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Wnt/Fz interactions in the developing Central Nervous System

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A thesis submitted for the degree of Doctor of Philosophy

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Thesis Abstract

Wnt signalling is central to many early developmental processes including embryonic patterning, programmed cell death and cell migration. Recent studies have implicated Wnt signalling in the formation of neuronal connections in the nervous system. *Wnt7a* regulates synapse formation in the cerebellum by inducing presynaptic differentiation characterized by axonal remodelling and presynaptic assembly. A very similar gene, Wnt7b regulates dendritic development in hippocampal neurons, but can also trigger axonal remodelling. These different responses are due to the activation of different signalling pathways. We aim to identify the receptors triggering these processes in neurons.

Intracellular signalling by Wnts is initiated by the activation of their seven transmembrane receptors Frizzled (Fz). Three known Wnt signalling pathways may be activated: the canonical, the planar cell polarity and Calcium pathways. Ten Fz receptors and 19 Wnts have been identified in the mouse genome. To begin to address what Fz receptors are used by Wnts, we examined the pattern of expression of *Wnt7a* and *Wnt7b* together with several *Fz* receptors during postnatal brain development. We found that *Wnt7a*, *fz7,* and *fz3* are expressed in the postnatal and adult cerebellum. *Wnt7b* and *fz3* are highly expressed in the postnatal hippocampus. These overlapping patterns of expression led us to investigate the ability of Wnt7a and Wnt7b to bind to the cell surface of HEK293 cells expressing the ligand-binding domain of Fz receptors. Binding of Wnt7a an -7b to Fz-3, -5, -7 and -8 was tested. Wnt7b binds Fz3 and Fz5, whilst Wnt7a binds Fz7 and Fz3. Signalling activity was then assessed by measuring TCF/LEF mediated transcription (Top-Flash assay) and by the increased levels of ß-catenin. Wnt-7a is able to activate the canonical pathway in Fz7 and Fz3/LRP6 transfected HEK293 cells. These studies highlight the idea that activation of the canonical / β-catenin pathway by Wnt7a can be mediated by Fz7 and the LRP6/Fz3 complex.

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Abbreviations used

A-P	Antero-Posterior
APC	Adenomatous polyposis coli
APS	Ammonium Persulfate
BSA	Bovine Serum Albumin
CA	*Cornu ammonis*
cDNA	complementary DNA
CK	Casein Kinase
CM	Conditioned medium
CNS	Central nervous system
CS	Chicken Serum
DEPC	Diethylpyrocarbonate
DG	Dentate Gyrus
DRG	Dorsal root ganglion
drl	derailed
DTT	Dithio-threitol
D-V	Dorso-Ventral
Dvl	Dishevelled
ECL	Enhanced chemiluminescence
EGL	External granular layer
FC	Foetal Calf
FCS	Fœtal Calf Serum
Fz	Frizzled
GC	Granule cell
GST	Glutathione-s-transferase
h	Hours
HA	Haemaglutinin
Hh	Hedgehog
HSPG	Heparin sulphate proteoglycan
IGL	Internal granular layer

ITS	Insulin Transferrin Sodium Selenite
LEF	Lymphocyte enhancer binding factor
LRP	Lipoprotein Related Protein
MAP	Mictrotubule Associated Protein
MCS	multiple cloning site
min	Minutes
NGF	Nerve growth factor
P	Post-natal age (days)
P/S	Penicillin Streptomycin
PBD	Pak Binding Domain
PBS	Phosphate Buffer Saline
PCM	Phase Combining System (for scintillation counting)
PDZ	PSD-95/Disc large/Zona occludens
PFA	Paraformaldehyde
RGC	retinal ganglion cells
RLU	Relative Light Units
RNA	Ribonucleic acid
Rpm	revolutions per minute
DNA	Deoxyribonucleic acid
SSH	Sonic Hedgehog
RT	room temperature
SDS	sodium dodecyl phosphate
sFRP	soluble Frizzled-Related Protein
SV	Synaptic Vesicle
TCF	T-cell factor
TEA	Triethanolamine
TEMED	(N,N,N,N -Tetramethyl-Ethylenediamine)
TGF	Transfortming growth factor
WIF	Wnt Inhibitory Factor
Wnt	Wingless and Nt-1

Chapter I

Introduction

I. Introduction

The central nervous system (CNS) of vertebrates is the most complex biological system of all. 10^{11} cells of defined identity are specifically interconnected in order to co-ordinate movement, memory storage, cognition as well as the maintenance of bodily functions. Although most neural cells have points in common, such as their morphology or their electrico-chemical properties, most of them express specific proteins and are precisely connected to their targets. Santiago Ramòn y Cajal, a pioneer neurobiologist, had already speculated on the importance of the preciseness of the connections in between neurons in his principle of connectional specificity (Ramòn y Cajal, 1906). Using the silver staining method developed by Golgi, it appeared to Ramòn y Cajal that each individual cell made specific connections, not only with selected post-synaptic target cells but also at specific locations on these cells' dendrites. Much of this complex circuit is generated during development. It is therefore not surprising that out the 80,000 genes of the human genome, 30,000 are expressed in the CNS. Understanding the interplay of genes which enables the formation of neural circuits is a fascinating area of research. Although many key developmental events are well understood, elucidating the molecular basis of axon guidance, target recognition and synapse formation remains an active field of research.

The focus of this thesis is the understanding of the molecular processes that contribute to the formation of neuronal circuits. In particular, could the role played by Wnt factors during neuronal development be clarified by establishing which specific receptors mediate Wnt signalling in neurons? In the following sections, important aspects of CNS development will be described. First, key aspects of neural development will be mentioned in order to bring synapse formation into context. In parallel, key gene families and signalling pathways regulating neural development will be introduced. Secondly, the Wnt signalling pathways, which are involved in events ranging from the generation of primordial CNS precursors to the establishment of functional synapses, will be explained in detail. In the third section, the characteristics of the different Wnt receptors will be clarified, with particular emphasis on how receptor/ligand pairs have been so far identified.

I. 1. The development of the nervous system

I. 1. 1. Early events of neural development

The mature CNS can be divided into six distinct brain regions and the spinal cord. All the cells that compose these structures a derived from common progenitor cells. The events that lead to the formation of the neural tube, which contains the progenitor cells of the CNS, is such a critical developmental event that it is very highly conserved throughout phylogeny. Indeed, the mechanism that regulate its formation and subsequent differentiation into different neuronal sub-population are conserved from *Xenopus* to higher mammalian organism such as the Mouse and Humans, as related events occur in the worm *Caernorhabditis elegans* and the fruit fly *Drosophila melanogaster*. Because the neural tube is easier to study than a more mature CNS, key signalling mechanisms were discovered by studying this critical developmental stage. Indeed, many signalling pathways regulating the formation and patterning of the neural tube also play key roles later aspects of development (Cayuso and Marti, 2005; Logan and Nusse, 2004).

In addition to being highly conserved, the mechanisms that regulate the formation and the patterning of the neural tube bare many similarities with subsequent events of neural development. For example, most changes in cell fate are induced by extra-cellular signals that trigger changes in gene expression within the cell. The interaction of signalling molecules with the cell surface receptors triggering the activation of specific transcription factors is a critical and recurrent event in development. The discovery of molecular mechanisms that occur during early neural development considerably contributed to our understanding of the signalling mechanisms occurring later in development. Studies in *Drosophila* or in chick identified signalling molecules that revealed to be critical in the development of all organisms, not only because of their roles early events in development, such a determination of cell fate, but also because of their implication in later aspects of development, such as cell survival or movement (Dormann and Weijer, 2003; Mehlen *et al.*, 2005). For example, Wnt factors are critical during many early developmental events such as tissue polarity establishment, cell fate determination and cell movements (Ciani and Salinas, 2005; Logan and Nusse, 2004). In

addition, their role in late neurodevelopment has recently emerged, in the context of axon guidance, dendritogenesis and synaptogenesis (Hall *et al.*, 2000; Rosso *et al.*, 2005). Although the mechanisms of Wnt signal transduction are well understood, understanding the specific molecules involved in particular events remains to this date a very active field of research.

Studies of the neural tube identified other key gene families and signalling mechanisms regulating its development into an array of neural precursors. Sonic Hedgehog comes into play to initiate the differentiation of cells of the neural tube into different neural progenitor subtypes. SHH, secreted from the notochord, a structure located ventrally to the neural tube, is able to induce the differentiation of cells located along the dorso-ventral axis of the neural tube (Roelink *et al.*, 1995). Cells close to the SHH source, which detect high levels of SHH, differentiate into floor plate cells, which will in turn express SHH themselves, in a process described as homogenic induction (Kalderon, 2005). Cells located more dorsally, which detect lower concentrations of SHH may differentiate in motor neurons or interneurons. SHH is thus a potent morphogen which has the capacity to induce different cell types according their position within a SHH gradient. Importantly, diffusion of SHH is limited by the post-translational addition of cholesterol moiety. These features are shared by other secreted factors such as Wnt factors which are also palmitoylated (Willert et al., 2003) and may function as an axon guidance molecule in a concentration-dependent manner (Schmitt et al., 2006). How do these differentiating cells sense the environment? How are the different signals integrated in order to trigger specific cellular responses?

The cell surface receptors expressed in specific cell populations are critical to the responses to SHH exposure, as well as to the other factors that regulate neural tube and spinal chord patterning, such as Bone Morphogenic Proteins (BMPs) and Wnt (Sasai and De Robertis, 1997). This highlights the fact that during development, cellular changes are a consequence of both intrinsic and extrinsic factors. Intrinsic factors such as which genes are expressed in the cell control the responsiveness of certain cells to specific cues. In turn, reception of extrinsic signals may affect the activation of transcription factors leading to changes in gene expression, ultimately resulting in changes in cell fate. In fact, in order the further tighten the control of the cell's response to extra-cellular ligands,

receptors for these ligands are often multimeric protein complexes. For example, Wnt and Hh signals are transduced by receptor complexes composed of two protein families (Nusse, 2003), but the precise function of each receptor component remains in some cases elusive.

The use of multimeric receptors transducing the signals from secreted proteins is a common feature of biology. These receptor complexes provide extra levels of substrate specificity and increased accuracy in the cellular response. In fact, enzymes and chaperones that contribute to the synthesis and expression of these receptors are often critical for signal transduction (Culi and Mann, 2003; Hsieh *et al.*, 2003; Kadowaki *et al.*, 1996)). For example, BMP receptors are dimers, with one subunit mediating binding of BMPs to the cell surface and the other subunit triggering the intracellular signal. SHH also relies on two receptor proteins, patched (ptc) and smoothened (smo), to activate a protein kinase cascade inside cells. Binding of Hh to Ptc relieves the inhibition of smo by ptc and activates signalling (Fig I.1) (Nusse, 2003). In the case of Wnt signalling, receptor complexes often contain receptors from the Frizzled gene family as well as receptors from the Lipoprotein Related Protein (LRP) family (Pinson *et al.*, 2000; Tamai *et al.*, 2000), in the case of "canonical" Wnt signalling. Alternatively, Ryk may act as a Wnt-receptor but is it not clear whether Ryk can function alone or requires additional receptors (Liu *et al.*, 2005; Lu *et al.*, 2004; Schmitt *et al.*, 2006). A number of soluble molecules, such as sFRP or WIF, bind Wnt or Wnt receptors and are known to negatively regulate Wnt signalling by disrupting receptor/ligand interactions (Fig. I. 1) (Kawano and Kypta, 2003). Amongst this wide number of possible signalling mechanisms, it is crucial to determine which receptors mediate Wnt effects in neurons. Establishing the composition of the receptor complexes mediating Wnt signalling in neurons is the main objective of this thesis.

The mechanisms by which the antero-posterior axis of the neural tube is patterned are similar to those used to pattern the dorso-ventral axis. Although different signals are utilised, such as Retinoic Acid and Fibroblast Growth Factors (FGF family), the

A. Receptors and regulatory factors for for Wnt signalling

B. Hh receptors and regulatory factors

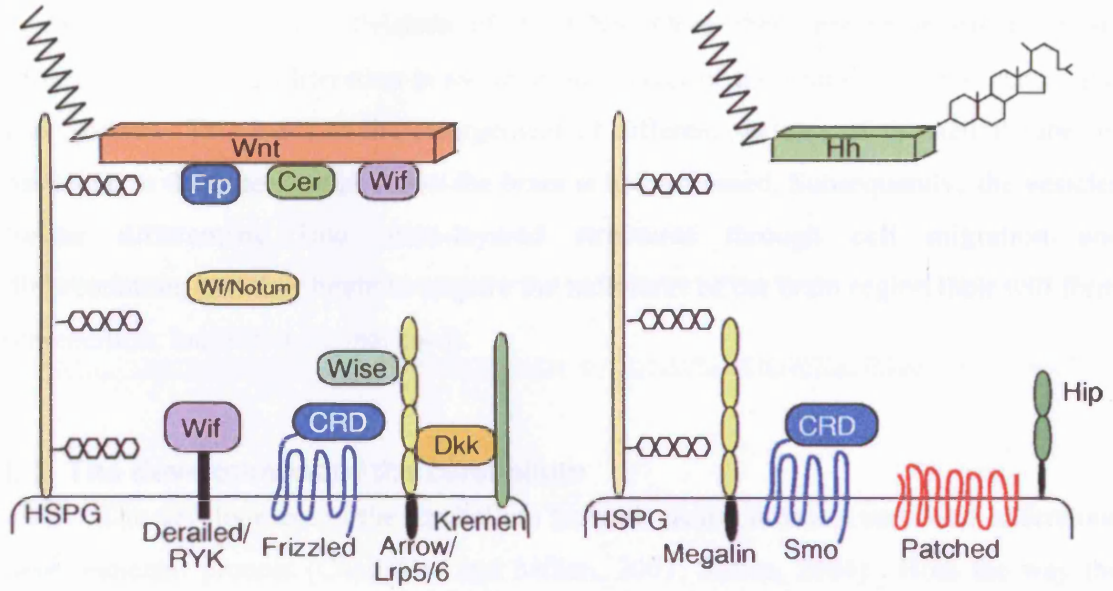


Figure I. 1 Receptor complexes and secreted factors regulate Wnt and Hh signalling

Wnt and Hh signalling have many biochemical and mechanistic similarities

A. Wnt signalling is mediated by three receptor families and regulated by a number of secreted factors. Frizzled are the main receptors for Wnt, which are palmitoylated. Fz bind Wnt via their CRD, which is also found in the secreted Wnt antagonists sFRPs. The LRP5/6/Arrow co-receptor is necessary for the activation of canonical/Wg signalling, and LRP5/6 function is inhibited by Dkk and Wise. Kremen may down-regulate the surface expression of LRP5/6 through Dkk. Ryk receptors may also function as Wnt receptors, they compete with WIF for Wnt binding. HSPG are also implicated in Wnt reception and transport.

B. Hedgehog signalling relies on Smoothen (Smo) and Patched. Hh is modified by the addition of a palmitoyl group at its N-terminus and a cholesterol at its C-terminus. HIP (hedgehog inhibitory protein) binds and inhibits its function. HSPG are also important for Hh reception. Megalin is related to proteins of the LRP family and is implicated in Hh internalisation and signalling. (Adapted from Nusse, 2003).

generation of specialised structures in the brain is similar to the way different neuronal populations are generated in the neural tube. Antero-posterior (rostro-caudal) differentiation of the neural tube occurs through the generation of vesicles which determine the major sub-divisions of the CNS. Once these precursor structures are formed, active cell proliferation in the innermost layer of the neural tube, the ventricular zone, occurs. This leads to the enlargement of different sections of the neural tube, in particular at the anterior end where the brain is being formed. Subsequently, the vesicles further differentiate into multi-layered structures through cell migration and differentiation, and thus begin to acquire the hallmarks of the brain region their will form (cerebellum, brain stem, spinal cord).

I. 2. The development of the cerebellum

The development of the cerebellum from the neural tube is a very well understood developmental process (Chizhikov and Millen, 2003; Sotelo, 2004) . Both the way the primordial A-P boundaries signal and the subsequent cellular differentiation, clonal expansion and migration of neural precursors are examples of how these processes occur in other brain regions.

I. 2. 1. The formation of cerebellar precursor subtypes

At around embryonic day 9.5 in the mouse, the neural tube is sub-divided along the AP axis into 4 main segments: the forebrain, the midbrain, the hindbrain and the spinal cord. Each of these segments is further subdivided into smaller segments. For example, the hindbrain is divided in 7 rhombomeres, and the cerebellum will arise from cells of the most anterior rhombomere. Signals from the isthmic organizer (IsO) which delimitates the hindbrain/midbrain barrier are particularly important in shaping the developing cerebellum. Studies of naturally occurring mutations and gene targeted disruption have shed considerable light on the roles of specific genes during development of the cerebellum.

Two secreted factors, FGF8 and Wnt1, are critical for the formation of the anterior boundary of the cerebellum. FGF8 was identified at the main IsO-derived factor

that is critical to the midbrain and cerebellar development, because *FGF8* mutant mouse have severe cerebellar defects (Liu *et al.*, 1999; Meyers *et al.*, 1998). Additionally, heparin beads soaked in recombinant Fgf8 induce cerebellar markers (Crossley *et al.*, 1996; Martinez *et al.*, 1999). Wnt1 signalling is also important in the isthmic region, mainly for its role in promoting cellular proliferation and sustaining *FGF8* expression (Dickinson *et al.*, 1994; Matsunaga *et al.*, 2002). In addition, many homeobox transcription factors of the *engrailed* and *Pax* families are important in specifying the fate of cells in the vicinity of the IsO. The formation of the posterior boundary of the cerebellum is less well defined, but IsO-derived FGF8 seems also to control the anterior boundary by inhibiting the expression of homeobox genes expressed in rhombomere 2, the rhombomere immediately posterior to rhombomere 1. Additional signals from the roof plate, notably from the TGF-ß family contribute to the specification of cell fates along the D-V axis, in a similar fashion to the role this gene family has in the spinal cord (Chizhikov and Millen, 2003).

