocerebellar pathway (Results: Chapter 2). Since BDNF is required for developmental CF innervation (Sherrard *et al.,* 2009) and Pax3 is important in neural development (Mayanil *et al*, 2001), we wanted to know whether these molecules are also involved in developmental

reinnervation or in normal olivocerebellar development, and whether Pax3 is part of an injury response programme. Establishing the molecules that take part in adult and developmental reinnervation will give insight on the (re)formation of this pathway, as well as possibly other pathways of the brain. Such information may give insight into mechanisms that could be applied for treatment of neural injury.

MATERIAL AND METHODS

Experimental Animals

Experiments were performed on Swiss mice under licence from the Comité National d'Ethique pour les Sciences de la Vie et de la Santé, in accordance with European Communities Council Directive 2010/63/EU and NIH guidelines. Pregnant Swiss mice were purchased from Janvier Labs and were sacrificed under isofluorane anaesthesia.

Organotypic Cultures

Experiments were conducted on the olivocerebellar pathway *ex-vivo,* cerebellar explants, extracted from mice embryos at embryonic day 14 (E14, DIV0), as previously described (Chédotal et al 1997; Letellier, *et al.*, 2009). Following the removal of the embryos from the uterus, their brain was dissected and the hindbrain, medulla, pons and the cerebellum were cleared of meninges and cultured on a millicell membrane (0.4μm pore, Merck Millipore, Tullagreen, Ireland) in 5%CO_2 at 35°C. The explant was placed so that the two hemicerebella plates, which are not fused at this age, were adjacent to the hindbrain but not in touch with each other. Culture media of the explants was changed every two days.

Explants were pedunculotomised at 9 DIV (equivalent post natal stage of P3), this procedure entailed the removal of two hemi-cerebella from one explant and their placement adjacent to the hemi-cerebella of a host explant that remained intact, so they can be reinnervated. The following day (10 DIV), the grafted hemicerebella of some explants were treated with 350 IU of neuraminidase Endo-N (AbCys, France) to remove PSA from NCAM.

Viral Transfections

At 6 DIV (P0) the hindbrain explants were injected in the inferior olivary region with either a pooled siPax3/hPax3/Pax3i/GFP lentivirus (LVsiPax3; Gentaur, Paris France) that knocks-down Pax3 expression by 70% (according to the manufacturer) for the experimental condition

or a lentiviral vector encoding enhanced GFP (LVeGFP) under the control of a Phosphoglycerate Kinase (PGK) promoter for the control. Injections took place using a glass micropipette attached to a 1μL Hamilton syringe held in a micromanipulator. In both conditions, explants were transfected with four 50nL injections in the inferior olivary area.

Immunohistochemistry and fluorescence microscopy

For immunohistochemical visualisation of olivocerebellar reinnervation, indirect immunofluorescence was performed 10 days following pedunculotomy. Explants were fixed at 19 DIV (P13) with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (PBS) 4°C for 4 hours. Non-specific binding sites were blocked for two hours prior to antibody incubation, using a 20% normal donkey serum solution in PBS which contained 5% gelatin, 0.25% Triton X-100, and lysine (PBST-G) (Letellier, *et al.* 2007). They were then incubated overnight with primary antibodies mouse monoclonal anti-calbindin (CaBP; 1:2000, Swant) and guinea pig anti-vesicular glutamate transporter 2 (VGLUT2; 1:3000, Chemicon) in PBST-G. The antibodies were chosen because VGLUT2 is expressed in climbing fibres terminals and in the cerebellum calbindin is a marker specific for Purkinje Cells (Letellier *et al.*, 2007). Following rinsing with PBS, the explants were incubated with secondary antibodies AMCA-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-guinea pig (both 1:200, Jackson ImmunoReseach Laboratory) in PBST-G for two hours. Explants were then washed and mounted on glass slides in Mowiol. The grafted hemicerebella were examined using epifluorescence microscopy (DM6000; Leica).

Reinnervation Analysis

Fluorescent z-stack images (45 per hemicerebellum) were taken of the grafted hemicerebella (n=6 for experimental conditions, n=8 for control condition). Images were captured in rows, starting from the edge closest to the host cerebellum and ending at the furthest edge. Typically, there were five rows in each hemicerebellum. Images were analysed using ImageJ and the co-localisation of CaBP and VGLUT2 was considered reinnervation. PCs with and without VGLUT2 were counted and reinnervation is presented as the percentage of PCs colocalised with CFs, i.e. (CaBP+VGLUT2)/CaBP cells. For each graft hemicerebellum, the mean reinnervation score was calculated for the first 2 rows (proximal zone) and rows 3-5 (distal zone).

qRT-PCR

The inferior olivary area was dissected from explants 6h (P3, 9 DIV), 24h (P4, 10 DIV) and 48h (P5, 11 DIV) following pedunculotomy at P3 (9 DIV), and at the same ages from non-pedunculotomised controls. For each time-point and condition n=6, although each point required pooled tissue from 3 explants. RNA was then extracted from the inferior olivary area using Trizol (according to manufacturer's protocol, Life Technologies (Chomczynski & Sacchi, 1987) and RNA concentration was measured by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) before being stored at -80°C. 200ng of total RNA was reverse transcribed in 20µl using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was amplified on a LightCycler® 480 (Roche Applied Bioscience, USA) in 10 µl reaction volume using SYBR Green I Master Mix (annealing temperature 58 °C, 50 cycles). TUBB5 was used as a reference gene and interest genes included ST8Sia-II, GAP-43, given its role in axon collateral sprouting (Dinocourt*, et al*., 2006) and Pax3 in order to identify its possible role in developmental reinnervation. Gene sequences were as follows:

GAP43:	Forward (F1): GAGTGAGCAAGCGAGCAGAAAAGA TM = 59,0
	Reverse (R1): CGGAAGCTAGCCTGAATTTTGGTC TM = 60,0
ST8Sia-II:	Forward (F1): AGCACAATGAACGTGTCCCAGAA TM = 59,1
	Reverse (R1): GAGCCAGGTTGCACCTTATGACA TM = 59,2
Pax3:	Forward (F1): AGCAAACCCAAGCAGGTGACA TM = 59,2
	Reverse (R1): AGGATGCGGCTGATAGAACTCACT TM = 59,6
TUBB5:	Forward (F1): GCTAAGTTCTGGGAGGTGATAAGCG TM = 58,0
	Reverse (R1): CCAGACTGACCGAAAACGAAGTTG TM = 58,0

All samples were amplified in triplicate and the mean was used to calculate gene expression in each tissue sample. Raw data were pre-processed with Lightcycler 480 software (Roche Applied Bioscience, USA) according to Pfaffl's equation. Target gene expression was normalised to the harmonic mean of 2 housekeeper genes

Statistics

Data were analysed for normality and homogeneity of variance and then groups were compared by ANOVA and post hoc Bonferroni test, using GraphPad Prism 7. For those that did not reach normality or homogeneity we used non-parametric ANOVA, Kruskal-Wallace and Dunns tests.