Cells of rhombomere 1 of the hindbrain respond to the signals mentioned above, in particular those of the BMP family. BMP activate transcription factors which trigger the differentiation of cells of rhombomere 1 into precursors to cerebellar granule cell, Purkinje cells or other neuronal or glial cell types. For example, the proneural *Math-1* transcription factor becomes activated by BMPs and plays a critical role in the initial specification of granule neurons as well as Pontine neurons (Ben-Arie *et al.*, 1997; Ben-Arie *et al.*, 2000). The gene *meander tail (mea)* was identified in a spontaneous mouse mutation and is critical to the development of granule cell progenitors. *Meander tail* mutants lack granule cells in the mature cerebellum and have a disorganised Purkinge cell layer (Ross *et al.*, 1990). The precise signals that induce the other precursor cell types in the region are unknown, but it is likely the activation of specific transcription factors is central to these events.

I. 2. 2. Cell migration

Once a particular cell progenitor has acquired its identity, the number of these cells is expanded by stimulating their proliferation whilst some of them migrate to establish themselves in their target regions. Cell migration in the developing cerebellum

occurs in two distinct phases. First, precursor neurons migrate to establish the nascent areas of the cerebellum (E13-E16). The best example of this migration is the lateral migration of granule cell precursors from the rhombic lip along the inner the surface of the cerebellar plate (Fig. I. 2). Their migration forms the external granular layer (EGL) of the cerebellum, where millions of granule cells will be generated until the end of the first post-natal week in the mouse.

The second phase of migration is when neurons reach maturity and the cell bodies migrate to their final location. The process by which granule cells of the EGL migrate inwards radially towards the IGL is well established and occurs in between P6 and P15 in the mouse. Pre-established Bergmann fibres create migratory tracks allowing granule cells to migrate radially from the EGL to the IGL (Altman and Bayer, 1985). Granule cells leave behind their axons in the EGL which will develop further to create the molecular layer, the place where GC axons synapse with Purkinje cell dendrites. Granule cells of the deeper region of the EGL migrate to establish themselves in a position deep to the Purkinje cell layer and begin to form the Internal Granule Layer (IGL) (Fig. I. 2).

Cell-cell communication within the emerging cerebellum is critical to differentiation and proliferation of cerebellar neurons (Sotelo, 2004). Purkinje cells are important for the proliferation of granule cells in the external granule layer (EGL). This became evident when mutant mouse with defects in Purkinje cell number (*Staggerer*) or location (*reeler*) showed defects in granule cell proliferation (Mallet *et al.*, 1976; Mariani *et al.*, 1977). SHH produced by Purkinje cells was later discovered to be the signal stimulating granule cell precursors proliferation (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999). In the absence of SHH, the SHH receptor Patched triggers a signalling cascade repressing the transcription of genes positively regulating cell proliferation. In cells which receive SHH, Patched signalling is inhibited and proliferation is thus stimulated. Proliferating granule cell progenitors also have an autocrine loop to sustain their proliferation. *Notched2* is specifically expressed by these neurons and activates a signalling cascade which is in part regulated by a gene whose expression in controlled by SSH signalling, *Hes1* (Solecki *et al.*, 2001).

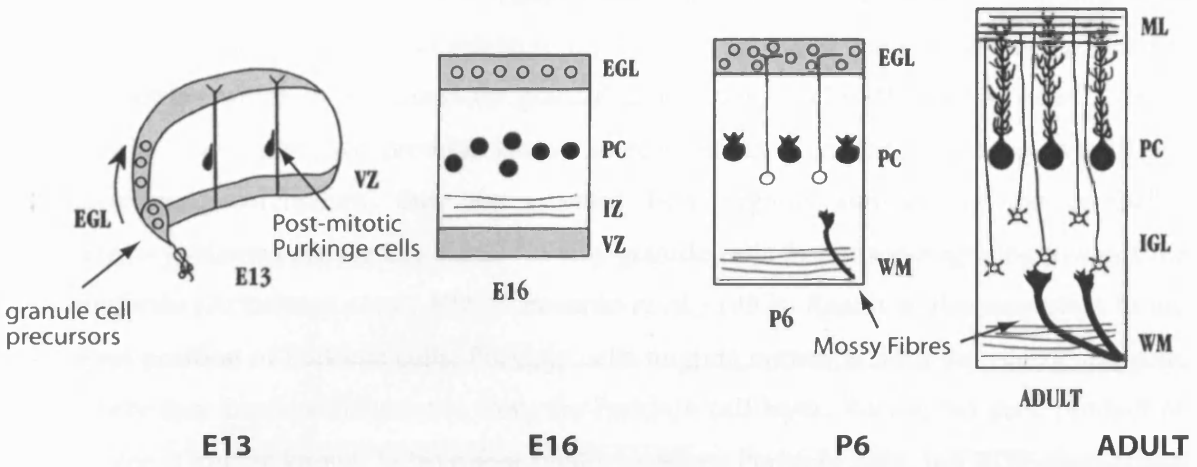


Figure I. 2. Cell Migration during cerebellar development

Cell migration during cerebellar development is an example of how complex brain structures are established

From E13-16, granule cell precursors migrate along the roof of the nascent cerebellum to form the EGL. At the same time, Purkinge cells become postmitotic and migrate from the VZ to their final location in the core of the cerebellum.
Postnatally, once sufficient numbers of granule cell have been generated in the EGL (around P6), post-mitotic granule cells migrate radially along to Bergmann glia. Granule cells leave behind their axons and establish themselves under the Purkinge cells layer.
After their migration, granule cells develop their axons further. These axons lie parallel to the roof of the cerebellum and form the molecular layer (ML). In the ML, parallel fibres synapse with Purkinge cell dendrites, which also arborise in later postnatal periods. As granule cells reach the IGL between P6-P22, mossy fibres originating from the Pontine nuclei also reach the IGL. Each mossy fibre terminal form a large, multi-synaptic contact with several granule cells.
EGL, External germinal layer; VZ, ventricular zone; WM, white matter; IZ, intermediate zone: IGL, internal germinal layer
Adapted from Hatten, 1999.

Secreted signals are also implicated in the signalling the end of migration. In the *rostral cerebellar malformation (rcm)* mutant, granule cells cells fail to stop migration once they reach the IGL (Ackerman *et al.*, 1997). It appeared that *rcm* encodes a receptor for netrins which is expressed by granule and Purkinje cells (Przyborski *et al.*, 1998). Netrins are a group of proteins known to regulate axon guidance (see below). In the developing cerebellum, they are secreted from regions surrounding the cerebellar territory. Netrins are the key signals to stop granule cells from over-migrating towards the midbrain (Ackerman *et al.*, 1997; Leonardo *et al.*, 1997). Reelin is also important to the final position of Purkinje cells. Purkinje cells migrate outwards from the ventricular zone where they have proliferated to form the Purkinje cell layer. Reelin, the gene product of *reeler*, a mutant known to have abnormally localised Purkinje cells, is a EGF-like repeats containing protein produced by cells located externally to the nascent Purkinje cells layer. Reeling signals to stop Purkinje cells migrating beyond these cells. (D'Arcangelo *et al.*, 1997). Neurons other than granule cells only begin their terminal differentiation once they have reached their final position. Neurons need to send out axons and dendrites to encounter synaptic partners. Secreted signals, transmembrane receptors and cell-adhesion play crucial role in the guidance of axons, the recognition of their targets and the formation of synapses.

I. 1. 3 Axonal guidance

Axon guidance is another example of a developmental event where cell surface proteins play a critical role. Indeed, once sufficient numbers of neuronal precursors have been generated and have migrated to a given region, neurons begin to terminally differentiate and start to extend their axons in search for their synaptic targets. Although many axons reach their target by adhering to other processes which have already reached their targets, the first axons extend to their targets as "pioneers". Furthermore, every individual axon needs to be capable of responding to molecular cues in the environment in order to reach its specific target(s). Although many receptor-ligand interactions regulating axon guidance are understood, the receptors mediating Wnt signalling in axons guidance and synaptogenesis are only beginning to be identified.

The growth cone is the dynamic tip of the growing axons, it is a specialised structure that enables axons to sense the environment and thus respond to guidance cues. Growth cones encounter two types of guidance factors: trophic factors and tropic factors. Trophic factors promote neuronal survival and often promote axonal outgrowth. Tropic factors act as either attractant or repellent signals that guide growth cones towards a particular direction. These guidance cues can be either secreted, associated with the extracellular matrix, or cell surface molecules (Figure I. 3). In many cases, guidance molecules are bi-functional in that they may induce the outgrowth or attraction of one type of neuron whilst triggering axonal retraction or repulsion in others. Once again, the receptors expressed at the growth cone play critical roles in enabling each axon to respond adequately to the signals encountered.

I. 1. 3. 1. The Netrins

One well studied family of bi-functional factors are the *netrins* (Livesey, 1999). Netrins are secreted glycoproteins that utilise receptors from the immunoglobulin superfamily to signal in neurons. Both *netrins* and their receptors (*unc-40* in *C.elegans* and *DCC* in vertebrates) are well conserved throughout phylogeny. Studies in *C. elegans* and *Drosophila* contributed to the identification of netrin receptor/ligand pair in different aspects of axon guidance and outgrowth (Culotti and Merz, 1998). Three genes in *C. elegans* were shown to collaborate in the guidance of spinal cord axons. The vertebrate netrins (netrin-1 and -2) were first identified as the factors mediating the attraction of commissural spinal neurons towards the floor plate, a source of signals defining the ventral midline (Hedgecock *et al.*, 1987; Tessier-Lavigne *et al.*, 1988). *netrin-1* and *-2* have some homology with laminins and were found to be homologous to the *C. elegans* gene *unc-16*. Further studies in the nematode revealed two members of the immunoglobulin superfamily, *unc-5* and *unc-40* were involved in the same axon guidance processes and thus appeared as potential netrin receptors (Hedgecock *et al.*, 1987; (Hedgecock *et al.*, 1990). *Unc-40* is homologous to the *DCC* gene in vertebrates. Both *unc-40* and *DCC* were shown to transduce the attractive properties of netrins in the dorso-ventral guidance of commissural neurons using function-blocking antibodies and knock-

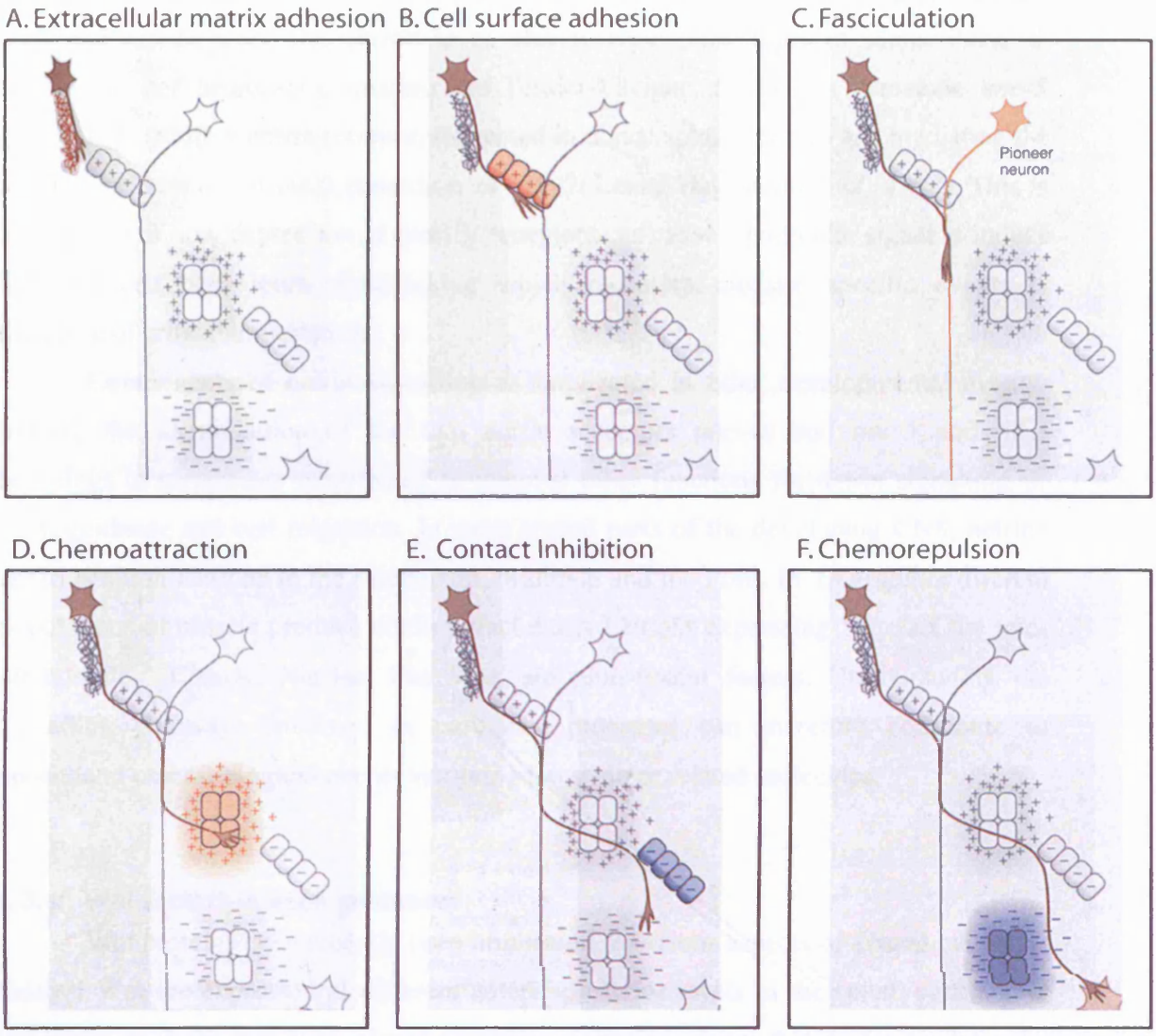


Figure I. 3. Axonal guidance mechanisms

Guidance of axons towards their specific synaptic target implies a wide range of signalling mechanisms

A growing axons (in brown) may encounter a number of guidance cues until it reaches its synaptic target.
A. First, extra-cellular matric components may stimulate growth.
B. Cell Surface adhesion molecules may regulate the direction or the rate of axonal growth.
C. The growing axon may fasciculate with a pre-existing track made by other neurons or glia. **D.** Secreted chemoattractant cues may diffuse from their source and attract the axonal growth cone.
E. Intermediate targets may express repellent cues at their surface and thus instruct the axon to move away.
F. Additionally, secreted chemorepellent cues may induce the turning of the axon.
As the neuron enters into the vicinity of its target, synaptogenic cues instruct the axon to slow down, arborise in some cases, enlarge its growth cone and begin to accumulate synaptic proteins.
(Taken from Kandel *et al., 2000)*

out strategies (Hedgecock *et al.*, 1990; Keino-Masu *et al.*, 1996). Interestingly, the floor plate and netrins were also shown to be able to repel other types of axons, those of trochlear motor neurons(Colamarino and Tessier-Lavigne, 1995). The nematode *unc-5* proved to be another netrin receptor, expressed in dorsal spinal neurons and mediating the repulsive effects of netrins (Leonardo *et al.*, 1997; Leung-Hagesteijn *et al.*, 1992). This is an example of how expression of specific receptors can cause a particular signal to induce different cell behaviours. Establishing which receptors mediate specific events is therefore of critical importance.

Components of netrin signalling as implicated in other developmental events. Indeed, the identification of the two netrin receptors *unc-40* and *unc-5* and their homologs in vertebrates contributed to uncover other functions for netrin signalling in axon guidance and cell migration. In more rostral parts of the developing CNS, netrins act to position neurons in the cerebellum, midbrain and the Pons. In *Drosophila* discrete populations of muscle produce netrin attract motor neurons expressing *frazzled*, the *unc-40* homolog. Clearly, Netrins, like Wnt, are pluri-potent factors. Understanding the signalling pathways involved in particular processes can therefore contribute to understand other biological events involving the same or related molecules.

I. 3. 2. Wnt factors in axon guidance

Wnt proteins have recently been implicated in various aspects of axonal guidance. Several Wnt are expressed at different anterio-posterior levels in the spinal cord and it has come to light that they signal to axons migrating along this rostro-caudal axis. Recently, the discovery of a receptor mediating axonal guidance of *Drosophila* commissural neurons, *Derailed* (*Drl*) (*Ryk* in mammals), subsequently revealed that Wnt signalling through these receptors was a new mechanism of axon guidance (Yoshikawa *et al.*, 2003). Wnt and Ryk have been implicated in mediating neuronal outgrowth as well as guidance at the *Drosophila* midline and along the mammalian cortico-spinal tract (Liu *et al.*, 2005; Yoshikawa *et al.*, 2003). Yet, in other circumstances such as the guidance of commissural axons as they exit the spinal cord and turn towards the brain, a more classical Wnt/Fz pathway has been implicated (Lyuksyutova *et al.*, 2003). Very recently, it emerged that Wnt3/Ryk/Fz5 signalling may co-operate with EphrinB1-EphB signalling

to guide retinal axons along the medial-lateral body axis (Schmitt *et al.*, 2006). This paper revealed a fascinating aspect of Wnt signal transduction, where Wnt3 acts as a repulsive signal at high concentrations through the Ryk receptor, whilst acting as an attractive cue at lower concentrations, via Frizzled receptors (Schmitt *et al.*, 2006). Clearly, the role for Wnt signalling in axon guidance is a rapidly evolving field of research which is uncovering exciting new facets to our understanding of both axon guidance and Wnt signalling.

I. 1. 3. 3. Other secreted guidance cues

In the context of axon guidance, many other receptor/ligand play critical roles in shaping the paths taken by neurons. An important family of guidance cues are the *semaphorins*. Semaphorins use two classes of receptors: The neuropilins, which also belong to the immunoglobulin superfamily, and the *plexins*, which are homologous to *semaphorins* themselves (Fujisawa, 2004). There are 15 known *semaphorins*, some of which are secreted whilst other are anchored to membranes. Another class of guidance cues identified in the context of retinal axon guidance, are the *ephrins*, which signal through a large class of tyrosine kinase receptors, the eph kinases (Orioli and Klein, 1997). Ephrins and the eph receptors possess the unique particularity that they may function as receptors and ligands, respectively (Klein, 2004). Both *semaphorins* and *ephrin* gene families have points in common with *netrins* and *Wnt* in the way they are also involved in different developmental events.

I. 1. 3. 4. The role of the extra-cellular matrix and cell surface proteins

Growing axons also specifically recognise substrates encountered within the extra-cellular matrix (ECM) (McKerracher *et al.*, 1996). Two large gene families contribute to this substrate specificity. The integrins can encode a multitude of dimeric receptors through the association of one of 16 alpha chains and one of 8 beta chains. This receptor diversity contributes to the detection of the appropriate laminin substrate present in the ECM. In fact, laminins are heterotrimers taken from 5α, 4ß and 3γ genes and

therefore a great number of specific substrate can be generated from this ECM constituent alone.

Cell-cell adhesion is another mechanism contributing to axon guidance, fasciculation of growing neurons and target recognition (McKerracher *et al.*, 1996). Cadherins are an important family of cell-adhesion molecules (Ranscht, 2000). This receptor family comprises more than 100 members, which create calcium dependent homophilic interactions. Cadherins are glycosylated which may add a further level of specificity to their interactions. Outgrowth and guidance controlled by these signals detected involves the cytoskeleton. Both integrins and cadherins are indirectly coupled to actin and thus provide a link between the extracellular environment and the growth cone's cytoskeleton. Clearly, the potential for guidance cues within the CNS is enormous and understanding the link between cues, receptors and cellular responses is a very active field of research.

Intracellular signalling cascades couple the signals received by cell-surface receptors to changes in cytoskeletal dynamics. At the growth cones, localised changes in Ca^{2+} levels are known to affect kinases such as calmodulin-dependent protein kinase II and phosphatases such as calcineurin (Bolsover, 2005). Ultimately, these enzymes may affect cytoskeletal dynamics by affecting the phosphorylation status of Microtubule Associated Proteins (MAPs) (Dickson, 2001). In parallel, signalling cascades leading to the activation of small GTPases of the Rho family such as Rac and Rho may also affect the cytoskeleton . Clearly, a combination of signalling events occurring at the growth cone regulate is progression. The receptors expressed at the growth cone play a key role a triggering these responses. Understanding the signalling pathway controlled by individual receptors is critical in order to clarify the molecular processes that lead to precise axonal pathfinding.

With so many families of guidance cues, receptors and signalling pathways, it remains today a daunting task to explain the molecular basis of the guidance of particular axons. Nonetheless, the identification of receptor/ligand pairs involved in specific aspects of guidance enabled to sub-divide axon guidance in localised signalling events induced by different kinds of ligands. The astonishing complexity of inter-neuronal connectivity is achieved by the specific expression of cell surface receptors, often multimeric, in order

to respond with a high degree of specificity to the extra-cellular cues encountered. The final stages of neurodevelopment are target recognition and the formation of synapses. Although some molecules involved in guidance are implicated in this process, other signals come into play in order to trigger the differentiation of axons and dendrites into functional synapses.

I. 1. 4. Synaptogenesis

The last step in the formation of neuronal circuits is target recognition and the formation of a functional communication structure: the synapse. Much of the early work that contributed to our understanding of how synapses formed came from studies at the neuro-muscular junction. Nonetheless, in recent years, many factors organising pre- and post-synaptic differentiation (NMJ) of central neurons have been discovered. It appears that a combination of priming and inducing factors co-operate to first prepare growing axons to form a synapse and in turn stabilise the connection established. Although the activity of a given synapse contributes to its stabilisation, synaptic activity does not seem to be critical to the initial formation of synapses since neurotransmission-deficient mutants are able to form synapses (Varoqueaux *et al.*, 2002; Verhage *et al.*, 2000). Therefore, intercellular communication is central to target recognition and synapse formation. Which are the molecules regulating synaptogenesis? How are extra-cellular signals converted into cellular responses?

Three aspects of the synapse need to be specialised for transmission to occur efficiently. On the pre-synaptic side, synaptic vesicles containing neurotransmitters must accumulate near an area containing the machinery necessary for fast voltage-induced exocytosis: the active zone. Similarly, the post-synaptic zone apposed to the active zone must accumulate neurotransmitter receptors and the signalling components in order to efficiently transmit the signal in the target cell. Finally, the extra-cellular space in between the pre- and post-synapse, the synaptic cleft, has to be equipped with the enzymes that will degrade excess neurotransmitter in order to ensure the signal is short-lived. The establishment of these structures at the neuro-muscular junction are well understood and generated an early model for post-synaptic differentiation. In the CNS, many signalling events leading to both pre- and post-synaptic differentiation have

emerged. In the case of Wnt signalling, the receptors mediating synaptogenesis remain so far unknown.

I. 1. 4. 1. Lessons from the NMJ

The establishment of neuro-muscular junction occurs in several well-established steps, and bares the hallmarks of synaptogenesis in other parts of the central nervous system. Indeed, both the motor neurons and the muscle differentiate in a stepwise fashion in order to make an efficient synapses (Sanes and Lichtman, 1999). Bi-directional signalling occurs between the neuron and its target. At the NMJ, the signalling mechanisms triggered are well-understood, from the receptor/ligand interactions involved to the signalling cascades activated. Neuronal activity also plays a role in the final steps of the junction's development. The assembly of NMJ is therefore considered as a model example of synapse formation and is summarised in Figure I. 4 (Sanes and Lichtman, 1999).

At the time motor neurons reach their targets the myotubes, these myotubes express low levels Acetylcholine Receptors (AChR) evenly throughout their surface. Yet, signals emanating from the incoming motor neurons cause AChR to accumulate in the vicinity of the nerve terminal. McMahan and colleagues identified a large proteoglycan secreted by motor neurons that was responsible for AChR clustering: Agrin (McMahan, 1990). Agrin signals to a receptor called muscle-specific kinase (MuSK) although the co-receptor MASC (myotube-associated specificity component) is also involved, because MuSK cannot directly bind agrin (Sanes *et al.*, 1998) (Figure I. 4).. Activation of MuSK leads to the activation of a cytoplasmic protein called Rapsyn which directly binds AChR and is responsible for their accumulation at synaptic sites (Sanes and Lichtman, 1999). Importantly, a component playing a central role in Wnt signalling, dishevelled, binds MuSK and this interaction is necessary for agrin-mediated AChR clustering (Luo *et al.*, 2002). It appears the Dishevelled may relay the signal to downstream effectors such as PAK1, which may in turn contribute to raspyn's activation. Therefore, both dishevelled and rapsyn is necessary for agrin-mediated AChR clustering. The discovery that

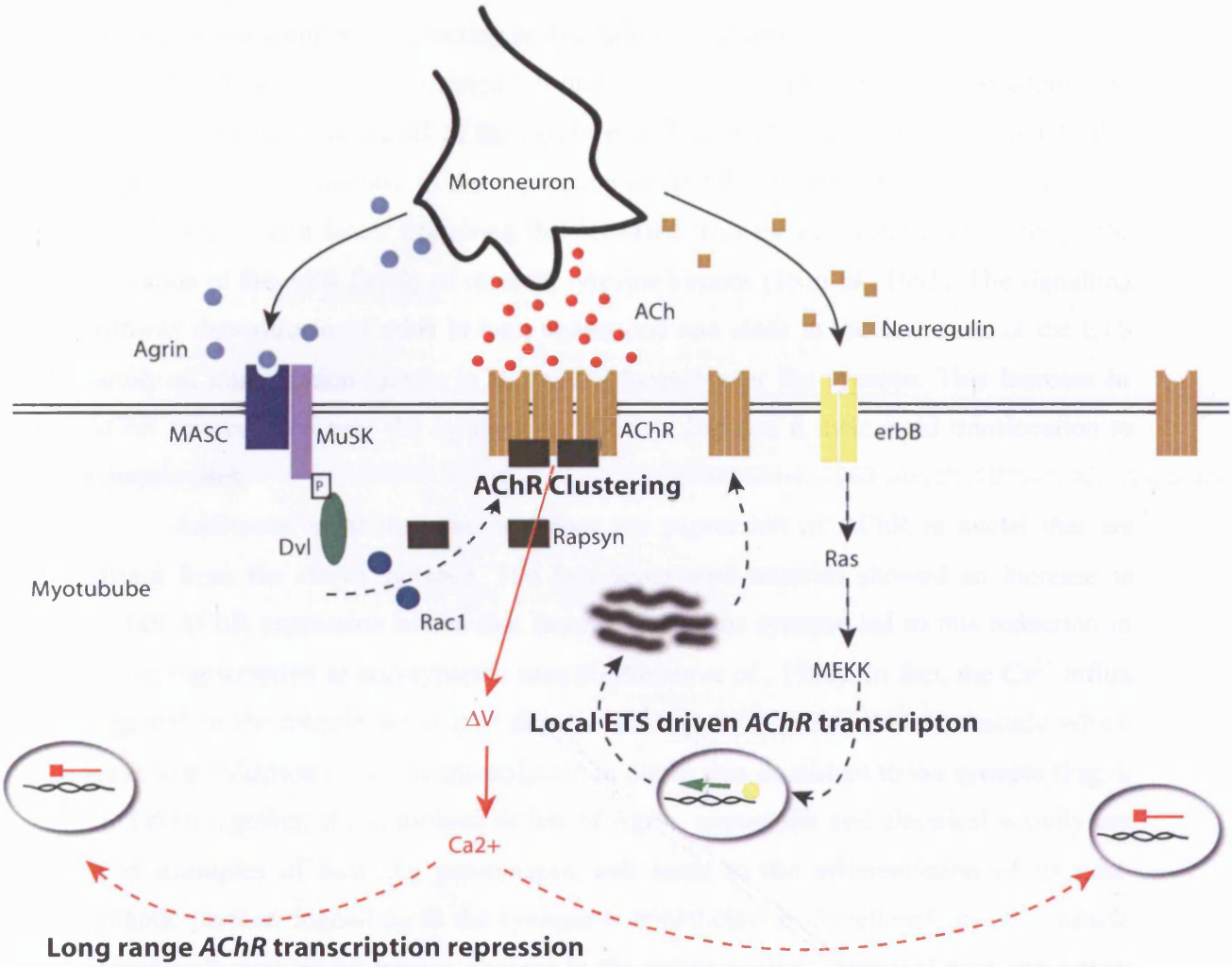


Figure I. 4. Motoneuron-derived signals drive post-synaptic specialisation of the NMJ

Development of the neuro-muscular junction into an efficient synapse is an example of axon-induced specialisation of a post-synaptic target

Agrin secreted by the motoneuron binds a co-receptor complex composed of MASC and MuSk at the myotube surface. Upon agrin reception, MuSK becomes phosphorylated which enables MuSK to bind to Dishevelled (Dvl). Dvl relays the agrin signal to intracellular effectors such as Rac1 which lead to the activation of Rapsyn, which contributes to **AChR clustering** via a direct interaction.
The motoneuron also produces **Neuregulin** which binds and activates erbB receptors. A signalling cascade involving Ras and MEKK is triggered, leading to the activation of ETS transcription factors. Consequentially, **AChR sub-units are transcribed locally** in the nuclei situated close to the pre-synaptic target.
The **depolarisation** induced by ACh reception leads to a depolarisation throughout the myotube. The resulting release of Calcium triggers a kinase cascade which **inhibits AChR transcription in nuclei located away from the synapses**.
Ultimately, the combination of these processes lead the polynucleated myotube to locally express and cluster AChR near synaptic sites, whilst reducing the numbers of extrasynaptic AChR.
Adapted from: Sanes and Lichtman, 1990 and Luo *et al.*, 2002

dishevelled plays a role in organising post-synaptic structures was the first examples of a Wnt signalling component affecting post-synaptic structures.

Myotubes are polynucleated but only receive few inputs, therefore in addition to clustering pre-existing AChR of the myotube surface, motor neuron-derived signals also trigger a localised increase in the expression of AChR sub-units (Fig. I. 4). Neuregulin was identified as a factor triggering this localised increase in transcription, through the activation of the erbB family of receptor tyrosine kinases (Jo *et al.*, 1995). The signalling pathway downstream of erbB in well understood and leads to the activation of the ETS family of transcription factors in the nuclei located near the synapse. This increase in AChR transcription near the synapse is important because it their rapid translocation to synaptic sites.

Additional signalling events reduce the expression of AChR at nuclei that are distant from the active synapse. The fact denervated muscles showed an increase in overall AChR expression hinted that fact activity at the synapse led to this reduction in AChR transcription at non-synaptic sites (Goldman *et al.*, 1988). In fact, the Ca^{2+} influx triggered in the muscle when it is depolarised triggers a protein kinase cascade which leads to a reduction in AChR transcription in nuclei that lie distant to the synapse (Fig. I. 4). Taken together, the combined action of Agrin, neuregulin and electrical activity are good examples of how the pre-synaptic cell leads to the differentiation of its post-synaptic partner. Signalling at the synapse is nonetheless bi-directional, and the muscle expresses factors which trigger changes in the motor neuron's terminal as it encounters its target.

Although axons of motor neurons deprived of target muscle do differentiate to some extent and are able to release neurotransmitters, muscle-derived factors are non-the-less important to the maturation of the incoming neurons (Lupa *et al.*, 1990). Less information is available about the identity of these retrograde signals, but cell adhesion molecules and factors that remain embedded in the synaptic basal lamina have been shown to instruct motor neurons where to form synapses (Sanes and Lichtman, 1999). Cell adhesion molecules such as N CAM and N-Cadherin could mediate cell-cell recognition in some circumstances. Alternatively, the diverse array of laminin trimers that can be encountered in the basal lamina could also contribute to this target-derived

signalling. Clearly, bi-directional signalling is critical to the formation of all synapses. At central synapses, many target-derived signals have been found, and their signalling mechanisms have sometimes been understood.

I. 1. 4. 2. Synaptogenic signalling at central synapses

The formation of synapses in the CNS occurs over a very long period of time starting during embryogenesis and that continues in post-natal life. In fact, the processes underlying memory storage are thought to have common points with synapse formation. For example, the activation of Ca^{2+}-dependent signalling cascades, the accumulation of of AMPA receptors at the synapse and changes in dendritic spine morphology are events which are both observed during synapse formation and associated with synaptic plasticity (Malenka and Bear, 2004; Turrigiano and Nelson, 2004; Zhang and Linden, 2003). Therefore understanding these important developmental events could also shed light on higher cognitive processes.

Growing axons encounter a series of potential synaptic partners on their way to their destination but only stop and begin to form functional synapses with a high degree of specificity. In a process that has some similarities with axon guidance, the growth cone encounters both secreted and cell-surface molecules that instruct it to terminally differentiate into a pre-synaptic terminal. As it can be expected from what we know of the NMJ, the axon itself also influences the synaptic differentiation of the dendrite at the point of contact (Akins and Biederer, 2006; Waites *et al.*, 2005). Although many misconnected or inefficient synapses will subsequently be eliminated, the process of synapse formation is thought to be a highly specific process. This selectivity is achieved by precise receptor-ligand interactions between growing axons and signals produced by their targets (Akins and Biederer, 2006). Understanding the molecules responsible for specific aspects of neuronal maturation is a key challenge to complete our understanding of CNS development.

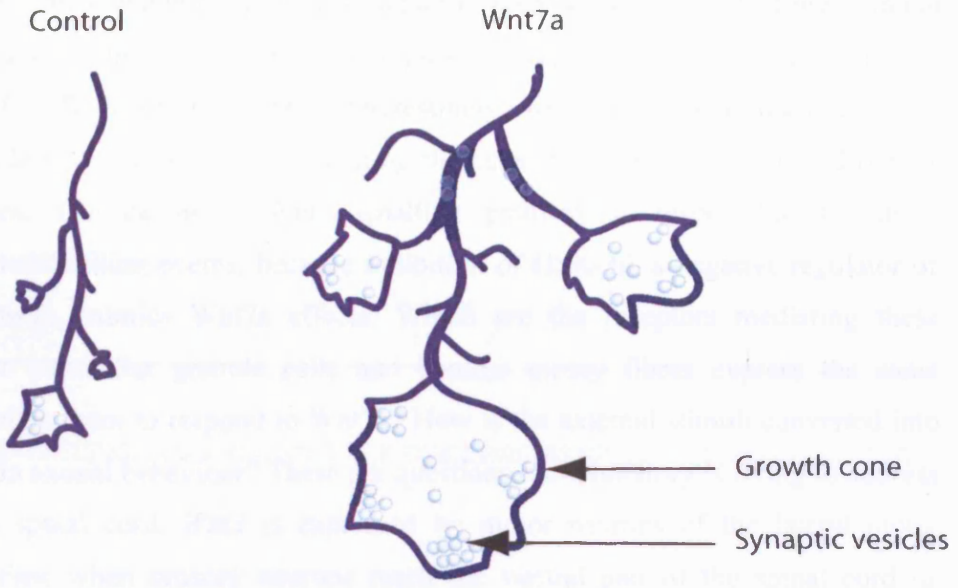
I. 1. 4. 2. 1 Diffusible signal prime neurons before synapse formation

Growing axons encounter diffusible signals as they arrive towards their target. Some of these signals are referred as "priming signals" because they instruct the neuron to accelerate its maturation and "get ready" to form a synapse with a partner in the vicinity. For example, some axons branch out as their reach their targets and most neurons need to accumulate synaptic vesicles in order to be able to assume synaptic transmission. Wnt3 signalling has been implicated in the terminal arborisation of neurons in the spinal cord (Krylova *et al.*, 2002). Wnt7a is known to trigger cytoskeletal remodelling in growing axons and is also able to trigger the accumulation of synaptic vesicles in cerebellar neurons (Hall *et al.*, 2000; Lucas and Salinas, 1997). Other factors produced by neurons, such as FGF family members and BDNF, trigger similar changes, (Alsina *et al.*, 2001; Umemori *et al.*, 2004). Signals emanating from glia, such as Thrombospondin-1 also contribute to making neurons enhance their capacity to form synapses (Ullian *et al.*, 2004). These priming molecules share the fact that when they are added to neuronal cultures, they increase the overall level of synaptic markers but do not seem to convey specific information regarding the recognition of synaptic partners. Nevertheless, only specific neurons respond to these cues. In the case of Wnt signalling in neurons, how specific Wnt factors affect different aspects of the behaviour of specific neuronal behaviour is still poorly understood. Elucidating which receptors mediate specific cellular is a pre-requisite to deciphering intracellular signalling mechanisms of Wnt signalling in neurons.