RESULTS

With its capacity to generate axon collaterals that form an alternative path that functionally compensates following its injury, the immature rodent olivocerebellar pathway (OCP) provides a useful model to study mechanism underlying intrinsic developmental post lesion plasticity (Sherrard *et al*., 1986; Sugihara *et al*., 2003; Willson *et al*., 2007). We used our *ex vivo* model of the OCP (Chédotal *et al*., 1997; Letellier *et al.,* 2009), to study modifications of gene expression post-Px and PC reinnervation.

Pax3 upregulation in the inferior olivary region after pedunculotomy in developing hindbrain explants

To assess whether the molecules involved in developmental post-lesion plasticity of the OCP overlap with those identified in BDNF-induced reinnervation in the mature system, we performed gene expression analysis on the inferior olive of lesioned-coculture explants, selecting target genes based on observations described in Results: Chapter 2. We induced post-lesion repair in hindbrain explants by placing a denervated hemicerebellum in contact with an intact host hemicerebellum, at the equivalent of post-natal day 3 (P3, 9 DIV; Fig 3.2A), so that CFs in the host could grow into the denervated graft hemicerebellum and reinnervate the denervated PCs, as occurs *in vivo* (Zagrebelsky et al., 1997; Sugihara et al., 2003; Wilson et al., 2007).

Results from qRT-PCR indicate that neither *gap43* nor *sia2* change their expression during the 3-day period of the experiment (9-12 DIV; equivalent P3-P6), nor was there any change in expression between olivary regions from lesioned and intact explants (Fig. 3.2B). This may be simply because both GAP43 and Sia2 are highly expressed in the developing cerebellum (Console-Bram *et al.,* 1996; Ong *et al.,* 1997) and small post-lesion changes were not detectable. In contrast, *pax3* shows a developmental expression change in control tissue, significantly increasing between developmental stages P3 and P4 (ANOVA, $F_{4,23}$ = 4,632, $p<0.01$; post-hoc P3 vs P4, $p<0.05$; Fig. 3.2D). Interestingly, Pax3 mRNA expression was rapidly upregulated 6 hours following Px (ANOVA, $F_{2,13}$ = 4,672, $p<0.03$), with the ratio between the two conditions indicating a 2-fold increase of Pax3 expression 6h following pedunculotomy. No difference was seen between the experimental and control condition after 24 and 48 hours, indicating that this rapid increase of Pax3 expression 6 hours after injury plateaus in the two following time points; and the developmental increase of Pax3 in control tissue at P4 brought expression in the 2 groups to the same level.

Post-lesion reinnervation after pedunculotomy in immature hindbrain explants requires PSA-NCAM

As Pax3 was upregulated acutely after pedunculotomy, as occurs following lesion and BDNF treatment in the mature system, we decided to examine molecules implicated in that process: PSA-NCAM and Pax3 (see Results: Chapter 2).

To examine whether PSA-NCAM plays a permissive role for post-lesion plasticity during early developmental stages, grafted hemicerebella in contact with host explants were treated with Endo-N to remove PSA at 24h and 7days post lesion. The percentage of reinnervated PCs was measured after 10 days. Control explants at this stage have robust spontaneous reinnervation post-Px, achieving over 50% PC reinnervation; a result which is similar to *in vivo* (Sherrard et al 1986; Zagrebelsky et al 1997). Grafted hemicerebella treated with EndoN had reduced PC reinnervation (ANOVA $F_{(3,34)} = 33.3$, $P<0.0001$) in both proximal and distal regions (Fig 3.3). This result confirms a similar requirement for PSA-NCAM in post-lesion repair during BDNF-induced repair later in development (Results Chapter 2).

Knock-down of olivary Pax3 expression decreases post-lesion reinnervation of PCs in developing explants

In order to identify whether the observed post-lesion increase of Pax3 was a part of the olivary reinnervation process and therefore necessary for olivocerebellar reinnervation, we compared reinnervation in lesioned explants transfected with control lentivirus (LVeGFP) and those transfected with a knock-down virus (LVsiPax3) which reduced Pax3 expression by 70% (manufacturers data). Compared to the extensive reinnervation in LVeGFP explants those transfected with LVsiPax3 had significantly decreased reinnervation compared to controls (ANOVA $F_{3,28} = 14.7$, $p<0.0001$; Fig 3.4A) and this was most striking in the proximal zone. When this data was examined in more detail, with each 250μm zone being kept separate, the significant difference between groups remained (LVsiPax3 vs. LVeGFP; two-way ANOVA $F_{1,50} = 33.3$, $p<0.0001$). Although reinnervation decreased with increasing distance from the host-graft junction in the LVeGFP control group ($F_{4,29} = 11.2$, $p<0.0001$), this was not the case in the LVsiPax3 group ($F_{4,21} = 0.34$, $p=0.85$; Fig 3.4B), with reinnervation being globally reduced throughout the graft hemicerebellum. This suggests that Pax3 specifically promotes axon collateral formation and that once formed their elongation is unchanged, as PC reinnervation was not further reduced with increasing distance of axonal extension.

DISCUSSION

In this study we investigated mechanisms of post-lesion repair in the developing olivocerebellar pathway. Our results indicate that PSA-NCAM is necessary for this repair. In addition, Pax3 plays an important role in this reinnervation: we observed a rapid transient increase in Pax3 expression following olivocerebellar lesion at the time when CF reinnervation is beginning; and showed that Pax3 depletion at the time of pedunculotomy reduces the capacity of the developing inferior olive to reinnervate denervated PCs. These data together with our results in the mature system (Chapter 2, Article I), suggest that post-lesion repair in both the developing and mature olivocerebellar path use the same injury-response processes.