I. 1. 4. 2. 2. Wnt signalling in synapse formation

Several Wnt factors have been implicated in the terminal differentiation of axons as they reach their targets. *Wnt7a* is expressed by cerebellar granule cells at the time when mossy fibre axons reach the IGL of the cerebellum and initiate synapse formation (Lucas and Salinas, 1997). In fact, mossy fibre explants grown *in vitro* respond to Wnt7a by thickening their processes and enlarging their growth cones (Figure I. 5. A). Additionally, the synaptic vesicle protein synapsin-1 accumulates along axons and at growth cones (Fig. I. 5. A). Since these changes are the hallmarks of the change that

A. Schematic diagram of cerebellar cultures exposed to Wnt7a.



B. Schematic diagram of electron micrographs of mossy fibre - granule cells synapses in *wt* and *Wnt7a -/-* mouse

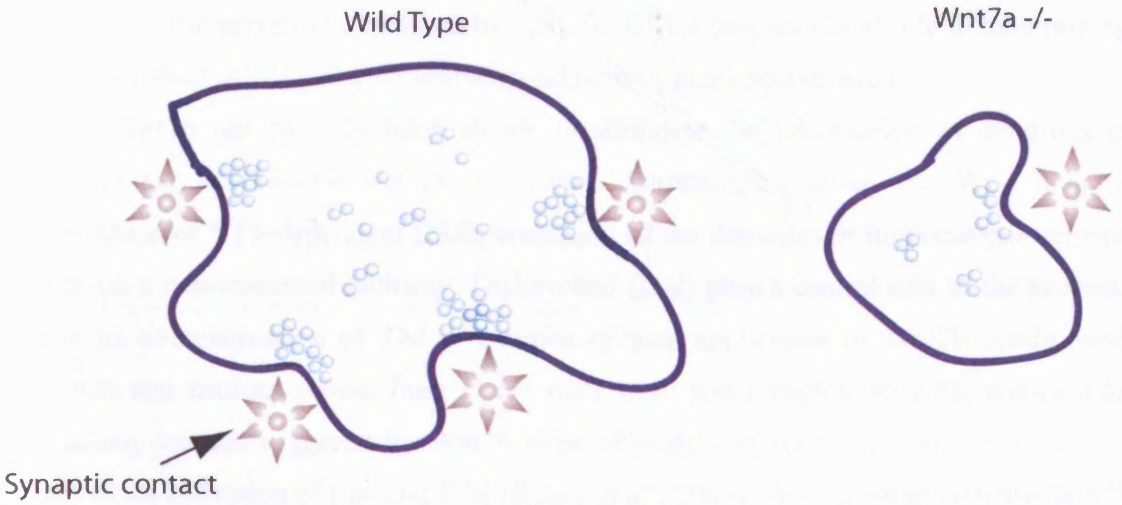


Figure I. 5. Wnt signalling affects developping axons

A. In the absence of Wnt7a, neurons have small growth cones, few branches and accumulate few synaptic markers such as synapsin-1. In contrast, neurons exposed to Wnt7a conditionned medium have enlarged growth cones, thicker axons and accumulate synaptic markers at growth cones and along axons.

B. In the developping cerebellum of wild type mice, mossy fibre terminals are large and complex. Individual "glomerular rosettes" make several synapses with granule cell targets. In contrast, in mutant mice lacking *Wnt7a* , mossy fibre terminals are smaller, less complex and make fewer synaptic contacts.

precede the formation of synapses, Wnt7a is understood to be a synaptogenic factor. In fact, in *Wnt7a-/-* mice, the multisynaptic structure formed between a mossy fibre terminal and several granule cells are smaller, less complex and have reduced levels of synapsin-1 staining (Fig. I. 5. B) (Hall *et al.*, 2000). Interestingly, Wnt7a triggers similar changes in cultured granule cell neurons, thus illustrating the capacity of Wnt7a to act in different neuronal types. The canonical Wnt signalling pathway is responsible for these synaptogenic remodelling events, because inhibition of GSK-3ß, a negative regulator of the Wnt pathway, mimics Wnt7a effects. Which are the receptors mediating these responses? Do cerebellar granule cells and pontine mossy fibres express the same receptors enabling them to respond to Wnt7a? How is the external stimuli converted into such changes in axonal behaviour? These are questions our laboratory is trying to address.

In the spinal cord, *Wnt3* is expressed by motor neurons of the lateral motor column at a time when sensory neurons reach the ventral part of the spinal cord to synapse with motor neurons. Wnt3 affects the terminal arborisation of a specific class of sensory neurons, NT-3 dependent Dorsal Root Ganglia (DRG), but does not affect NGF-dependent neurons (Krylova *et al.*, 2002). How is this specificity achieved? It is likely that the precise receptors expressed by specific DRGs play a critical role in determining which neuronal sub-population will respond to the signals encountered.

Wnt7b has recently been shown to stimulate the arborisation of dendrites of hippocampal neurons (Rosso *et al.*, 2005). Surprisingly, unlike the Wnt3-induced arborisation of NT3-dependent DRG, branching of the dendrites of hippocampal neurons relies on a non-canonical pathway. Dishevelled (Dvl) plays a central role in the process, since its overexpression of *Dvl* in neurons mimics application of Wnt7b conditioned medium and neurons grown from *Dvl-/-* mice have less complex dendritic arbors. The signalling cascade triggered by Wnt7b must ultimately affect the cytoskeleton since it leads to the activation of Rac and JNK (Rosso *et al.*, 2005). Yet, in some systems, Wnt7b was shown to induce axonal remodelling in a manner similar to Wnt7a, thus probably using the canonical pathway (Salinas *et al.* unpublished). In fact, a recent paper demonstrates that both Wnt7b and Wnt7a affect the clustering of synaptic vesicle markers such as VAMP2 in mossy fibres (Ahmad-Annuar *et al.*, 2006). How do two closely related Wnt factors, such as Wnt7a and Wnt7b, trigger different signalling pathways in

different cells whilst sharing common effects in other cells? Once again, elucidating the receptors that mediate the effects of Wnt7a and Wnt7b is critical in order to understand these processes better. Identifying the receptors responsible for specific neuronal behaviours would enable to study their intracellular signalling mechanism in much more depth.

I. 1. 4. 2. 3. Cell surface molecules mediate target recognition

Cell-surface adhesion molecules have been implicated in target recognition and in some cases, synaptogenesis. Cadherins and proto-cadherins are localised at synapses and can mediate target recognition by homophilic interactions. For example, interconnected neurons of the limbic system were shown to express a specific cadherin (Bekirov *et al.*, 2002). Yet, cadherins seem only to mediate target recognition and not trigger synaptogenesis because in chick lacking certain cadherins, neurons miss their target but remain capable of forming synapses (Inoue and Sanes, 1997). Therefore additional signals must come into play to trigger synaptogenic differentiation once an axon has encountered a matching partner.

A cell adhesion molecule, SynCAM is able to promote synapse formation in neurons which encounter a cell expressing SynCAM (Biederer *et al.*, 2002). A non-neuronal cell expressing SynCAM was shown to trigger accumulation of active zone markers and most strikingly, the recycling of membrane indicative of active synaptic vesicle exo- and endo-cytosis occurring at these sites. Furthermore, neurons expressing dominant-negative *SynCAM* mutants were unable to differentiate their pre-synaptic terminal correctly (Biederer *et al.*, 2002). More studies are now being carried to evaluate the degree of specificity that SynCAM signalling exhibit.

At the same time Wnt proteins were shown to induce synaptic changes in cerebellar neurons, a cell adhesion molecule called neuroligin was shown to induce contact-induced synaptogenic changes (Scheiffele *et al.*, 2000). Cells expressing neuroligin may induce the formation of active zones as seen by the accumulation of pre-synaptic markers and the recycling of the dye FM1-43, labelling zones of active exo-and endo-cytosis (Dean *et al.*, 2003; Scheiffele *et al.*, 2000). Neuroligin signalling is mediated through ß-neurexin, the neuroligin receptor, which becomes clustered upon

neuroligin binding. This clustering event is associated with the formation of active zones. The neuroligin/ß-neurexin pair came under the spotlight again recently with the discovery the ß-neurexin induced the local clustering of post-synaptic PSD-95 and NMDA receptors (Nam and Chen, 2005). To date, this is the only example of a cell surface molecule pair able to trigger both pre- and post-synaptic differentiation. It raises the interesting possibility that other cell adhesion molecules could also be involved in bi-directional synaptogenic signalling. Otherwise, like agrin at the NMJ, the anterograde signals that mediate post-synaptic differentiation have so far been mainly secreted factors.

I. 1. 4. 2. 4. Post-synaptic differentiation of central neurons

One well established secreted signal that is able to trigger the post-synaptic differentiation of neurons is Narp (neuronal activity-regulated pentraxin)(O'Brien *et al.*, 1999). Narp is able to bind to the extra-cellular domain of AMPA-type glutamate receptors and trigger their clustering. Over-expression of Narp in spinal cord neurons or in 293 cell co-cultured with spinal neurons leads to AMPA-receptor clustering (O'Brien *et al.*, 1999). Interestingly, Narp activity is specific to excitatory synapses on dendrites of inhibitory neurons, once again illustrating the high degree of specificity that neurons exhibit during their response to the signals they encounter (Mi *et al.*, 2002).

Another factor affecting post-synaptic differentiation belongs to the ephrin family of axonal guidance molecules. The receptor of EphrinB, EphB, is able to bind the extra-cellular domain of NMDA receptors and application of EphrinB leads to both EphB and NMDA receptor to cluster (Dalva *et al.*, 2000). Yet, other post-synaptic elements such as the PSD-95 (post-synaptic density 95kDa) scaffold protein are unaffected by EphrinB. Each of the signals mentioned here are trigger precise changes in specific neuronal populations. Clearly, a combination of signalling events is triggered in developing neurons in order to establish neuronal networks. Yet, the molecular basis of individual signalling events must first be understood in order to the possible interactions between signals and pathways.

It is clear that much progress remains to be made in our understanding the molecular processes that regulate synapse formation. Nonetheless, as we discover more

synaptogenic signals and their receptors, numerous *in vitro* assays are available to test the function of the proteins identified. For example, neuronal morphology and synaptic protein accumulation can be observed using fluorescent microscopy, and synaptic activity can either be visualised by FM1-43 dye recycling or by electrophysiological methods. In parallel, the generation of genetically engineered animal models may reveal the *in vivo* functions of some of these genes. Subsequently and using similar approaches the signalling cascades that regulate the neuronal response to these signals will be studied. In the case of Wnt signals and their role in synaptogenesis, the receptors transducing Wnt signals in neurons are up-to-now unknown. Identification of the receptors mediating the synaptogenic effect of Wnt7a in the cerebellum is the main objective of this thesis.

I. 2. Wnt signalling

The Wnt signalling pathway, alongside SHH, BMP, FGF and several other key signalling pathways, is a well characterised and fundamental signalling system involved in many aspects of development. The importance of Wnt signals during development is highlighted by the many developmental disorders associated with mutation of Wnt pathway components (Logan and Nusse, 2004). Furthermore, the identification of Wnt signalling pathway components as oncogenes revealed that Wnt signalling is also important in maintaining the homeostasis of adult tissues. Because of Wnt implication in so many aspects of biology, each component of its signalling pathway has been studied, and even today new proteins continue to be found to be implicated in these complex signalling pathways.

I. 2. 1. The Wnt gene family

The human genome project has identified and localised 19 *Wnt* genes. Wnt proteins share 35% amino-acid sequence identity, but *Wnt* gene sharing the same numeral such as Wnt7a and Wnt7b can share up 79% nucleotide sequence identity. Wnt contain an amino-terminal signal sequence and are involved in intercellular signalling, indicating that they

are secreted proteins. The crystal structure of Wnt proteins remains mostly unknown because of their notorious insolubility, which hinders many biochemical studies of Wnt factors. The recent discovery that Wnt proteins are palmitoylated explains in part this insolubility (Willert *et al.*, 2003). Interestingly, palmitoylation is necessary for Wnt function, probably because it contributes to its cell surface anchoring and limits its diffusion to a few cell diameters (van den Heuvel *et al.*, 1989). Wnts are also glycosylated, but mutagenesis of the sites of glycosylation suggested that this modification is not essential for the signalling activity (Mason *et al.*, 1992). The only structural information known is that folding may rely on intramolecular disulfide bonds because Wnts exhibit highly conserved Cystein residues at very defined positions (Miller, 2002).

The *in vivo* importance of Wnt factors during development is highlighted by the strength of the phenotypes caused by mutations of *Wnt* genes (see the Wnt homepage). Many homozygous mutations of *Wnt* genes are lethal. Many of these mutations affect CNS development, both at early and later stages. For example, Wnt3 is involved in gastrulation whilst Wnt7a is involved in patterning of the limbs and synapse formation (Hall *et al.*, 2000; Liu *et al.*, 1999; Parr and McMahon, 1995). The importance of Wnt signals in the formation of CNS structure is highlighted in *Wnt1* and *Wnt3a* mutants. Mouse lacking *Wnt1* fail to develop a hindbrain and a cerebellum, whilst *Wnt3a* mutants lack the hippocampus (Lee *et al.*, 2000; McMahon and Bradley, 1990). Analysis of mutants for other *Wnt* genes revealed that Wnt signalling is critical in many aspects of development such as limb and kidney development, as well as controlling the proliferation of specific cell types and thus acting a tumor suppressor (Liang *et al.*, 2003; Parr and McMahon, 1995; Stark *et al.*, 1994; Yamaguchi *et al.*, 1999). In order to clarify the way Wnts trigger this plethora of events, the specific receptors for individual Wnt ligands must be identified.

Upon reception of Wnt to the cell surface, three signalling pathways can be activated. The receptors expressed by a given cell play a key role in triggering these diverse cellular responses. Several families of Wnt receptors have been identified and these constitute key branching points for the different Wnt signalling pathways. How have Wnt receptors been discovered? How are the different Wnt signalling pathway

initiated? Which Wnt receptors signal in neurons? These questions will be addressed in more depth in the third part of the introduction and throughout this thesis. Nonetheless it can be mentioned that first and best established Wnt receptors are the Frizzled family of sevenpass transmembrane receptors (Logan and Nusse, 2004). These were first identified in genetic screens in *Drosophila* and subsequently shown to bind Wnt and mimic various aspect of Wnt signalling (Bhanot *et al.*, 1996). In some instances, Frizzled require the assistance of a co-receptor of the lipoprotein family, Lipoprotein related proteins 5 and 6 (LP5/6) (Arrow in *Drosophila)* in order to activate the best established Wnt signalling cascade also known as the "canonical" Wnt pathway. The canonical pathways involves the stabilisation of ß-catenin and transcriptional activation of genes controlled by the TCF/LEF (T-Cell Factor/Lymphoid enhancer factor, to be referred as TCF) family of transcription factors. As mentioned above, in axon guidance, a novel type of receptor has been discovered, Ryk (derailed in *Drosophila*). Other cell surface proteins have been shown to be able to bind Wnt, such as Ror receptors, and activate non-canonical signalling cascades (Oishi *et al.*, 2003). Several lines of evidence suggest that Wnt proteins are endocytosed upon binding their receptors (Gonzalez *et al.*, 1991; van den Heuvel *et al.*, 1989) and endocytosis could play a part in some aspects of Wnt signalling (Bejsovec and Wieschaus, 1995). Clearly, much remains to be elucidated regarding the precise molecular interactions that regulate the signalling cascades activated by Wnt receptors.

I. 2. 2. The Wnt signalling cascades

Different signalling outcomes may be detected in response to a given Wnt. The cell type is a critical factor that determines which signalling pathway may be activated, but other factors, such as the presence of extracellular modulators of Wnt signalling, are also important. The response to Wnt signals can be divided into three pathways: The "canonical" pathway, the Planar Cell Polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Figure I. 6). Although it is possible that several pathways are activated at once, most research has focused on studying these pathways independently.

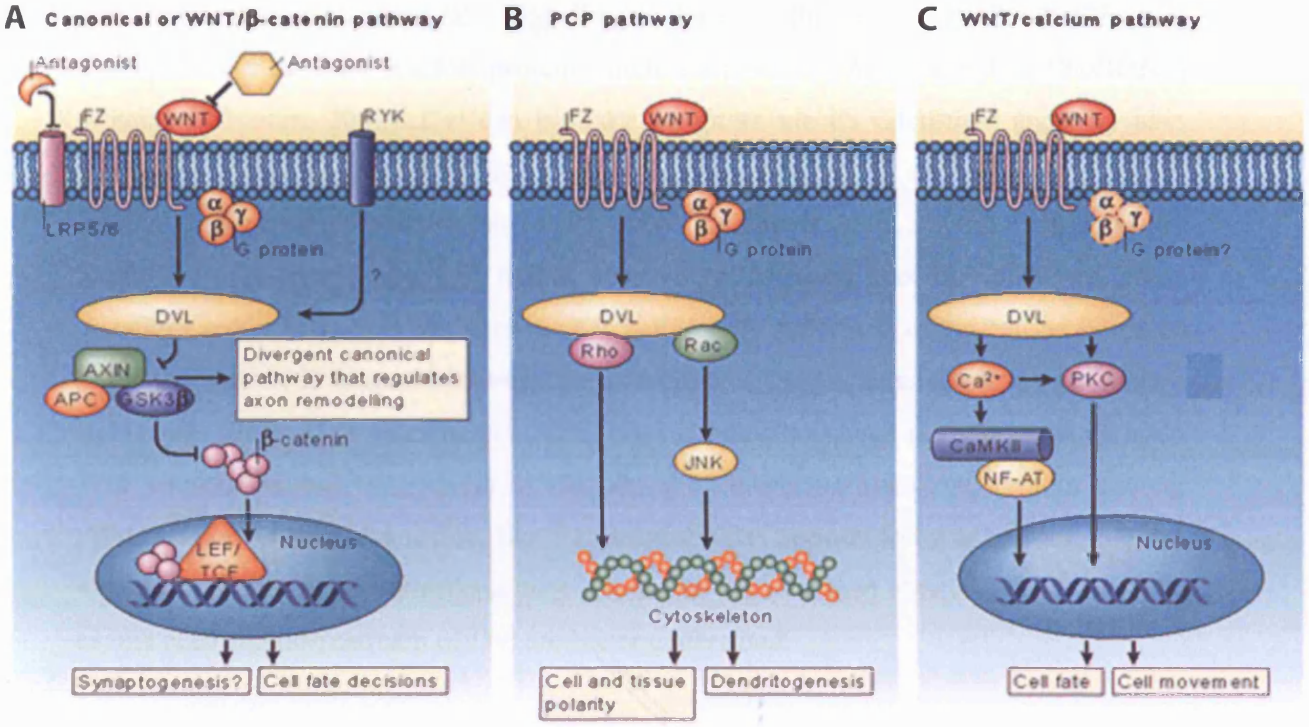


Figure I. 6. The Wnt signalling pathways

Reception of Wnt at the cell surface can trigger a variety of intracellular events but is regulated by different extracellular inhibitors

A. The canonical Wnt pathway is activated upon Wnt binding to the sevenpass transmembrame receptor Frizzled and the LRP5/6 co-receptor. The Ryk receptor may also act to activate this pathway. Through trimeric G-proteins, Dvl is activated. Activated Dvl inhibits the activity of the β–catenin destruction complex composed of Axin, APC, GSK3β and numerous protein kinases and phosphatases. GSK3β's inhibition results in β–catenin accumulation, which can in turn translocate to the nucleus and act as a transcriptional co-activator alongside TCF. Additionally, GSK3β inhibition can affect the activity of Microtubule Associated Proteins (MAP) such as MAP-1B and thus regulate cytoskeletal dynamics. Extracellular inhibitors may regulate this pathway, either by blocking Wnt binding to Frizzled (sFRP) or to LRP5/6 (Dkk).

B. The Planar Cell Polarity pathway also relies on Fz, trimeric G-proteins and Dvl. Although the mechanism of activation of this pathway is in some cases unclear, the outcome is the activation of small molecule GTPases, such as Rho, Rac, and JNK. These molecules affect the cytoskeleton, for example by regulating actin polymerisation, and also stimulate the development of dendritic arbors.

C. The Wnt Calcium pathway. Reception of certain Wnt at Fz receptors can trigger Calcium release from intra-cellular stores in a G-protein dependent manner. Calcium-dependent enzymes such as CamKII and PKC are activated. In turn the signalling cascade activated may affect cell fate or cell movements by both cytoplasmic or nuclear events.

Taken from Ciani and Salinas, 2005.

I. 2. 2. 1. Dishevelled

The component that lies most upstream in the Wnt signalling pathway is Dishevelled (Dvl). In fact, apart from Frizzled receptors, Dvl is the only protein to have been implicated in all three Wnt signalling pathways (Sheldahl *et al.*, 2003; Wharton, 2003). Dvl is a modular scaffold protein which is known to bind no less than 18 different proteins (Wharton, 2003). Dvl can bind Fz receptors via its C-terminus and may also dimerise via its DIX domain with axin, another scaffold protein at the heart of the ß-catenin destruction complex (Itoh *et al.*, 2000; Umbhauer *et al.*, 2000). Although the function of its three conserved domains has been assigned specific functions, many questions around Dvl function remain. It is particularly striking that Dvl can be localised at the membrane, in intracellular vesicles, as well as in the nucleus (Axelrod *et al.*, 1998; Itoh *et al.*, 2005; Umbhauer *et al.*, 2000). Dvl is phosphorylated at multiple sites upon Wnt stimulation but the precise consequence of these modifications remain unclear (Willert *et al.*, 1997). Therefore, like Fz receptors, Dvl appears to be at the core of Wnt signal transduction, but its precise function has so far remained elusive. Thankfully, the events occuring downstream of Dvl are better understood.

I. 2. 2. 2. The canonical / ß-catenin Wnt signalling pathway

The canonical / ß-catenin Wnt pathway is the best understood signalling pathway induced by Wnt factors. The hallmark of this canonical pathway is the elevation of cellular ß-catenin levels, which ultimately affect the transcription of genes controlled by the TCF/LEF transcription factors. Nonetheless, the transcriptional output may vary according to the cell type. The critical role for this signalling pathway in development and the maintenance of tissue homeostasis is highlighted by the number of developmental defects and cancers caused by mutation of Wnt factors or component of the signalling machinery (Logan and Nusse, 2004). In the following section, the main components of Wnt signal transduction within the cell will be described.

I. 2. 2. 2. 1. ß-catenin

The central component of the canonical Wnt pathway is ß-catenin. ß-catenin is a bi-functional protein implicated in both Wnt signal transduction and cell adhesion. During Wnt signalling, ß-catenin cytoplasmic accumulation leads to its translocation to the nucleus, where it functions as a transcriptional co-activator (Huber *et al.*, 1996). In cell adhesion, ß-catenin indirectly links cell surface cadherins to the actin cytoskeleton (Kemler, 1993). Yet, the two processes can occur largely independent of each other, because cadherin loss-of-function does not hyperactivate Wnt signalling and conversely, Wnt signalling does not generally affect cell adhesion (Caca *et al.*, 1999; Gottardi and Gumbiner, 2004).

The pool of cytosolic ß-catenin is dynamically regulated and ß-catenin may translocate to the nucleus if it accumulates inside the cell. A multi-protein complex described as "the destruction complex" regulates ß-catenin stability. Indeed, in the absence of Wnt signalling, ß-catenin is phosphorylated which earmarks it for ubiquitination and subsequent degradation by the proteasome. Upon reception of Wnt signals, ß-catenin is no longer targeted for degradation because the kinases of the destruction complex, GSK-3ß in particular, are inhibited or removed from the destruction complex. As ß-catenin accumulates in the cytoplasm, it may translocate to the nucleus and activate gene transcription through TCF transcription factors (Fig. I. 6. A).

The destruction complex is composed of two kinases and a phosphatase brought together with ß-catenin by the two scaffold proteins Axin and APC. ß-catenin is first phosphorylated by casein kinase Ia (CKIa) which enables it further phosphorylation at three sites by GSK-3ß. These phosphorylation events lead βTrCP to ubiquinate ß-catenin and thus target it for degradation by the proteasome. A phosphatase present in the destruction complex, protein phosphatase 2A (PP2A) may modulate the phosphorylation status of ß-catenin. The critical event leading to Wnt-induced ß-catenin's stabilisation is the removal of GSK-3ß from the destruction complex caused by GBP/frat, which competes with axin for GSK-3ß binding (Li *et al.*, 1999). This event effectively inactivates GSK3ß and leads to the accumulation of ß-catenin. The use of chemical inhibitors of GSK-3ß, such a Lithium or BIO (see materials and methods), are common tools to chemically stimulate the canonical pathway. GBP/frat recruitment to the

destruction complex by Dishevelled was shown to desintegrate the complex, and thus appeared as a critical link between Dishevelled and ß-catenin in this Xenopus system (Li *et al.*, 1999). Yet, mouse mutants lacking all *frat* family members have no obvious defects in canonical Wnt signalling, thus raising questions about the previously established role of frat. Thus, despite the fact many inter-molecular contacts have been established, some molecular mechanisms that lead to ß-catenin stabilisation remain to be established.

Many components of the destruction components interact with either LRP5/6 or Frizzled, the two receptors required to activate this pathway. The finding that Axin binds LRP5/6 and that Dvl binds Fz hints that these components may be recruited at the membrane in some instances (Logan and Nusse, 2004). In fact, recent studies demonstrate that the Axin recruitment to LRP5/6 may be a crucial event in the regulation of the activity of the destruction complex (Tamai *et al.*, 2004). Once again, identifying which specific receptors control given Wnt-mediated processes will enable a more detailed study of the complex events occurring at the membrane during Wnt signalling.

The outcome of ß-catenin's stabilisation and its translocation to the nucleus is a change in gene expression. ß-catenin associates with DNA binding proteins of the TCF/LEF family. In the absence of ß-catenin in the nucleus, TCF functions as a repressor of Wnt-target genes through an interaction with Groucho (Cavallo *et al.*, 1998). Groucho's function is mediated by interactions with histone deacytelases, and thus TCF/Groucho repress Wnt-target genes by affecting the local chromatin structure (Chen *et al.*, 1999). In contrast, as ß-catenin enters the nucleus, it forms a complex with TCF and converts the complex into a transcriptional activator. It is thought that ß-catenin binding to TCF removes Groucho from the complex and instead recruits the histone acetylase CBP/p300 or other chromatin remodelling enzymes. Other nuclear components affect ß-catenin function in the nucleus. Some factors affect ß-catenin binding to TCF or the formation of the TCF/CBP complex, such as Chibby or ICAT (Tago *et al.*, 2000; Takemaru *et al.*, 2003). TCF is also subject to regulation by Nemo which phosphorylates TCF and affects its DNA binding affinity (Ishitani *et al.*, 2003). Therefore expression of Wnt target genes is controlled by both the DNA-binding properties of TCF and changes in chromatin structure.

The genes that are activated by Wnt signalling may vary according to the cell type. Wnt signalling often affects cell proliferation and thus cell cycles regulators such as cyclinD1 are direct transcriptional targets of Wnt signalling (Tetsu and McCormick, 1999). In addition to the direct TCF-target genes induced by Wnts, a cascade of genes may in turn be induced by these TCF-target genes. This indirect activation of "secondary" target genes enables a broad range of transcriptional response to Wnts and also permits the fine tuning of the cellular response to Wnts. Feedback loops that up- or down-regulate components of the Wnt signalling pathway are also a common feature in most cells responding to Wnts. Interestingly, many genes affected by Wnt signalling are receptors from either Fz, LRP or HSPG gene families (Baeg *et al.*, 2001; Cadigan *et al.*, 1998; Wehrli *et al.*, 2000; Willert *et al.*, 2002). Receptor down-regulation is perhaps used to reduce signalling in activated cells or permit the diffusion of Wnt ligand towards cell further away from the Wnt source (Logan and Nusse, 2004). It thus appears that the transcriptional consequence of Wnt signalling has evolved to provide complex, cell specific responses. Wnt signalling is thus able to fine-tune itself within the cell and the environment, in addition to triggering a precise cellular response.

In addition to having this critical role at regulating gene transcription, the canonical / ß-catenin pathway affects cytosolic events. Of particular interest is how does canonical Wnt signalling affect neuronal cytoskeleton? Although the main consequence of canonical signalling is a transcriptional response, the effect of Wnt on the neuronal cytoskeleton can be mimicked by GSK-3 inhibitors (Hall *et al.*, 2002; Lucas and Salinas, 1997). Therefore, canonical Wnt signalling is not limited to a transcriptional response and a "divergent" canonical pathway has emerged (Ciani *et al.*, 2004). Dishevelled is central to the microtubule stabilisation induced by Wnt, since its expression mimics the effects caused by Wnt7a (Hall *et al.*, 2000). Neurons expressing *Dvl1* have larger growth cones and thicker axons (Ciani *et al.*, 2004). This is due to an increased number of stable microtubules which loop at enlarged growth cones. Dvl's role in microtubule stabilisation is also seen in differentiated neuroblastoma cells, where *Dvl1* expression protects stable microtubules from depolymerization by nocodazole (Krylova *et al.*, 2000). Strikingly, these cytoskeletal remodelling events require neither ß-catenin nor transcription, whereas inhibition of GSK-3 mimics the effect of Dvl on microtubule stability. Therefore, Wnt

signalling to microtubules involves a localised regulation of GSK-3 activity by Dvl and Axin, both of which bind microtubules (Ciani *et al.*, 2004). In turn, changes in GSK-3 activity modulate microtubule stability through the direct phosphorylation of MAP-1B, a microtubule associated protein, by GSK-3 (Goold *et al.*, 1999). These findings provided the molecular basis explaining how Wnt could directly regulate neuronal shape and behaviour.

1. 2. 2. 3. The Wnt/Ca2+ pathway

Originally, Wnts were classified in two-categories: Transforming or non-transforming. When *Wnts* were expressed in epithelial cells, some were able to alter cell morphology and growth characteristics whilst other did not affect them (Wong *et al.*, 1994). In addition, these "transforming" Wnts were able to induce the formation of secondary axis when expressed in *Xenopus* embryos. Typical "transforming" Wnt were *Wnt1*, *Wnt3a* and *Wnt8*. With time, transforming Wnt became labelled as "canonical" Wnts as they emerged to be able to activate the ß-catenin pathway when over-expressed (Shimizu *et al.*, 1997). On the other hand, other Wnts, such as Wnt-5a, showed little or no ability to induce these changes and we thus labelled as "non-transforming". Yet, some non-transforming *Wnts* were found to affect cell movements when over-expressed in *Xenopus* embryos (Moon *et al.*, 1993). Clearly, the Wnt gene family was not involved in only one process and it appeared likely different signalling cascades could be triggered by different Wnts.

The first clue that this alternative Wnt signalling pathway involved calcium signalling was the observation that over-expression of 5HT1c serotonin receptor in *Xenopus* embryos displayed a similar phenotype that *Wnt-5a* over-expression (Ault *et al.*, 1996; Slusarski *et al.*, 1997). Since the 5HT1c receptor was known to stimulate Ca^{2+} release from intracellular stores, Wnt and Frizzled proteins that were known to be weakly "transforming" proteins, we tested for their ability to influence calcium signalling. Wnt5a and RFz2 were shown to affect calcium transients when over-expressed in *Xenopus* embryos (Slusarski *et al.*, 1997). Calcium release involved the phosphatidylinositol pathway and G-proteins because it was sensitive to inositol phosphatases and pertussis toxin. The transforming Wnt8 was unable to trigger the same response as Wnt5a. rFz1

triggered a much lower calcium response than RFz2 (Slusarski *et al.*, 1997). Therefore it appeared that separate signalling pathway could be affected by specific Wnt/Frizzled combinations.

Cells are equipped with many Calcium-sensitive enzymes and the response to Wnt5a and RFz2 was shown to activate Protein Kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) (Kuhl *et al.*, 2000; Sheldahl *et al.*, 1999). The precise events occurring downstream of these two enzymes remain unclear, but in the same way than activation of the canonical pathway can lead to various cellular responses, this pathway can surely affect various cellular processes (Fig I. 6. C). The calcium pathway is implicated in cell movements during gastrulation, in cell adhesion and heart development (Kuhl *et al.*, 2001; Sheldahl *et al.*, 2003; Tada *et al.*, 2002; Veeman *et al.*, 2003). Strikingly, in some circumstances, activation of this pathway inhibits ß-catenin's effect on gene transcription (Slusarski *et al.*, 1997). Clearly, a better understanding of individual signalling pathways, as well as cross-talk between pathways, will clarify the cellular consequence of Wnt signalling activation.

1. 2. 2. 4. The planar cell polarity pathway

Frizzled and *Dishevelled*, two key components of the Wnt signal transduction machinery, were first described as *Drosophila* mutants for the direction of hair growth in the developing wing. Indeed, in addition to a classical apical-basal polarity, some epithelia are also polarised along the plane of the epithelium, a polarity known as Planar Cell Polarity (PCP). In the *Drosophila* wing, such a polarity is important in order to guide the uniform direction of hair growth towards the distal end of the wing. PCP signalling establishes a cortical mark on cells in order to assist their organisation and function. The coordinate organisation of fish scales, bird feathers, retinal cells, as well as the stereocilia of the inner ear are all generated by PCP signalling.

Many components of the PCP pathway were identified in *Drosophila*. In particular within cells of the developing wing, defects in PCP signalling affect the orientation, subcellular localisation or number of hair growing from given cells. *Frizzled* and *Dishevelled* play central roles in PCP signalling, but all the other PCP genes are distinct from the genes involved in canonical signalling. If *dFz1* is mutated in the

Drosophila, hairs of the developing wing form at the centre of the cell and fail to point distally (Wong and Adler, 1993). Epistasis experiments revealed that Dishevelled is immediately downstream of Fz in PCP signalling, but lies upstream of other PCP genes (Krasnow *et al.*, 1995). Dishevelled is therefore considered as a possible branching point for these two pathways, but our understanding of it mode of activation and its function remains so far elusive. Additionally, different Frizzled may preferentially activate one pathway or the other. Although dFz1 and dFz2 can partially compensate for each other in the canonical pathway, dFz1 is exclusively required for PCP signalling whilst dFz2 has no role in PCP and cannot compensate for dFz1 (Bhanot *et al.*, 1999; Strutt, 2003).

One critical aspect of PCP signalling is asymmetric distribution of polarity molecules. Fz and Dvl accumulate at the distal membrane whilst two other central PCP factors Strabismus (aka Van Gogh) and Prickle localise on the proximal side (Fanto and McNeill, 2004). Flamingo, a member of the protocadherin family, is localised on both proximal and distal sides of the cell. Using inter-cellular homophillic adhesion, flamingo contributes to the stabilisation of this asymmetric distribution (Usui *et al.*, 1999). The number of genes involved exclusively in PCP and not in canonical signalling illustrates the importance of a tight regulation of the signalling events occurring downstream of Fz/Dvl.

Downstream of this asymmetrically localised signalling complexes lie small GTPases that control the growth of actin bundles which give rise to hairs in the *Drosophila* wing (Fig. I. 6. B). Mutations of Rac, RhoA and the Rho effector *Drok* lead to multiple wing hairs (mwh) phenotypes, a common indicator of PCP defects. The activation of Rho and this signalling cascade requires Daam1, a molecule binding the PDZ domain of dishevelled (Habas *et al.*, 2003). A parallel pathway does not require Daam1 but leads to the activation of Rac and ultimately stimulates the JNK kinase pathway. Quantification of active Rac in cells can therefore be used as a read-out for the activation of this pathway. The DEP domain of Dvl is required for the activation of this Rac/JNK pathway (Habas *et al.*, 2003; Veeman *et al.*, 2003). In addition, cells treated with microtubule-disrupting drugs such as vinblastine give rise to multiple hairs without defects in orientation (Turner and Adler, 1998). Therefore the microtubule cytoskeleton appears to function to localise factors to promote hair initiation at precise locations.

Furthermore, two PCP mutants *inturned (in)* and *fuzzy (fy)* have an enhanced phenotype when mutant wings are treated with microtubule-disrupting drugs (Adler and Lee, 2001). *in* and *fy*, may thus collaborate with Rac to regulate microtubule dynamics (Eaton *et al.*, 1996).