Pax3 is involved in developmental reinnervation

The importance of Pax3 in axonal outgrowth or synapse formation has never been previously established. However our observations of a post-lesion increase in Pax3, and a decrease in reinnervation when Pax3 is knocked down, point to the role of Pax3 in axonal outgrowth and developmental reinnervation following injury. Although these findings are novel, Pax3's role in tumour invasiveness, as identified in paediatric alveolar rhabdomyosarcoma (ARMS; Brown *et al.*, 2016), is consistent with our data. ARMS is a highly aggressive skeletal muscle malignant tumour (Brown *et al.,* 2016; Lam *et al.,* 1999) characterised by an oncogene translocation that results in much higher transcriptional activation by mutated Pax3 (Lam *et al.,* 1999). The process of tissue invasion by cancer cells requires the extension of cellular processes, into which the cytoplasm migrates (Mehlen *et al.,* 2011); this process is similar to the extension of filopodia by the axon growth cone during axonal growth (Mehlen *et al.,* 2011).

Innervation and reinnervation mechanisms; similarities between immature and mature systems

The results described in Chapter 2 show that the addition of BDNF to the grafted mature hemicerebellum after pedunculotomy induces an increase of Pax3 in the inferior olivary area, as we have demonstrated for the immature system. We have also shown that inferior olivary Pax3 overexpression is capable of inducing CF reinnervation in the mature olivocerebellar path in the absence of BDNF. Therefore, the very rapid increase of Pax3 expression post pedunculotomy appears to be linked to the initiation of CF reinnervation in both immature and mature brains.

The findings that the mechanisms underlying both developmental and mature reinnervation are similar, still leaves the question of whether these mechanisms are a recapitulation of normal developmental processes of the olivocerebellar pathway. Given the role of Pax3 in developmental processes such as the formation of the neural tube (Degenhardt *et al.,* 2010), as well as its increased expression in the inferior olive at the age at which CF-PC synapses are forming (P3-4; Altman, 1972), it could be suggested that both developmental and mature reinnervation are a recapitulation of events taking place during normal development. However, not all events during reinnervation of the mature system are the same. Notably, in experiments overexpressing Pax3 in the ION of mature explants, PCs are not multi-reinnervated by new CFs (Chapter 2, Article I) as they are during development (Lohof *et al,* 2005). Furthermore, when reinnervation in the mature system takes place following the addition of BDNF, it does so with a lack of multi-reinnervation (Letellier *et al.,* 2007, 2009), indicating a difference between normal developmental processes and reinnervation processes, as it is the state of PC maturation which controls its innervation processes. This suggests that the developmental mechanisms are similar but altered in reinnervation, and that in the olivocerebellar pathway Pax3 plays a role of an injury-response gene, primarily involved in axonal outgrowth. In order to better understand the role of Pax3 in innervation vs. reinnervation, future experiments might include the overexpression or knockdown of the factor earlier during development (E17), when CFs first enter the cerebellum (Chédotal and Sotelo, 1993) and observe the processes of CF and PC development.

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FIGURE LEGENDS

Figure 3.1 Post-pedunculotomy olivocerebellar reinnervation *in vivo*

A: Following left inferior cerebellar pedunculotomy in the perinatal period (*; e.g. P3) the axotomised pathway degenerates and new axons arising from the intact inferior olive cross the cerebellar midline to reinnervate denervated Purkinje cells (dotted line).

B: After unilateral pedunculotomy in the adolescent or adult brain similar transcommissural olivocerebellar reinnervation can be induced by growth factors such as BDNF. This reinnervation is less extensive than that induced during development.

* = Px site; ION = inferior olivary nucleus. (Modified from Willson et al 2008)

Figure 3.2 Changes in inferior olivary gene expression in the reinnervating inferior olive following pedunculotomy and coculture.

A: Schema showing how the pedunculotomy and coculture procedure was done. The two hemicerebellar plates are removed from one explant (dotted lines) and placed adjacent to the cerebellum of another (blue arrows). Climbing fibre axons (grey lines) sprout collaterals (red dotted arrows) into the denervated graft tissue. The green square outline the 2 inferior olives.

B: Relative expression of *gap43* 6h, 24h and 48h in intact normal controls (basal) and after lesion and coculture 9 DIV, equivalent to P3 (PxP3). The ratio of lesioned to basal expression (i.e. fold change, on the right) does not change at all (grey dotted line represents when the gene is expressed identically in the 2 conditions).

C: Relative expression of *sia2* 6h, 24h and 48h in intact normal controls (basal) and after lesion and coculture 9 DIV (PxP3). The ratio of lesioned to basal expression (on the right) does not change.

D: Relative expression of *pax3* during development (left side) increases significantly between 9-10 DIV (equivalent P3 to P4; $F_{4,23} = 4{,}632$, $p<0.01$) and remains relatively stable until 21 DIV (P15). Post pedunculotomy (right side) *pax3* expression increases very rapidly ($F_{2,13} = 4{,}672$, $p<0.03$), so that at 6h post-lesion there is a 2-fold greater *pax3* expression (lower panel) compared to controls and then remains stable. Yellow circle indicates a significant fold change (χ^2 test, $p<0.05$).

Within group comparisons: ## = $p<0.01$; between group comparisons: * = $p<0.05$.

Figure 3.3 Reduction of PSA-NCAM reduces Purkinje cell climbing fibre reinnervation
Explants were pedunculotomised at 9DIV (P3) and treated with EndoN to lyse PSA from NCAM. The amount of CF-PC reinnervation was significantly reduced by this treatment (ANOVA $F_{3,34} = 33,33$, $p<0.0001$) in both proximal and distal zones. *** = $p<0.0001$

Figure 3.4 Pax3 knockdown decreases inferior olivary axon growth and Purkinje cell reinnervation throughout the graft hemicerebellum
A: After lesion and coculture at 9 DIV, Purkinje cell reinnervation was reduced in the proximal zone of explants treated with LVsiPax3 to knock-down Pax3 expression (ANOVA $F_{3,28} = 14.7$, $p<0.0001$). *** = $p<0.0001$
B: In LVeGFP-lesioned explants, there is extensive reinnervation proximally which decreases with distance from the host-graft junction (One-way ANOVA $F_{4,29} = 11.22$, $p<0.0001$). After Pax3 knockdown, CF-PC reinnervation is decreased throughout the cerebellar plate with no proximal-to-distal gradient (One-way ANOVA $F_{4,21} = 0.34$, $p=0.86$). % PC reinnervation in LVeGFP explants compared to row1 or 2: ## = $p<0.01$; ### = $p<0.001$.