Finally, an even more complex PCP signalling event is the fact PCP mutants have non-cell autonomous effects. Indeed, clones of *Fz* and *Strabismus* mutant cells affect the direction of hair growth in the surrounding wild type cells (Taylor *et al.*, 1998; Vinson and Adler, 1987). The mechanism of this non-cell autonomous effect is still controversial, but the release of an unknown secreted factor, factor X, has been brought forward to provide missing links in this phenomenon. Newly found PCP genes, the atypical cadherin *fat (ft)* and *dachous (ds)* were found to be essential to PCP signal in the developing eye, the wing and the abdomen (Adler *et al.*, 1998; Yang *et al.*, 2002). These proteins have been proposed to regulate Fz activity but also to control the production of factor X through their interactions with the transcriptional repressor Atrophin (Fanto *et al.*, 2003; Yang *et al.*, 2002). Clearly, non-canonical Wnt signalling pathways must be studied in more depth to confirm the many plausible hypotheses brought forward by studies in the wing and eye.

I. 2. 2. 5. Cross-talk between Wnt pathways

One last critical point to clarify is the cross-talk between canonical and non-canonical Wnt pathways. Indeed, some reports indicate that non-canonical signalling may inhibit canonical signalling *in vivo* (Topol *et al.*, 2003; Torres *et al.*, 1996). One striking observation in *Xenopus* embryos is that over-expression of the non-canonical *Wnt5a* can block the secondary axis formation induced by *Wnt8* over-expression (Torres *et al.*, 1996). In the *Wnt5a* knock-out mouse, domains of Wnt/ß-catenin signalling controlling limb formation are expanded (Topol *et al.*, 2003). A recent paper demonstrated that Wnt5a could inhibit Wnt3a-induced TCF-transcription in cell lines (Mikels and Nusse, 2006). Strikingly, the cell surface receptors expressed play a critical role in this process. The Ror2 receptor is mediating this inhibitory action of Wnt5a, but if LRP6 and Fz4 are co-expressed, cells respond to Wnt5a by activating TCF-mediated transcription. Interestingly, this cross-talk seems to occur downstream of ß-catenin,

because Wnt5a does not affect the Wnt3a-induced increase in cellular ß-catenin The mechanism enalbing this inhibitory effect of Wnt5a remain unclear, as Wnt5a did not induce a calcium wave or require pertussis toxin-senstive G-proteins (Mikels and Nusse, 2006). Nevertheless, this study was the first to study interactions between two Wnts of such antagonistic consequence. Surely, a better understanding of signalling at the point where the signalling cascades diverge is critical to understanding these processes.

I. 3. Wnt Receptors

The response of a cell to extra-cellular factors relies on the specific cell-surface receptors it expresses. In the case of Wnt signalling, in addition to the 10 mammalian *Frizzled* genes, the first Wnt receptors to be identified, three gene families have also been implicated in binding and transducing Wnt signals. The *LRP5* and *LRP6* genes (*Arrow* in Drosophila) are now well established as necessary components of the canonical Wnt signal transduction machinery, functioning as Wnt-co-receptors alongside Fz. Ryk, an atypical receptor tyrosine kinase, has recently been implicated in transducing Wnt signals in axonal guidance (Liu *et al.*, 2005; Lu *et al.*, 2004; Schmitt *et al.*, 2006). A third class of Wnt receptors belonging to the Ror family of receptor tyrosine kinase have been implicated in cell migration in *C. elegans* and convergent extension mouvements during *Xenopus* embryogenesis, two processes controlled by non-canonical Wnt signalling pathways (Forrester *et al.*, 2004; Hikasa *et al.*, 2002). In mammalian cells, Ror2 was shown to mediate Wnt-5a signalling leading to the activation of the JNK pathway in mammalian cells (Oishi *et al.*, 2003). Clearly, the 19 *Wnts* trigger diverse intracellular responses using cell-specific receptor complexes.

Which receptor complexes mediate Wnt signalling in developing neurons? How do these receptors co-operate to trigger the different Wnt signalling cascades? Most past research has focused on studying the roles played by individual receptors. Yet, it has become apparent that Wnt reception involves multi-protein complexes, and therefore several receptor components must be studied in parallel in order to gain a better understanding of how Wnt signals are transduced across the membrane.

The receptors mediating the synaptogenic effects of Wnts in the developing cerebellum and hippocampus are to this date unknown. Since canonical signalling has been implicated in the Wnt7a-induced changes occurring in the developing cerebellum, it is likely that a Fz/LRP co-receptor complex is implicated. On the other hand, Wnt7b dendritogenic effects in the hippocampus are mediated by non-canonical pathways, therefore it is possible that other receptor complexes than Fz/LRP may be implicated in this process. What are the specific receptor components mediating these effects? In order to understand the possible signalling mechanisms occurring, Wnt binding to its receptors and how these receptors connect to the downstream signalling machinery will be described. Furthermore, the role of Wnt proteins in bringing different receptor components together, as it does for the Fz/LRP complex, will be highlighted.

I. 3. 1. Frizzled receptors : the "canonical" Wnt receptors

The *Frizzled* gene was first identified in *Drosophila* mutants with defects in the cell polarity of their wing (Vinson and Adler, 1987). Analysis of the *Fz* sequence revealed it was a seven-transmembrane receptor and that it had numerous homologues across species, and thus gave rise to the *Frizzled* gene family (Vinson *et al.*, 1989; Wang *et al.*, 1996).

I. 3. 1. 1. Frizzled protein domains

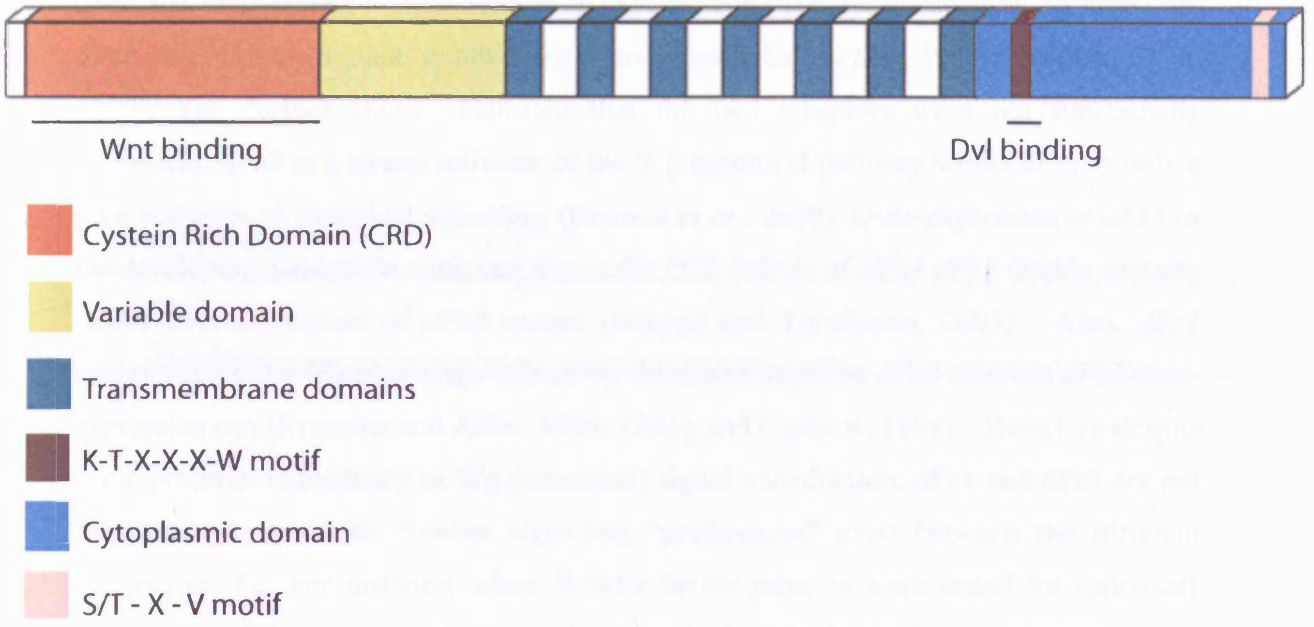
In a search for *Fz*-related sequences, Bhanot and colleagues identified *dFz2* in the *Drosophila* genome, which shared 33% sequence identity with *Fz* (to be referred from now as *dFz1*). The Frizzled gene family share a conserved pattern of 10 cystein residues in its extracellular N-terminal domain as well as a S/T-X-V sequence at its C-terminal (Figure I. 7. A). Bhanot and colleagues showed that dFz2 could bind and transduce Wingless (Wg), the *Drosophila* Wnt, signalling. Transfection of *dFz2* conferred Wg-responsiveness to S2 as seen by an increase in intracellular levels of armadillo (ß-catenin). In addition, 293 cells expressing either *dFz2* or its extracellular Cystein-Rich Domain anchored to the membrane via a GPI anchor (*GPI-dFz2CRD*) could retain Wg at their

surface (Bhanot *et al.*, 1996). This study was the first to demonstrate binding and signalling between a Wnt and a Fz receptor. It is also one of the only publication to show binding of a Wnt to a full-length Fz. From this point onwards, Frizzled receptors were thought to be the main transmembrane proteins responsible for Wnt signal transduction.

A subsequent publication by Nusse and co-workers compared the capacity of six mammalian frizzled (mFz3,4,5,6,7,8), one Xenopus Frizzled (XFz8) and dFz2 in their ability to bind XWnt8 (Hsieh *et al.*, 1999). XWnt8 was only able to bind a subset of these receptors indicating the specificity of Wnt/Fz binding. In addition, the authors devised a solid-phase binding assay using a fusion protein consisting of the Fc domain of human immunoglobulin fused to the CRD of different frizzled (FzCRD-IgG). This construct was used to demonstrate that XFz8CRD-IgG could precipitate XWnt8. The authors also quantified the affinity of the interaction between XWnt8-AP (Alkaline Phosphatase) fusion protein and XFz8CRD-IgG immobilised on protein-A agarose beads. The affinity of the binding was estimated to be of 9 ±2nM (Hsieh *et al.*, 1999). This data confirmed the hypothesis that the CRD domain was critical for Wnt binding to Fz.

The crystal structure of the CRD domains of Fz and the secreted Wnt antagonist sFRP3 was recently published (Figure I. 7. B) (Dann *et al.*, 2001). The CRD domain lacks homology with other protein folds, and thus may constitute a new family of ligand-binding fold. The CRD is composed mainly of α-helices and all its cystein residues form disulfide bonds. A binding assay of CRD mutants and the mapping of solvent-exposed residues highlighted a single region at the CRD surface responsible for Wnt binding. Interestingly, CRD crystals we often found as dimers. The dimer interfaces are highly complementary and are conserved between Fz8 and sFRP3. These data therefore suggested that Frizzled receptors may function as dimers (Dann *et al.*, 2001).

A. Frizzled receptor structure



B. Crystal structure of a Fz8CRD dimer

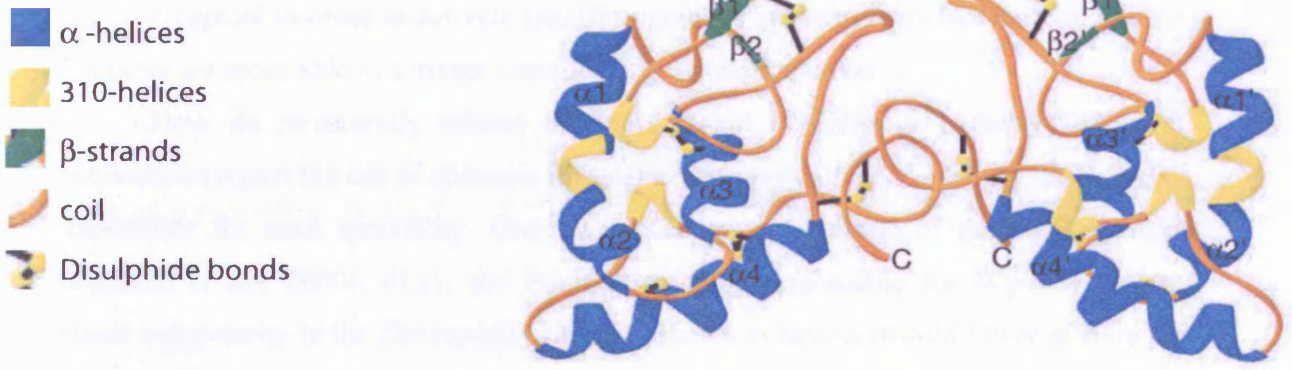


Figure I. 7. Structure of Frizzled receptors

Several domains of Frizzled are known to play specific roles in Wnt signal transduction

A. Frizzled receptors are composed of an conserved extra-cellular domain responsible for Wnt binding, a variable domain, seven transmembrane domain and a cytoplasmic portion Dishevelled binds Fz at a conserved motif immediately after the seventh transmembrane domain.
B. The crystal structure of Fz8CRD revealed the peptide tended to form dimers, suggesting Fz receptor dimerisation could be mediated by the CRD domain. The FzCRD is mainly α-helical with all 10 cystein residues forming disulfide bonds. The fold of the CRD does not have clear stuctural homologs.
Adapted from: A. The Wnt homepage (Nusse, Stanford). B: Dann *et al.*, 2001.

The study of the roles played by different domains of Frizzled came in the context of establishing the roles of dFz1 and dFz2 during the development of *Drosophila* wing. *dFz1* and *dFz2* appear to be redundant in canonical Wg signal transduction, because only *dFz1, dFz2* double mutants exhibits *Wg* phenotypes (Bhanot *et al.*, 1999; Chen and Struhl, 1999). Yet, further studies indicated that the two receptors were not functionally equivalent. dFz2 is a strong activator of the Wg canonical pathway whilst dFz1 is only a poor activator of canonical signalling (Boutros *et al.*, 2000). Over-expression of *dFz1* in the developing *Drosphila* wing can rescue the PCP defects of *dFz1 dFz2* double mutants whilst over-expression of *dFz2* cannot (Strapps and Tomlinson, 2001). Also, *dFz1* cannot rescue the *Wg*-phenotype caused by dominant negative *dFz2* whereas *dFz2* over-expression can (Krasnow and Adler, 1994; Zhang and Carthew, 1998). Therefore despite some potential redundancy in Wg (canonical) signal transduction, dFz1 and dFz2 are not functionally equivalent. Similar signalling "preferences" exist between the different mammalian *Fz*. For instance, when Wnt-Fz fusion proteins were tested for canonical signalling activity when co-transfected alongside LPR6, Wnt-Fz4 and Wnt-Fz5 fusion proteins showed to more potently activate canonical signalling than Wnt-Fz7 or Wnt-Fz3 (Holmen *et al.*, 2002). Clearly, Fz receptors may associate with particular Wnt ligands and co-receptors in order to activate specific signalling pathways *in vivo,* and specific Fz receptors are more able to activate canonical signalling than others.

How do structurally related receptors signal to different pathways? Several publications report the use of chimeric dFz1/dFz2 chimeras aimed to identify the domains responsible for such specificity. One study focused on the role of the CRD domain (Rulifson *et al.*, 2000). dFz1, the Frizzled receptor responsible for Wg-independent planar cell polarity in the *Drosophila* wing was shown to have a 10-fold lower affinity for Wg that dFz2 (Rulifson *et al.*, 2000). By exchanging the CRD of *dFz2* with the dFz1CRD domain (*dFz2-Fz1CRD*), the chimeric *dFz2-Fz1CRD* showed a reduced capacity to produce a Wg phenotype, characterised by ectopic wing margin bristles. Ectopic expression of the reverse construct, *dFz1-Fz2CRD*, produced an enhanced Wg-phenotype as compared to wild-type *dFz1* expression alone. On the other hand, this Fz chimeras did not affect the capacity of dFz1 to affect PCP signalling (Rulifson *et al.*, 2000). Therefore,

high-affinity Wg binding appears to be critical for Wg-mediated canonical signalling but dispensable for Wg-independent PCP signalling.

Another study focused on the role of the cytoplasmic sequences of Frizzled receptors in activating either canonical or PCP signalling (Boutros *et al.*, 2000). Using more complex dFz1/dFz2 chimeras, Boutros and colleagues highlighted a key role for cytoplasmic domains in determining pathway specificity. Indeed, the cytoplasmic tail of *dFz2* consistently activated Wg-armadillo signalling. In contrast, both the intracellular loops of the transmembrane domains and the cystoplasmic tail of *dFz1* were required to cause PCP gain-of-function phenotypes when over-expressed (Boutros *et al.*, 2000). Therefore, although the cytoplasmic tail of *Fz* is critical for canonical signalling, no specific *Fz* domain appears to be responsible for PCP signalling.

A study by Umbauer and colleagues identified a conserved protein motif in the C-terminus of Frizzled that is required for the activation of canonical signalling and Dishevelled binding (Umbhauer *et al.*, 2000). Through serial deletions and chimeric frizzled proteins, the Lys-Thr-X-X-X-Trp motif located C-terminal to the last transmembrane segment was demonstrated to be critical. This motif is responsible for both Wnt target gene induction, Dishevelled phosphorylation and membrane relocalisation in *Xenopus* embryos (Umbhauer *et al.*, 2000). The functional equivalence of the cytoplasmic domains of the three frizzled tested in this study indicate that specific interactions with other transmembrane or intracellular proteins must hold the key to establishing pathway specificity. What are these binding partners? By which mechanisms are they recruited? What is the function of Dishevelled recruitment at the membrane? Additionally, the role of Wnt ligands in bringing receptor complexes together appears to be a key event during signal transduction (Cong *et al.*, 2004; Liu *et al.*, 2005; Tamai *et al.*, 2000). Clarifying the molecular interactions at the cell membrane is critical to understand the way cells respond to Wnt/Fz signalling.

I. 3. 1. 2. *In vivo* Frizzled function

I. 3. 1. 2. 1. *Fz* Phenotypes in Mouse

Analysis of *Fz4* expression using a lacZ knock-in reporter found fz4 to be expressed in many regions of the CNS, including cerebellar Purkinje cells. The cerebellum of *Fz4* (-/-) mice develops normally until P18 but extensive cell death then occurs, notably in cerebellar granule cells. The defects observed in *Fz4* mutants highlights the importance of individual Fz genes in different developmental processes and hint the potential of therapies targeted at Wnt/Fz function.

The targeted mutation of *Fz3* in the mouse revealed a role for Fz in the development of the CNS (Lyuksyutova *et al.*, 2003; Wang *et al.*, 2002). Pre-natally, *Fz3* expression seems to be restricted to the CNS. At mid-gestation, *Fz3* is highly expressed in the cortex, the diencephalon and the brainstem. *Fz3* -/- newborn mice have a curled tail, an indicator of neural tube defects, flexed limbs and die of respiratory failure shortly after birth. Analysis of *Fz3* -/- embryos revealed for the first time that Fz signalling may be involved in axonal development. In *Fz3* -/-mice, several major axonal tracts of the forebrain, such as the thalamocortical and nigrostriatal tracts, are severely disrupted. The lack of appropriate connections leads to the death of many striatal neurons at late stages of gestation. In the developing spinal cord of *Fz3* mutants, commissural axons show anterior-posterior guidance defects (Lyuksyutova *et al.*, 2003). These two mouse mutants highlight that Fz are involved in different key aspects of CNS development. Understanding the identity and function of Wnt/Fz pairs is a challenge that could pave the way for neuronal regeneration therapies.

I. 3. 1. 2. 2. *Frizzled and disease*

Mutations of *Drosophila Frizzled* revealed some critical differences between *dFz1* and *dFz2*. What are the consequences of *Frizzled* mutation in mammals? Naturally occurring mutations of *Frizzled4* genes are responsible for familiar exudative vitreoretinopathy (FEVR) (Robitaille *et al.*, 2002). This disease is autosomal dominant and causes defects in vascularisation of the retina. It appears that FEVR is caused by a mutation in *Fz4* which causes Fz4 to oligomerise and accumulates in the ER. This highlights that feature that the functional expression of Frizzled can be difficult, a fact reported by other researchers in the field (Hering and Sheng, 2002).

Fz4 was later associated with another disease affecting the eye vasculature, Norrie's Disease, and found to be a receptor for the Norrin protein (Xu *et al.*, 2004). This constitutes the only well-established receptor-ligand pair involving a Frizzled in mammals. The targeted deletion of *Fz4* in the mouse leads to cerebellar degeneration and progressive deafness caused by defects in the peripheral auditory system (Wang *et al.*, 2001)

I. 3. 2. Frizzled receptor binding partners

In addition to binding Wnt factors, Frizzled are able to bind transmembrane co-receptors and intracellular signalling effectors. Recently, small trimeric GTPases have been implicated as the closest downstream effectors of frizzled signalling (Katanaev *et al.*, 2005), and their role may fill-in the gaps remaining regarding how frizzled are coupled to the different signalling pathways they trigger. Some reports have associated the capacity of certain Frizzled receptors to recruit Dvl to the membrane with their capacity to activate the PCP pathway (Axelrod et al., 1998). Yet, several other reports claim that frizzled receptors are able to recruit Dvl to the membrane when over-expressed, regardless of the pathway preferentially activated by the receptor (Boutros *et al.*, 2000; Umbhauer *et al.*, 2000). Although the function of Dvl's membrane recruitment is not clear, more and more evidence point to the fact that a receptor complex containing Dvl and Axin may be formed upon Fz/LRP association. As more molecular interactions between Wnt signalling pathway components are detected, the better we begin to understand how Wnt signalling cascades are triggered.

I. 3. 2. 1. Cytosolic Frizzled-binding partners

Frizzled being seven transmembrane receptors, it is not surprising they have been found to interact with a number of cytoplasmic molecules. Since it remains unclear how Wnt signalling is triggered upon Wnt binding, understanding which proteins physically associate with frizzled receptors is an important starting point to further studies. Two elements of the ß-catenin destruction complex have been shown to interact with Frizzled. Dishevelled binds Fz directly through is PDZ domain, and APC may be present in a

ternary complex consisting of PSD-95 and Frizzled (Hering and Sheng, 2002; Wong *et al.*, 2003). What could be the consequence of these interactions?

The direct binding of Dvl to Fz is critical because interfering with this interaction disrupts canonical signalling (Wong *et al.*, 2003). Indeed, chemical-shift perturbation NMR spectroscopy demonstrated that the PDZ domain of Dvl could bind the membrane proximal region of the Fz C-terminal tail. An earlier report had identified a conserved KTXXXW motif at the Frizzled C-terminal that was necessary for canonical pathway activation, and that activation was co-related with the phosphorylation and membrane re-localisation of Dvl (Umbhauer *et al.*, 2000). Wong and colleagues further studied the importance of this motif and confirmed the KTXXXW motif was critical for Dvl binding (Wong *et al.*, 2003). Interestingly, the authors reported that the Dvl inhibitor Dapper (Frodo), despite not sharing sequence homology with the Fz peptide interacting with Dvl, also binds to Dvl PDZ domain. This study therefore elucidated a novel mechanism for Wnt signalling inhibition by Dapper which disrupts Fz/Dvl binding, in addition to mapping the site of binding to the PDZ domain of Frizzled. Importantly, disrupting Fz/Dvl binding did not affect non-canonical Wnt signalling, suggesting that the weak interaction reported between Fz and Dvl only affected canonical signalling (Wong *et al.*, 2003). Therefore, Fz/Dvl interaction through Dvl PDZ domain appears critical to canonical signalling.

An interaction between certain Frizzled and PSD-95 was also reported (Hering and Sheng, 2002). This interaction is of particular interest to our laboratory since PSD-95 is known to be a central scaffolding molecule at central synapses. Not only does this paper demonstrate that PSD-95 co-clusters with Fz1-2 and -7 at the surface of COS7, but it also shows that PSD-95 facilitates the surface targeting of these Frizzled. Furthermore, in cells expressing *PSD-95*, *rFz2* and *APC*, the localisation of APC shifts from a filamentous pattern reminiscent of microtubules to a clustered staining co-localising with rFz2 and PSD-95 (Hering and Sheng, 2002). Clearly, these ternary complexes should be studied in more depth as they could be involved in both cytoskeletal and synaptic changes induced by Wnts. The observation made by Hering and Sheng goes along the idea of a multi-protein complex being assembled around Frizzled upon receptor activation. In

addition to numerous cytosolic interactions, Frizzled also interact with the trans-membrane co-receptors of the LRP family.

I. 3. 2. 2. LRP5/6 are necessary co-receptors for canonical Wnt signalling

Three papers published back-to-back in the journal Nature in 2000 put under the spotlight a novel transmembrane protein family essential to canonical Wnt signalling. Mutations of the genes *arrow* in *Drosophila* and *LRP6* in mouse phenocopy mutations of *Wnt* known to affect developmental programs through the canonical pathways (Pinson *et al.*, 2000; Wehrli *et al.*, 2000). In *Drosophila, arrow* is required cell-autonomously for cells to respond to Wg. The *arrow* phenotype is rescued by *dvl* expression but is not rescued by *Wg* over-expression (Wehrli *et al.*, 2000). Analysis of the arrow primary sequence reveals a high degree of conservation with mammalian LDL-receptor related proteins LRP5 and LRP6. Arrow/LRP5/6 are putative type I receptor proteins containing EGF-like and LDL-receptor repeats (Wehrli *et al.*, 2000) (Figure I. 8). It thus appeared likely that this novel component acted as a receptor for Wnt. It was in fact suggested to act as a co-receptor alongside Fz. Indeed, loss of *arrow* disrupts the wing margin, an identical phenotype to the *dFz1 dFz2* double knock-out. Additionally, the potentiation of Wg signalling when dFz2 is over-expressed is dependent on *arrow*. Taken together, this publication demonstrated that *arrow/LRP5/6* is a critical component of the Wg receptor complex. Importantly, the authors did not notice any defects in PCP signalling, therefore indicating that arrow/LRP5/6 function is limited to canonical/ß-catenin signalling (Wehrli *et al.*, 2000).

Mutations of *LRP6* in the mouse leads to developmental defects which reflect a combinations of defects observed by the mutations of individual Wnt genes (Pinson *et al.*, 2000). *LRP6* homozygous mutants have severe defects in the development of their caudal body parts, a phenotype hypomorphic to *Wnt3a* mutants. LRP6 mutants also have limb patterning defects like *Wnt7a* mutants and have similar phenotypes to *Wnt1* mutants with regards to mid/hindbrain development (Pinson *et al.*, 2000). All these *LRP6* phenotypes are a consequence of defects in canonical signalling, thus indicating that *LRP6* played a critical role in canonical Wnt signal transduction. Yet, *LRP6* phenotypes are less severe

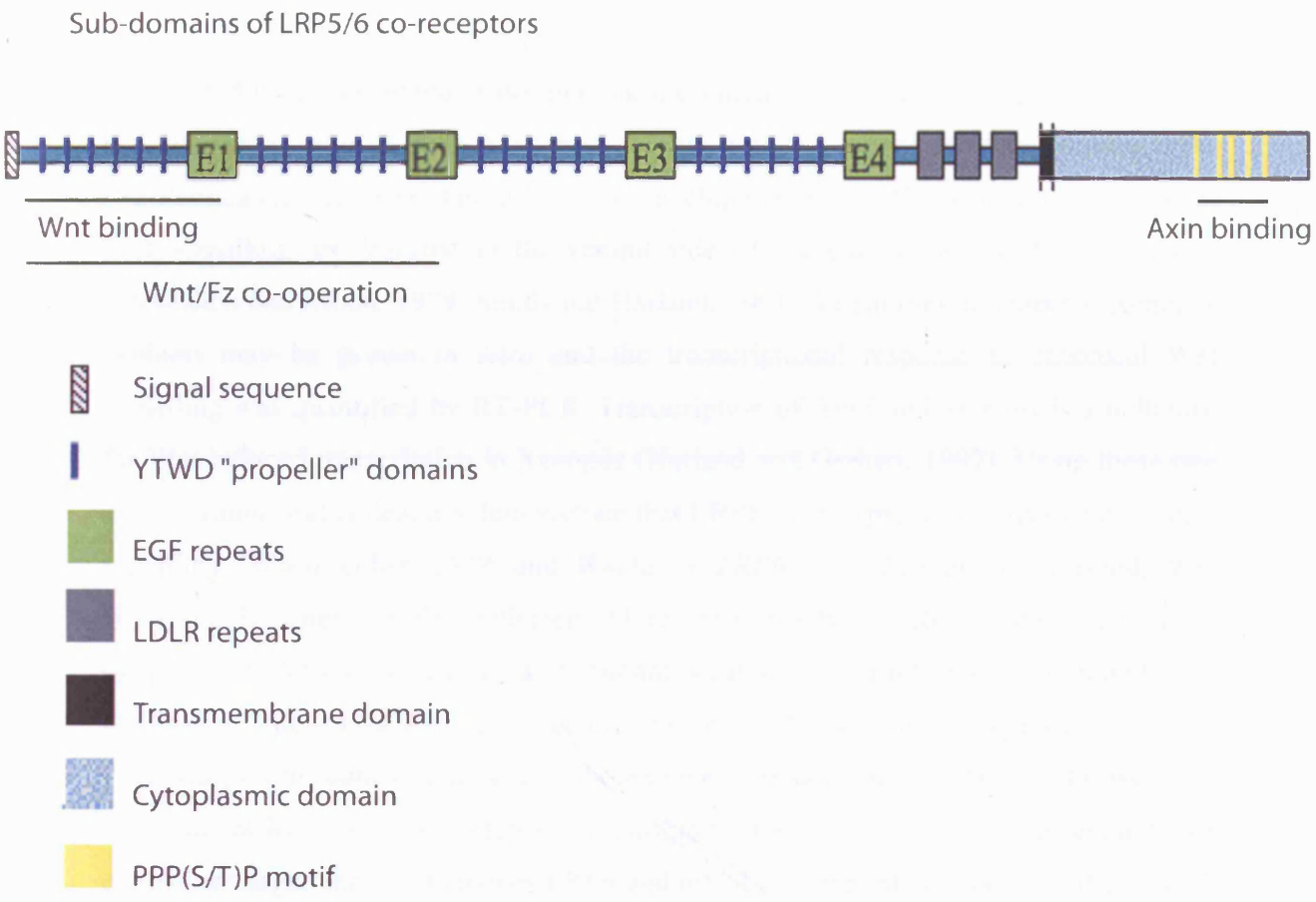


Figure I. 8. Structure of Arrow/LRP5/6

Recent research has begun to clarify the role of certain sub-domains of the LRP5/6 Wnt co-receptors

LRP5/6 have a number of repeated motifs which are homologous to other known proteins. LRP5/6 contain three EGF repeats, three LDLR repeats and three arrays of YTWP propeller domains. A conserved PPP(S/T)P is repeated five times at the C-terminal and is responsible for Axin binding.
Sources: the Wnt homepage (Nusse, Stanford).

than *Wnt7a* or *Wnt1* mutants, probably because of a redundant role played by *LRP5* that was not addressed in this study (Pinson *et al.*, 2000).

A third paper aimed at deciphering the function of the different protein domains in *LRP6* (Tamai *et al.*, 2000). A common *in vivo* assay for canonical Wnt signalling is the axis duplication occurring during Xenopus development when RNAs activating canonical Wnt signalling are injected in the ventral side of the embryo at the four-cell stage (McMahon and Moon, 1989; Smith and Harland, 1991). In parallel, transfected Xenopus explants may be grown *in vitro* and the transcriptional response to canonical Wnt signalling was quantified by RT-PCR. Transcription of *Xnr3* and *siamois* is a hallmark for Wnt-induced transcription in Xenopus (Harland and Gerhart, 1997). Using these two assays, Tamai and colleagues demonstrate that LRP6 over-expression activated canonical signalling. When either *LRP6* and *Wnt5a* or *LRP6* and *hFz5* are co-injected, Wnt signalling is synergistically activated. More interestingly, a *LRP6* mutant lacking its cytoplasmic domain acted as a dominant-negative construct which inhibited axis duplication induced by Wnt-1, -2, -3a and -8 or hFz5. Tamai and colleagues go further in their analysis by demonstrating that the extracellular domain of LRP6 could associate with mFz8CRD in a Wnt-dependent fashion. The result of this co-precipitation experiment argue that Wnt enables LRP6 and mFz8 to come into contact and thus acts to bring together the two core elements of the receptor machinery in order to trigger a response(Tamai *et al.*, 2000). The fact Wnt signalling could be activated by Wg bringing together Arrow and dFz2 was confirmed by experiments using an Arrow/dFz2 fusion protein linked at their cytosolic domain (Tolwinski *et al.*, 2003). When expressed in transgenic flies, the Arrow/dFz2 fusion induces more ectopic bristle formation on the wing than the independent expression of *arrow* and *dFz2*. Taken together, these findings were a major breakthrough in understanding the molecular basis of Wnt signalling at the membrane. These data highlight the importance of the Fz/LRP6 complex formed through extra-cellular interactions, in which the cytosolic domain of LRP6 plays a key role in intracellular signal activation. Subsequent experiments clarified further the functional consequence of the Fz/LRP6/Wnt ternary complex assembly.

The role of arrow/LRP5/6 extracellular and cytoplasmic domains in signal transduction was investigated by a number of groups. When the N-terminal extra-cellular

domain of Arrow/LRP5/LPR6 is deleted, LRP6ΔN, is constitutively active, suggesting the extracellular domain may regulate signalling activity (Liu *et al.*, 2003; Mao *et al.*, 2001; Tamai *et al.*, 2004). In contrast, a LRP6 protein lacking the intracellular domain (LRP6ΔC) is inactive, which demonstrates the key role for this domain in signal transduction (Tamai *et al.*, 2000). Furthermore, LRP6ΔC acts as a dominant-negative, indicating that other parts of the protein may associated with signalling complexes but inhibit their capacity to signal (Tamai *et al.*, 2000). It appeared that Axin, the central scaffolding component around which the ß-catenin destruction complex is formed, binds to the C-terminal of arrow/LRP5/6 (Liu *et al.*, 2003; Mao *et al.*, 2001; Tamai *et al.*, 2000; Tolwinski *et al.*, 2003). This interaction provides a direct link between a receptor complex containing LRP5/6 and the destruction complex which regulates ß-catenin levels. In fact, Wnt-signalling triggers the phosphorylation of LRP6 at a conserved PPP(S/T)P motif which is repeated five times in arrow/LRP5/6 intracellular domains (Tamai *et al.*, 2004). Importantly, phosphorylation of LRP6 creates a high affinity binding site for Axin, which binds to LRP6 preferentially when it is phosphorylated. If an LRP family member, which is unable to affect signalling when over-expressed, is modified to contain these PPP(S/T)P motif, the modified LRP protein becomes able to trigger canonical signalling. Therefore this peptide appears to function as a inducible docking site for Axin which is critical for LRP6 function in Wnt signalling (Tamai *et al.*, 2004). Axin being present in low amounts within the cell, its recruitment to LRP5/6 at the membrane could titrate the amount of axin available to nucleate ß-catenin destruction complex, and thus inhibit the formation of the destruction complex. Although many questions remain, such as the identity of the kinase or phosphatase that affect the phosphorylation state of LRP6, the Wnt-induced recruitment of Axin is a major step forward in understanding Wnt signalling. Indeed, the mechanisms by which Axin is recruited to LRP5/6 is similar to cytokine and receptor tyrosine kinase signalling.

The consequence of Axin's Wnt-induced recruitment at the membrane has so far not reached a consensus within the field. Two possible mechanisms for Wnt/LRP/Fz signalling can be put forward. The "co-recruitment" model argues that LRP6 recruit Axin whilst Fz recruit Dvl, and that these elements are brought together by Wnt binding at the cell surface to both LRP6 and Fz. In this model, the physical proximity of Axin and Dvl

leads to the inactivation and/or dissociation of the ß-catenin destruction complex. The "parallel signalling" model implicates that it is possible to experimentally activate two branches of the pathway, but that both of these pathway branches are required for functional Wnt signalling *in vivo*. One branch is based on LRP6/Axin interactions and the other is the Fz/Dvl/Axin interactions. Over-activation of either of these two branches can lead to ß-catenin stabilisation without the requirement for the other branch. It is proving very difficult to establish categorically the molecular basis of signalling at this time. Nonetheless, Wnt-mediated Axin recruitment at LRP6 is clearly a critical step in regulating the activity or stability of the destruction complex. New findings implicating G-proteins in Fz signal transduction may clarify what happens immediately after Fz receptors are activated.