Figure 3.1

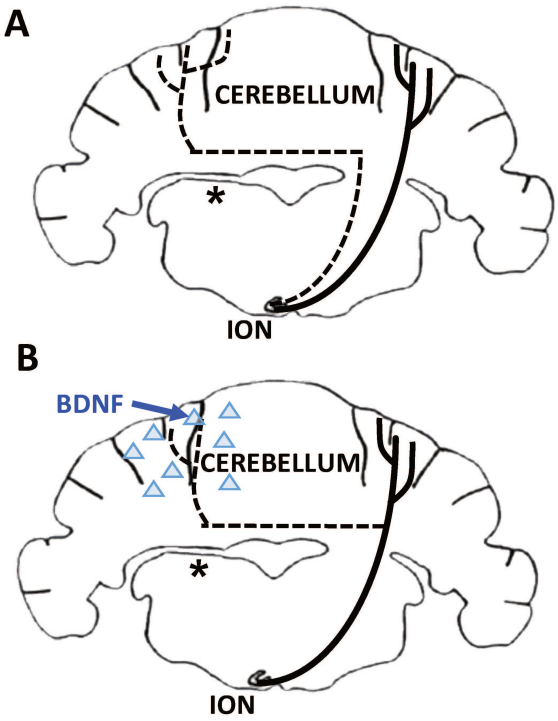


Figure 3.2

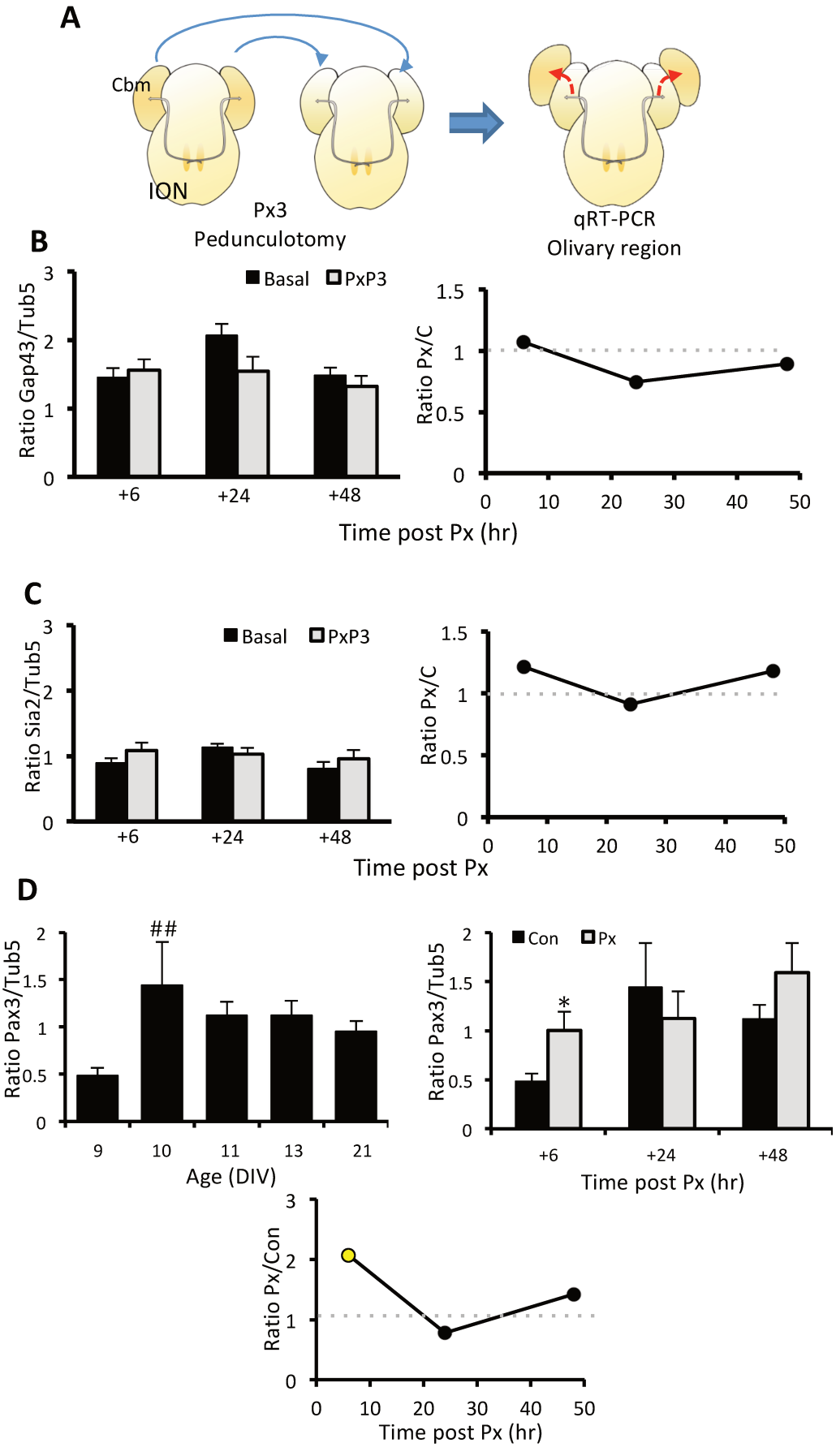

A
Cbm
ION
Px3
Pedunculotomy
qRT-PCR
Olivary region
B
Basal
PxP3
Ratio Gap43/Tub5
Ratio Px/C
+6
+24
+48
Time post Px (hr)
C
Basal
PxP3
Ratio Sia2/Tub5
Ratio Px/C
+6
+24
+48
Time post Px
D
##
Ratio Pax3/Tub5
9
10
11
13
21
Age (DIV)
Con
Px
*
Ratio Pax3/Tub5
+6
+24
+48
Time post Px (hr)
Ratio Px/Con
Time post Px (hr)

Figure 3.3

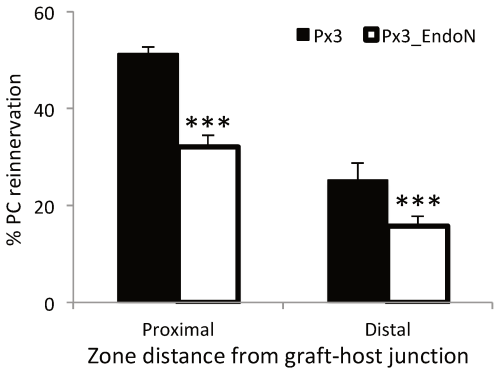
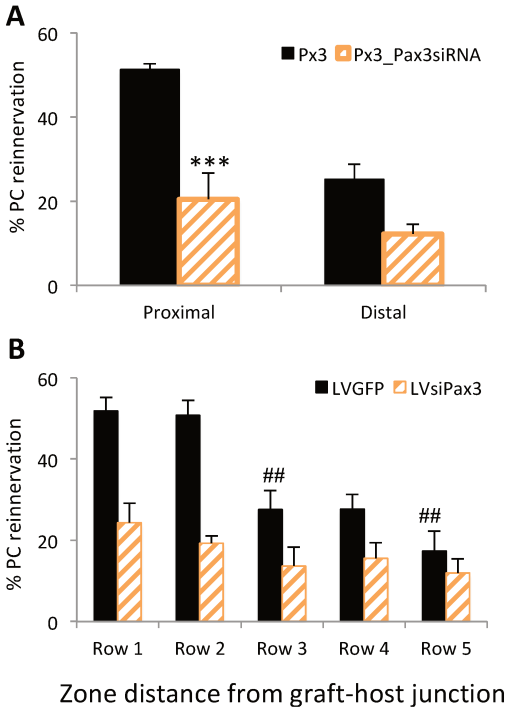

60
40
20
0
% PC reinnervation
Px3
Px3_EndoN
***
***
Proximal
Distal
Zone distance from graft-host junction

Figure 3.4


A
60
40
20
0
% PC reinnervation
Px3
Px3_Pax3siRNA
***
Proximal
Distal
B
60
40
20
0
% PC reinnervation
LVGFP
LVsiPax3
##
##
Row 1
Row 2
Row 3
Row 4
Row 5
Zone distance from graft-host junction

CHAPTER 4

General discussion, conclusion and perspectives

Chapter 4 – Discussion, conclusion and perspectives

I - Discussion

I.1- Intrinsic post-lesion plasticity of mature olivocerebellar axons is potentiated by exogenous BDNF into the target cerebellar tissue

We have used our ex vivo model of the olivocerebellar path (OCP) to study the responses of olivary axons (CFs) to the presence of denervated PCs, and the changes in these responses produced by the addition of BDNF. After a lesion of the OCP, the intrinsic capacity of CFs to produce collateral sprouts is considerably enhanced after the addition of BDNF into the denervated target area. Our morphological studies have shown that transverse CF collaterals of mature CF arbors are activated in the presence of a denervated grafted hemicerebellum. The elongation of these collaterals is induced over a limited distance, which is insufficient to allow much CF reinnervation of PCs within the denervated hemicerebellum. However BDNF addition into denervated hemicerebellum increases the growth of transverse branches and allows their elongation over greater distances. These observations suggest that (1) transverse CF collaterals participate in the intrinsic post-lesion plasticity of the mature OCP by responding to signals from the denervated PCs; and (2) BDNF treatment boosts these signals which potentiates the growth of CF branches in the intact host cerebellum so that they grow far enough to reinnervate the deafferented PCs.

Transverse branches are part of normal CF arbor structure in the mature OCP (Sugihara *et al.* 1999), and show dynamic behavior, extending and retracting but not forming synapses (Nishiyama *et al.*, 2007). It has been suggested that CF horizontal branches form collaterals within the molecular layer that are responsible for reinnervating neighboring denervated Purkinje cells after neurotoxic lesion to the mature ION and partial CF ablation from the cerebellum (Aoki & Sugihara 2012; Rossi *et al*. 1991). These horizontal branch collaterals are believed to be regulated by chemo-attractant signals released by denervated PCs (Strata & Rossi 1998). Rossi and co-workers (1991) found that CF branches induced by partial ION ablation only elongate over a few hundred μm, suggesting that the distance from the original CF arbor is a limiting factor. This idea is supported by the observation that intact mature CFs cannot reinnervate the deafferented hemicerebellum following unilateral pedunculotomy of

the adult OCP (Sherrard & Bower, 2001; Dixon & Sherrard, 2006; Willson *et al.*, 2008), in which ION axon collaterals need to grow long distances and cross the cerebellar white matter, which is a not a growth-permissive substrate.

Whether similar "transverse-type" branches are present on other CNS axon terminals is unknown. However, *in vivo* imaging studies of adult rodent and primate brains have identified axonal motility in different regions of the mature intact CNS, including the neocortex (De Paola *et al.*, 2006; Stettler *et al.*, 2006). In the cortex the degree of axon motility was found to be dependant on neuron type, with axons from layer 6 pyramidal cells showing high structural plasticity of their axon terminal branches and boutons (De Paola *et al.*, 2006). This motility could be related to a neuron's capacity to produce a post lesion plastic response. In support of this view, Canty *et al*., (2013) were able to show that individual transected layer 6 axons in the adult mouse brain were able to regrow, while axons from other cortical layers could not. Although the regenerating layer 6 axons did not reach their denervated targets (possibly because the single axon lesion does not generate a strong response from denervated targets or because the targets are too far away, e.g. in the brainstem or spinal cord), the correlation between intrinsic axon motility and post-lesion plasticity may provide new insights into mechanisms of post-lesion neural circuit repair.

The effects of BDNF injection into the denervated cerebellar tissue and the structural response of intact host CFs provide insight into how the intrinsic plasticity of remaining axons can be modulated by signals from the target area. Although BDNF has a well-known role in developmental axonal growth, branching, and synapse formation (Cohen-Cory *et al.*, 2010), the effects do not occur in the same manner in the mature CNS following injury. From studies performed in different models of CNS lesion, especially after spinal cord injury, it is known that the addition or overexpression of BDNF around the damaged tissue (i.e. close to injured axons) does not induce substantial re-growth of injured axons (Liu *et al.*, 2011). However our *ex vivo* observations (article I) show that the local application of BDNF into the denervated hemicerebellum induces CF reinnervation of PCs by increasing the number and length of CF collateral sprouts within the intact host tissue. There are two possible explanations for this effect. First, that the exogenous BDNF diffuses to CF terminals and directly activates them. This hypothesis is unlikely because when we looked for c-Fos expression in our *ex vivo* model following addition of BDNF into the grafted hemicerebellum, we found its expression confined to graft tissue, and not in the adjacent intact host explant; this suggests a restricted local effect of applied BDNF (article I). This is supported by the high density of truncated

TrkB receptors in the cerebellar cortex (Rico *et al.*, 2002; Sherrard *et al.*, 2009), and BDNF's tendency to adhere to cells and the ECM (Park & Poo, 2013) which reduces the likelihood of diffusion into intact tissue. Moreover, the small volumes of BDNF injected into the denervated hemicerebellum *in vivo* (Sherrard & Bower, 2001; Dixon & Sherrard, 2006; Willson *et al.*, 2008) are at some distance from the intact hemicerebellum (1-2 mm in the mouse) and therefore the amount of BDNF diffusing to the intact side is likely to be minimal. The second alternative is that that exogenous BDNF modulates signaling from the denervated target PCs. This modulated signaling from the target cells would then increase the plasticity demonstrated by intact CFs in the host cerebellum. Further studies are needed in order to identify a molecular link between BDNF and denervation-related cues, but the observed rapid formation and elongation of CF branches after BDNF treatment into denervated hemicerebellum, compared to non-treated denervated controls, supports the idea of a potentiation of the PC "call" to these CF branches.

I.2- BDNF-induced reinnervation mechanism involves afferent PSA-NCAM and transcription factor Pax3

The optimisation of the explant OCP model allows us to differentiate between contributions of the afferent CF axons and target PC neurons at the molecular level during BDNF-induced reinnervation in the mature OCP. By manipulating, pharmacologically and/or genetically, the expression of our candidate molecules in each synaptic component of the OCP we were able to identify the participation of PSA-NCAM and Pax3 in the post-lesion plasticity induced by exogenous BDNF.

I.3- BDNF-induced PSA-NCAM expression is necessary for their reinnervation of PCs

As mentioned previously (Chapter 1, **III.2**), intrinsic post-lesion CF plasticity activated by neurotoxic olivary lesion or grafted cerebellar tissue is only sufficient for reinnervation of immediately adjacent denervated PCs. However after addition of exogenous BDNF to the denervated hemicerebellum post-pedunculotomy, the intact OCP can extend transcommisural collaterals through the cerebellar white matter and reinnervate denervated PCs (Sherrard & Bower, 2001; Dixon & Sherrard, 2006; Willson *et al.*, 2008). In our *in vitro* system we showed that PSA-NCAM increases principally in the ION after BDNF injection into the

denervated hemicerebellum; we suggest that this molecule (PSA) is important for enhancing the plastic potential of the afferent CF in the mature OCP. Notably, axon elongation and growth through a non-permissive environment before reaching the appropriate denervated PCs is dependent on the expression of PSA-NCAM on or near the growing CF collaterals (cf reinnervation when PSA-NCAM is increased in either the cerebellum or inferior olive; Article I, Figures 2.3 & 2.4). Our two main observations in support of this hypothesis are: (1) injection of exogenous BDNF into an isolated hemicerebellum does not increase levels of PSA-NCAM therein, but PSA-NCAM does increase if the hemicerebellum is in contact with an intact host cerebellum from which reinnervating OCP axons can invade; (2) the overexpression of the enzyme ST8SiaII in the host ION is sufficient to induce CF-PC reinnervation; (3) overexpression of this enzyme in the target cerebellum induces much less reinnervation than when it is expressed in the ION (Article I, 20% versus 30%, $p < 0.01$; compare Figures 2.3 C and 2.4 B).

The expression of PSA-NCAM in collateral branches has been suggested to play a role in their elongation and appropriate target cell recognition during development of different neuronal circuits (Bonfanti, 2006; Gascon *et al.*, 2007), probably by regulating cell-cell or cell-substrate interactions (Rutishauser, 2008). This role has been evaluated in post-lesion mechanisms which become activated in the peripheral nervous system (PNS) following injury: expression of PSA-NCAM on regrowing motor axons is necessary for crossing the lesion site (inhibitory environment), and for selective reinnervation of their denervated muscle targets (Franz, 2005). Interestingly, it has been shown that PSA-NCAM expression in re-growing axons can be further modulated externally by electrical stimulation over damaged peripheral nerves, which allows even more regenerating axons to correctly reach their denervated targets (Franz *et al.*, 2008).

Unfortunately not much is known about the cellular mechanisms regulating the addition of PSA-NCAM to the membrane of growing axons during development or in adult state; this information would help to better understand how the BDNF-mediated post lesion plasticity of mature OCP regulates the PSA-NCAM levels in CF branches. Recently, Van Battum and co-workers (2014) have shown that PSA-NCAM expression in the growth cone membrane of mossy fibres in the developing mouse hippocampus is mediated by secretory vesicles containing PSA-NCAM and another IgCAM, L1. Membrane targeting of these vesicles is regulated by the enzyme MICAL-1, and is essential for the proper targeting and final lamina-

specific organization of mossy fibre afferents onto their target CA3 pyramidal neurons. Further investigation of this molecular pathway in our system could elucidate regulatory plastic mechanisms activated in the mature post-lesion OCP.

I.4- Pax3 transcription factor has a novel role in BDNF-induced reinnervation of the OCP

I.4.1- ION activation after addition of exogenous BDNF into the denervated hemicerebellum

Regenerative plastic responses following lesion in mature PNS axons, as well as in CNS axons of dorsal root ganglion neurons, require a cell body-mediated upregulation of regrowth associated genes (Mar *et al.*, 2014; Van Niekerk *et al.*, 2016a). In contrast, intrinsic collateral sprouting responses are thought to be locally regulated along the axon, without a cell body reaction (Smith & Skene, 1997; Carulli *et al.*, 2004), and controlled by target-derived mechanism (Caroni, 1997). In our ex vivo explant model of the mature OCP (article I), we found evidence for local regulation of a response in the CF axon terminal: when a denervated hemicerebellum is placed in contact with an intact explant, CFs locally grew transverse branches and there were no changes in expression of growth associated-genes in the ION region. However we also showed that if BDNF is added to this denervated hemicerebellum, a rapid acute response is found in the afferent ION tissue, which includes the upregulation of the transcription factor Pax3, and correlates with long-range CF sprouting and appropriate reinnervation of denervated PCs. These novel findings suggest that BDNF-induced plasticity in the mature OCP is controlled by the ION and involves, amongst others, the activation of Pax3.

I.4.2- BDNF-induced reinnervation depends on transcription of Pax3 in the ION

Our finding that Pax3 is upregulated in the mature ION following addition of BDNF into the deafferented hemicerebellum introduces a novel role for this transcription factor related to axonal function in mammalian neural circuits. This study shows for the first time an axon-related role for Pax3, specifically in a post-lesion plastic mechanism. Whether Pax3 is involved in other physiological axonal functions is still unknown.

Functions of Pax3 during development are well described, particularly in the mammalian

neural crest (Monsoro-Burq, 2015), but its continued expression in the adult nervous system is less well understood, especially whether it is involved in axonal plasticity. Pax genes' expression in the adult CNS may have a key role regulating cellular responses to a dynamic environment (Blake *et al.*, 2008). In fact, continued Pax3 expression in adult progenitor cells is identified with their ability to respond to environmental signals (Lang *et al.*, 2005), which in turn activate the re-expression of the Pax3 gene in differentiated cells (Kioussi, *et al.*, 1995). In addition, in *C. elegans* where axon regeneration occurs spontaneously, a genetic screening study searching for candidate genes involved in this intrinsic process, identified a role for Pax3 in the (normal) process of invertebrate axonal regeneration. RNAi against Pax3 impaired the regenerative capacity of GABAergic axons (Nix *et al.*, 2014), as indeed we observed in our mammalian glutamatergic system (Article I). Together with our observations, we propose that, in axons, Pax3 functions to augment an environmental-induced cell response (i.e. increasing the initial intrinsic CF-TB sprouting) and thus promote axon elongation and/or target selection.

We observed that BDNF treatment and overexpression of Pax3 in ION neurons have additive effects on CF reinnervation of PCs. This suggests that Pax3 overexpression could activate multiple plasticity pathways, as expected for a transcription factor, which reinforce the output of the BDNF-induced mechanism. Concurrently, BDNF-TrkB signalling also induces multiple intracellular signalling cascades, and the current data suggest that each driver (Pax3 or BDNF) primarily activate different but complementary paths. Examples of such signalling paths that are involved in axonal outgrowth include: mTOR and PI3K-GSK3-Smad1 in CNS axon regeneration (Saijilafu *et al.*, 2013; Chen & Zheng, 2014); and cAMP activation can allow re-growing axons to overcome a myelin-related inhibitory environment (Qiu *et al.*, 2002; Gao *et al.*, 2004). Fully understanding these pathways, and their links with Pax3 (if any), will require considerable further investigation; but transcriptome analysis for Pax3 targets during mouse myogenesis have identified several Ephrin genes (ligands and receptors) regulated by Pax3 expression (Lagha *et al.*, 2010). This is of special interest given the role of the Eph-ephrin signalling pathway in axon guidance and target selection, mediating both chemorepulsive and attractive interactions during development of neuronal circuits (Cramer & Miko, 2016). Ephrin ligands are expressed by Purkinje cells and their eph receptors by ION neurons (Sotelo & Chédotal, 2005), although more studies needs to be done to confirm their function in the formation of the OCP.

Given that the intrinsic plastic regrowth of CNS neurons is limited by cell-autonomous and cell-extrinsic factors, it has been proposed that additive treatments, manipulating both cellular and environmental effects, are needed to maximize post lesion repair in different CNS injury models. Given our data showing that that Pax3 increases environmental permissiveness through PSA-NCAM, yet also actively induces olivary axonal outgrowth (cerebellar LV-Pax3 was not effective), further investigation is needed to examine whether Pax3 could bridge these two processes.

I.5- Proposed model for BDNF-induced reinnervation in mature OCP

Our work and published data suggests that Purkinje cells lacking CF innervation release "denervation cues" which activate GAP43-mediated local growth of CF transverse branches (Grasselli *et al.*, 2011; Allegra Mascaro *et al.*, 2013). How CFs detect these denervation signals is unknown, but it has been proposed that constitutively-expressed GAP43 also mediates a target sensor response in afferent neurons, activating a signalling pathway to detect changes in their environments (Wehrlé *et al.*, 2001). Further studies of different pathways downstream of GAP43-activation in presence of denervation cues will allow the dissection of the role of GAP43 in either growth or sensor-related mechanisms.

The addition of BDNF into the denervated hemicerebellum could potentiate target-related denervation signals, which spread to the intact hemicerebellum and activate a cascade of events in CF branches. This signal would rapidly propagate to the cell body of ION neurons, leading to the activation of pro-regrowth transcription program, which involves the upregulation of Pax3. In PNS axons, axotomy induces calcium influx into the axoplasm, which leads to the propagation of calcium waves (mainly released from internal stores) to the soma, activating transcription of regenerative programs (Mar *et al.*, 2014). Even though in our *ex vivo* model afferent CFs are undamaged, fast $Ca2^{+}$ transients induced by action potentials have been observed in CF transverse branches, and are thought to mediate their dynamic motility (Nishiyama *et al.*, 2007). It will be interesting to evaluate in the future whether the $Ca2^{+}$ waves seen in undamaged CF transverse branches could play a role in rapid propagation of denervation signals to the cell body and the activation of an olivary axon-growth mechanism. The activation of post-lesion growth mechanisms in ION neurons leads to the further elongation and pathfinding of CF transverse branch collaterals. The final growth and target PC selection is probably mediated by the expression of PSA-NCAM in olivocerebellar

axon growth cones and/or in the extracellular matrix, which we show is necessary for reaching the appropriate denervated Purkinje cells and thus mediate BDNF-induced reinnervation.

I.6- BDNF-induced reinnervation of the mature OCP recapitulates some developmental plastic mechanisms

An advantage of the *ex vivo* OCP model is that hindbrain explants conserve the main developmental stages of the OCP in culture (Letellier *et al.*, 2009). This feature has allowed us to reproduce the physical disconnection of the OCP during development (Px at P3), and compare the spontaneous plasticity mechanisms of these immature OCPs with the BDNF-induced post-lesion plasticity in the mature state. Interestingly, we have seen that reinnervation in both states (Px3 and Px15+BDNF) share molecular mechanisms: Pax3 has an acute and fast upregulation in the ION in presence of denervated PCs, and reinnervation depends on PSA-NCAM expression.

Previous *in vivo* studies have shown that BDNF-induced reinnervation of the mature OCP reproduces some of the anatomical features seen during developmental reinnervation, such as the formation of transcommissural collaterals from intact afferents, which cross the midline through the cerebellar white matter (Sugihara *et al.*, 2003; Dixon & Sherrard, 2006; Willson *et al.*, 2008). Furthermore, studying developmental reinnervation, Zagrebelsky and co-workers (1997) observed rapid terminal branch outgrowth from the uninjured CF terminal arbors close to the midline and their invasion into the deafferented hemicerebellum within 24 hours post lesion. This fast activation of spontaneous post-lesion plasticity following Px resembles our observations in the *ex vivo* model (Article I), where BDNF-mediated reinnervation in the mature OCP involves the rapid outgrowth and elongation of CF transverse branches. These anatomical correlations, in addition with our molecular findings, support the idea that the BDNF-induced reinnervation in the mature OCP recapitulates steps of developmental plasticity. However, the *in vivo* model is inevitably slightly different from our explants. After the initial CF horizontal branch sprouting, Zagrebelsky and co-workers (1997) also noted that these fibres did not persist, but were replaced by axons that crossed the midline in either the white matter or granular layer and remained until adulthood. Unfortunately, the technical difficulty of following thin reinnervating axon collaterals through the thick explant tissue (approx. 200 μm) means that our data cannot distinguish whether the

horizontal branch sprouting is simply an initial response that initiates a subsequent growth programme in the ION or is the onset of the definitive reinnervation programme.

Axon re-growth mechanisms following lesion have been suggested to be distinct from initial axon growth during development (Mar *et al.*, 2014). However, our findings and those of others suggest that post lesion plastic mechanisms activated in developmental or mature states do share some common pathways. Yaniv and co-workers (2012), using an invertebrate model of CNS lesion, have shown that the mTOR signalling pathway is involved during developmental axon regrowth and also in mature axonal regeneration following injury. Since Pax3 and PSA-NCAM are involved in both spontaneous immature post-lesion plasticity and the plasticity induced by BDNF in the mature OCP, further experiments exploring links between these molecules and the mTOR pathway will be interesting.

The extension of developmental plasticity window to mature states has been considered a main goal to promote recovery from injury in the adult brain (Harel & Strittmatter, 2006; Nahmani & Turrigiano, 2014). Thus understanding BDNF-induced reinnervation mechanisms is important to unravel alternative pathways that could be tested in different CNS circuits and promote approaches to re-establish plasticity after injury. In this context, it is important to recognize the different amounts of CF reinnervation to PCs during developmental reinnervation versus BDNF-induced reinnervation (Article I and II; and *in vivo* data: Angaut *et al*., 1985; Sherrard *et al.,* 1986; Zagrebelsky *et al.,* 1997; Dixon & Sherrard, 2006; Willson *et al*., 2008). Although BDNF-induced reinnervation of the mature OCP shares some molecular mechanisms with developmental post-lesion plasticity, it induces less CF reinnervation. This can be explained in part by the fact that extrinsic factors dramatically change during maturation, and are considered to be important regulators in closing the developmental plastic window (Harel & Strittmatter, 2006; Geoffroy & Zheng, 2014). Even though we have suggested that the expression of PSA-NCAM helps the reinnervating CFs branches to elongate through non-permissive areas and reach denervated PCs, other extracellular matrix (e.g. CSPGs) and myelin-related proteins necessarily will interfere with this process. As mentioned in the introduction myelin-derived proteins signal through multiple receptors to inhibit growing axons and collapse growth cones; these events may compete with post-lesion growth-promoting signals.

An interesting observation from our *ex vivo* model is the large number of elongating transverse branches per CF activated after the BDNF treatment. Whether all of these branches are able to extend longer distances remains to be evaluated, but our reinnervation data suggest that, of the activated transverse branch population, only some are able to reach the denervated PCs. On the other hand, practical technical factors in our model led us to administer BDNF only once, the day after the unilateral injury to the OCP. Whether sustained BDNF treatments at different days post Px could further reinforce the signalling paths shown by our data, and/or recruit other cascades, to increase post-lesion plasticity in the mature OCP remains to be tested.

II- Conclusions and perspectives

During this thesis we have shown that the intrinsic post-lesion plasticity of the mature olivocerebellar pathway *in vitro* is mediated by climbing fibre transverse branches, and this is potentiated by the addition of exogenous BDNF into denervated hemicerebellum in order that they reinnervate the denervated tissue. The mechanism underlying the BDNF-induced CF reinnervation to PCs involves the activation of the transcription factor Pax3 in the afferent inferior olive and upregulation of the cell-adhesion-related molecule PSA-NCAM. Furthermore, this BDNF-activated post-lesion mechanism in the mature olivocerebellar pathway recapitulates steps of the spontaneous post-lesion plasticity observed early in development. The physiological function of this intrinsic OCP plasticity and growth potential is thought to be related to CF activity-dependent competition with parallel fibres in order to conserve their post-synaptic territory on PC dendrites. Thus CFs need to retain a plastic potential to maintain their function, but can also use it in post-lesion compensatory responses (Carulli *et al.*, 2004).

As part of this process, we have identified a novel role for Pax3 in post-lesion plasticity of the OCP. Studies linking Pax3 to axonal outgrowth are very limited, but our observation that Pax3 is only upregulated in association with reinnervation (spontaneously after Px during development when reinnervation occurs spontaneously, article 2; and in the mature system following BDNF which induces reinnervation, article 1) suggests that this transcription factor may be an injury-response gene rather than part of OCP development. Future studies on Pax3's downstream targets, and which are activated during CF-PC reinnervation, will help to dissect the plastic mechanisms involved in compensatory axon collateral outgrowth following neural circuit injury.

Clearly this study raises further questions about post-lesion plasticity and its relation to normal development and the normal functions of the various components studied here. To test whether a single plastic mechanism that is required to maintain CF territory on PC dendrites is also related to post-lesion reinnervation, one could test molecules involved in either process and see how they affect the non-related mechanism; and vice versa, e.g. down regulating the expression of Pax3 in the ION and evaluating the physiological synaptic territory of CFs over PC dendrites. Also, remodelling of CF terminal arbours and synapses has been correlated to learning and memory-related processes (Yuste & Bonhoeffer, 2001; Carulli *et al.*, 2004). It would be interesting to evaluate, in animal models under enriched environments or trained conditions, the dynamism of CF transverse branches and whether they develop synaptic properties, to try to unravel their physiological functions. At the molecular level as well, many questions remain to be answered: What are the BDNF-TrkB signals that potentiate CF terminal sprouting (which takes place before Pax3 and PSA-NCAM are upregulated)? Are they PC-related cues or are other cerebellar neurons involved? To what extent is Pax3 involved in normal OCP development? To unravel these questions the *ex vivo* model of the OCP gives the advantage of having an easy access to our region of interest to then perform expression-change studies, followed by pharmacological or genetic manipulation of different candidates molecules in the region of interest, and further imaging and electrophysiological approaches to evaluate morphological and functional related roles.

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