I. 3. 2. 3. Trimeric GTPases are downstream of Frizzled

Seven transmembrane receptors are very often coupled to trimeric GTPases, yet, until recently, G-proteins had not been directly implicated in Wnt/Fz signal transduction. Pertussis toxin (Ptx) is often used to disrupt G-protein signalling by uncoupling G-proteins to transmembrane receptors. A recent report from Katanaev and colleagues used Ptx do demonstrate that a G-protein (Go) was implicated in Wnt/Fz signal transduction (Katanaev *et al.*, 2005). In this paper, the authors tested the ability of Pertussis toxin (Ptx) to affect PCP signalling in the *Drosophila* eye, and noticed that Ptx was able to attenuate Fz signalling. The authors investigated whether tissue-specific expression of *G-oa47A* (*brokenheart*) mutants (to be reffered as Go), the Ptx target in the fly, could affect various aspect of Wnt signalling. Firstly, expression of *Go* hypomorphic alleles (*Go[007]*) affected both Wg signalling and PCP signalling. *Go[007]* induced a reduction in Wg-target gene expression and disruption of the wing margin, two canonical Wg phenotypes. Additionally, *Go[007]* displayed a very strong PCP phenotype, with up to five hairs growing from mutant cells. Secondly, over-expression of Go led to a localised increase in Wg-target gene expression. Expression of *Go* mutants which are locked in either GTP- or GDP-bound states (*Go-GTP* and *Go-GDP*), showed that only *Go-GTP* showed a similar phenotypes that *Go* over-expression, thus suggesting that Wg/Fz signalling may induce an exchange of GDP for GTP in Go. Fz and Go appeared to co-operate in a common

pathway since crosses between *Fz* and *Go* heterozygous flies displayed stronger PCP phenotypes that individual mutants. This synergism is a clear indicator that both Fz and Go signal in a common pathway. Finally, epistatic experiments suggest that Go-GTP can signal independently of Fz but requires arm (ß-catenin), Sgg (GSK-3ß) and Dsh (Dvl). Therefore *Go* lies downstream of *Fz* and upstream of Dvl and ß-catenin. (Katanaev *et al.*, 2005). These important data shed new light on a new aspect of Wnt/Fz signal transduction. G-proteins are located immediately downstream of Fz in the signalling cascade. Fz may act to promote the GTP bound state of Go, an event which is required for the activation of both the canonical and PCP pathways.

I. 3. 3. Wnt-mediated axonal guidance and new Wnt-receptors: the Ryk receptor family

Another receptor gene family, the atypical receptor tyrosine kinases *Ryk* were recently found to mediate axonal guidance induced by Wnts. The *Drosophila Ryk, derailed (drl)* had been implicated in the guidance of embryonic ventral nerve cord axons. *drl*-expressing axons cross the midline along the Anterior Commissure (AC) and avoid the Posterior Commissure (PC). Misexpression of *drl* in neurons normally following the PC causes them to re-direct their axons along the AC (Bonkowsky *et al.*, 1999). Since *drl* contained a WIF domain in their extracellular portions, and that WIF domain were known to bind Wnt, a genetic screen was carried out screening for mutations that would suppress the phenotype of *drl* ectopic over-expression. *dWnt5* was found to genetically and physically interact with *drl* and was thus put forward as a potential drl ligand guiding axons along the AC. Analysis of ectopic exression of *dWnt5* suggest that dWnt5 acts as a repellent signal to *drl* expressing neurons. Interestingly, *dFz1 dFz2* double mutants showed no axonal guidance defects, which led the authors to propose that Drl could function as an independent Wnt receptor, although it is possible other *dFz* receptors, whose function remain elusive, play a role (Yoshikawa *et al.*, 2003).

Studies in mammals nevertheless suggest that Ryk could function either as a Wnt co-receptor alongside Fz (Liu *et al.*, 2005; Lu *et al.*, 2004) or compete with Fz for the same Wnt ligand (Schmitt *et al.*, 2006). Liu and colleagues suggest that Ryk and Fz form a complex, because co-immunoprecipitation experiments show that a ternary

Wnt1/Ryk/Fz8CRD complex is formed when the proteins are over-expressed in cell lines (Lu *et al.*, 2004; Tamai *et al.*, 2000); however, the existence of this ternary complex needs to be demonstrated in normal tissue. Ryk is also shown to bind Dvl via their respective PDZ domains. Analysis of the synergism between Wnt3a and Ryk, indicate that Ryk co-operate in transducing Wnt3a canonical signalling, as indicated by measuring the transcription of the luciferase gene expressed under the TCF/LEF promoter (Topflash). Furthermore, Ryk siRNA transgenic mice were generated. Ryk siRNA transgenic mice die shortly after birth and displayed defects in axonal trajectories and fasciculation. Finally, Wnt3a-mediated axonal outgrowth of DRG explants was severely reduced in explants from Ryk siRNA mice, showing that Ryk was critical to some aspects of Wnt3a signalling (Lu *et al.*, 2004). Although these data highlight that Ryk may be required in canonical signalling, it is not clear whether Ryk is able to signal alone or whether it requires to be assiociated with a Fz to signal. A recent study revealed a fascinating twist into the roles of Ryk and Fz in axon guidance (Schmitt *et al.*, 2006). In the context of medial-lateral guidance of RGC axons in the chick optic tectum, high concentrations of Wnt3 mediated axon repulsion via Ryk whilst lower concentrations of Wnt3 mediated axonal attraction through frizzled receptors. Differential expression of Ryk along the dorso-ventral axis of the retina contributes to the guidance of different RGC populations. Axons of ventral RGC which express high levels of Ryk move laterally away from the Wnt3 source. In contrast, dorsal RGC expressing lower amounts of Ryk tend to send their axons medially towards the source of Wnt3. In addition, EphB/EphrinB2 signalling provides an additional mechanism of RGC axon guidance in the tectum (Schmitt *et al.*, 2006). Here again, it cannot be excluded that Fz may participate in Wnt/Ryk signal transduction. Nonetheless, Ryk appears as a key player in Wnt mediated axonal guidance. Once again, these discoveries highlight that the functions of Wnt during neuro-development is tightly regulated by a number of potential receptors. The function of Ryk as a receptor implicated in canonical signalling is another important point to address in our search for the receptors that mediate Wnt7a-induced synaptogenesis.

1. 3. 4. The Ror family of receptors

A final class of potential Wnt receptors, the Ror family of receptor tyrosine kinases, have received little attention to this date. Yet, *Ror* mutants in *C.elegans* and *Xenopus* have defects in either neuronal precursor cell migration or in morphogenetic movements (Forrester *et al.*, 2004; Hikasa *et al.*, 2002). These two findings are intriguing because cell migration in *C.elegans* is a consequence of canonical signalling whilst *Xenopus* convergent-extension mouvements constitute the archetype non-canonical Wnt pathway. Although Ror were at first orphan receptors for these events, it was suggested Ror functioned by affecting Wnt-signalling because Ror contain Fz-like CRD domains (Oishi *et al.*, 2003). In fact, in mammals, Ror2 -/- and Wnt5a -/- mice exhibit similar phenotypes (Oishi *et al.*, 2003). *In vitro* assays indicate that the Ror2CRD can bind Wnt5a and that they functionally synergise to activate a non-canonical pathway leading to JNK activation. In *Xenopus,* Ror2 and Wnt5a were also able to synergise to regulate convergent-extension movements, suggesting a functional link between Ror receptors and non-canonical signalling induced by Wnt5a. Interestingly, despite the fact Ror have active cytoplasmic kinase domains, it is their CRD domain which appear most important to their activity (Oishi *et al.*, 2003). Once again, this shows that regulation of Wnt binding at the cell surface is clearly a key step during Wnt signal transduction.

A recent publication from the laboratory of Roel Nusse revealed a new fascinating twist in Wnt signal transduction through Ror receptors (Mikels and Nusse, 2006). In this paper, the authors demonstrate that Wnt5a is able to inhibit Wnt3a-induced TCF-mediated transcription. This inhibitory activity of Wnt5a is enhanced when Ror2 is expressed. Interestingly, the CRD domain of Ror receptors is required for this activity. Suprisingly, the response to Wnt5a can be overturned in cells co-expressing LRP5 and Fz4. Indeed, Fz4 and LRP5 co-expression confers the cells the ability to activate the canonical pathway in response to Wnt5a, whereas co-expression of Fz4 and LRP6 did not permit Wnt5a to activate signalling (Mikels and Nusse, 2006). Clearly, these findings put the spotlight on the importance on the specificity of receptor complexes which enable Wnt factors to elicit precise response in different cells.

I. 3. 5. Wnt/Fz pairs and non-Wnt ligands

In addition to the numerous receptors implicated in Wnt signal transduction, the diversity in the range of consequences induced by Wnt signals arises from the fact mammals express 19 different Wnt genes. Therefore, we must now describe how specific Wnt/Fz ligand/receptor complexes have been established. Indeed, to decipher which *Fz* receptor(s) mediate *Wnt7a* signalling in the cerebellum and *Wnt7b* in the hippocampus, examples can be taken from other studies.

One of the most striking features of Wnt signalling is that very few receptor/ligand pair of physiological relevance have to date been assigned. Only in *Drosophila* has it been possible to prove that Wg signals mainly via dFz2 (Rulifson *et al.*, 2000; Zhang and Carthew, 1998). Yet, *dFz1* is able to compensate for *dFz2* absence and therefore dFz2 and dFz1 are known to be redundant Wg receptors (Bhanot *et al.*, 1999; Chen and Struhl, 1999). Indeed, redundancy between Fz receptors is common place and makes the analysis of *Fz* mutant phenotypes difficult to assign to a defect in the signalling induced by a particular Wnt. Additionally, Wnt signalling being such an key pathway during development, mutants for components of Wnt signalling often die at very early stages of development (Liu *et al.*, 1999; Monkley *et al.*, 1996). Therefore complex strategies will have to be devised in order to study genetic interactions within the Wnt pathway.

With the difficulty to generate *Wnt Fz* double mutants in mouse, studies of Wnt/Fz interactions often consist of binding assays followed by *in vitro* assays of Wnt signal transduction. Therefore, *in vivo* evidence assigning specific receptor/ligand pairs is extremely difficult and time-consuming in mammals. Gene expression data obtained by *in situ* hybridisation or from RT-PCR is extremely relevant, since it enables to point-out which *Fz* and *Wnt* have overlapping or complementary expression patterns. By putting together gene expression data with Wnt/Fz physical and functional interactions, it becomes possible to assign likely Fz/Wnt pairs that function during a particular developmental process. In fact, this is the approach we took to specify the Wnt/Fz interaction in the development of the hippocampus and the cerebellum. Gene expression data during post-natal development was first analysed. Subsequently, the *Fz* which were found to be expressed in the hippocampus and cerebellum were subject to binding and

signalling assays testing whether they could interact with Wnt7b and Wnt7a. How can Fz/Wnt binding be detected and measured? How can specific receptor/ligand pairs be tested for functional interactions? Different groups, led by Roel Nusse and his collaborators have developed a range of assays which have shed led light on the biochemistry of Wnt/Fz interactions.

I. 3. 5. 1. Physical Wnt/Fz interactions

Binding of Wnt to Frizzled receptors is a technically challenging task for several reasons. First, Wnt-being modified by the post-translational addition of a lipid, palmitate, it is difficult to produce large quantities of soluble Wnt-ligands for binding assays (Willert *et al.*, 2003). Secondly, Fz being transmembrane proteins and Wnt being lipid-modified, the detergent conditions necessary to solubilise these proteins may disrupt protein/protein interaction, and therefore make immunoprecipitation experiments difficult. Although many receptor/ligand interaction have been demonstrated by immunoprecipitation, Wnt/Fz binding has only been achieved with truncated receptors. Thirdly, Wnt binding to Frizzled leads to the endocytosis of the complex from the cell surface, it is difficult to detect large amounts of Wnt bound to the cell surface (Chen *et al.*, 2003; Dubois *et al.*, 2001). For these reasons, a series of constructs have been designed in order to facilitate the biochemical analysis of Wnt/Fz interactions.

Two FzCRD constructs are commonly used to assess Wnt/Fz binding. One construct involves anchoring the FzCRD to the cell membrane via a glycophosphatidylinositol anchor (GPI-FzCRD). This construct contains a myc epitope C-terminal to the GPI anchor in order to allow its immuno-detection (Bhanot *et al.*, 1996). Applying a concentrated conditioned medium containing a Wnt-Alkaline Phosphatase (Wnt-AP) to cells expressing GPI-myc-FzCRD, enables to qualitatively assess whether specific Wnt are able to bind to Fz (Hsieh *et al.*, 1999). The main advantage of using this construct for binding assays is that it enables the expression of CRD domains of different Fz proteins in a comparable fashion. Indeed, because of differences in Fz folding, surface localisation or stability, it is very difficult to detect binding of Wnt to full-length Fz, and impossible to compare binding of a Wnt to different full-length Fz expressed (Hsieh *et al.*, 1999). Wnt-conditioned medium is applied to living cells transfected with GPI-myc-FzCRD

which are subsequently prepared from immunodetection. Detection of membrane bound Wnt-AP occurs in an enzymatic reaction catalysed by the Wnt-AP. This method has been used by many groups and has assigned a number of Wnt/Fz interactions. For example, XWnt8 was shown to bind mFz4, mFz5, mFz8, XFz8 and dFz2 (Hsieh *et al.*, 1999), thus highlighting that Wnt factors may bind with similar efficiencies to a wide range of Fz receptors. Other reports following this protocol reveal that this lack of binding specificity between Wnt and Fz is widespread. The Drosophila Wnt, Wg, was shown to bind to the CRD of dFz1, dFz2, hFz5, mFz4, mFz7 and mFz8 (Bhanot *et al.*, 1996) (Table I. 1).

The other construct containing FzCRD enabling Wnt/Fz binding studies is the fusion of the heavy chain of human IgG with FzCRD (FzCRD-IgG) (Hsieh *et al.*, 1999). This construct enables immunoprecipiration of Wnt with FzCRD-IgG, through the interaction of the IgG portion of FzCRD-IgG with protein A. Such a construct is necessary because antibodies against specific Fz or Wnt suitable for immunoprecipitation or immunodetection were not commercially available at the time of this study. Fz6CRD-IgG was shown to precipitate with Wnt4 and XFz8CRD-IgG to precipitate with XWnt8 (Hsieh *et al.*, 1999; Lyons *et al.*, 2004) (Table I. 1). Furthermore, these FzCRD-IgG construct can be used in solid-phase binding assays to quantify the strength of FzCRD-Wnt binding. Indeed, protein A containing 96-well plates can be coated with FzCRD-IgG and concentrated Wnt-AP applied to test for binding. Although the determination of Wnt-AP concentration by comparative immunoblot is not very precise, the colour reaction generated by the alkaline phosphatase bound to the FzCRD-IgG enables the generation of Scatchard plots. With such a protocol, Hsieh and colleagues determined the affinity of XWnt8-AP for XFz8CRD to be of 9 ± 2nM (Hsieh *et al.*, 1999).

The main issue in these types of assays is the production of sufficient amounts of Wnt conditioned medium to reach the saturating conditions required for binding assays. Indeed, only a handful of Wnts are efficiently secreted as biologically active molecules in conditioned media. Wg, mWnt3a and XWnt8 are three Wnts with well established protocols for their production and purification (Hsieh *et al.*, 1999; van Leeuwen *et al.*, 1994; Willert *et al.*, 2003). Other Wnt factors are more difficult to produce as soluble factors and this has greatly hindered studies of Wnt/Fz interactions. This explains why many studies limit themselves to demonstrating functional interactions in transfected

cells or in RNA-injected developing Xenopus embryos. In fact, in some cases, the binding of a Wnt to a particular Frizzled does not correlate with the ability of the given pair to signal. For example, Wnt4 was shown to bind Fz6CRD yet it is not able to trigger a canonical response in canine kidney cells expressing *Fz6* (Lyons *et al.*, 2004). Therefore, assays evaluating the signalling capacities of Wnt/Fz pairs are necessary to confirm data obtained from binding assays.

Pair	Physical interaction	Functional interaction	Type of assay	Reference
hFz1/Wnt1	?	+	Topflash	(Gazit *et al.*, 1999)
hFz1/Wnt3	?	+	Topflash	(Gazit *et al.*, 1999)
hFz1/Wnt3a	+	+	IP, Topflash	(Gazit *et al.*, 1999)
hFz1/Wnt5a	+	-	IP, Topflash	(Gazit *et al.*, 1999)
rFz2/Wnt5a	?	+	Calcium	(Slusarski *et al.*, 1997)
mFz3/Wnt4	?	+	Mutant Explant	(Lyuksyutova *et al.*, 2003)
mFz3/Wg	-	+	CSB, ß-catenin	(Bhanot *et al.*, 1996; Takada *et al.*, 2005)
Fz4/Norrin	+	+	IP, CSB, Topflash	(Xu *et al.*, 2004)
mFz4/XWnt8	+	?	CSB	(Hsieh *et al.*, 1999)
mFz4/Wg	+	?	CSB	(Bhanot *et al.*, 1996)
mFz7/XWnt8	+	?	CSB	(Hsieh *et al.*, 1999)
hFz5/Wg	+	?	CSB	(Bhanot *et al.*, 1996)
mFz6/Wnt4	+	-	IP	(Lyons *et al.*, 2004)
mFz7/Wg	+	?	CSB	(Bhanot *et al.*, 1996)
mFz7/XWnt8	+	?	CSB	(Hsieh *et al.*, 1999)
mFz8/Wg	+	?	CSB	(Bhanot *et al.*, 1996)
mFz8/XWnt8	+	?	CSB	(Hsieh *et al.*, 1999)
rFz9/Wnt2	?	+	ß-catenin, Topflash	(Karasawa *et al.*, 2002)

Table I. 1. Mammalian Fz/Wnt pairs

A diversity of techniques have been employed to establish the physical and functional interactions between Wnt and Fz. This table summarises the established interactions between mammalian Wnt and Frizzled. In many cases, binding data had not yet been confirmed by functional data. In some instances, functional or genetic interactions are not supported by binding studies

Abbreviations :

+ : Postive interaction detected
- : No interaction detected
? : Interaction not tested to this date
Axis Duplication – RNA injection of in *xenopus* embryos
ß-catenin – tested for changes in cytosolic ß-catenin levels
Calcium - monitored calcium transients in zebrafish embryos.
CSB – Cell Surface Binding Assay
IP – Immunoprecipitation
Mutant explant – *in vitro* experiments done on primary tissues obtained from mutants
Topflash – Topflash assay measuring TCF/LEF transcriptional activation

I. 3. 5. 2. Functional Wnt/Fz interactions

There are three ways to monitor the activation of the canonical Wnt pathway in cultured cells. The most straight-forward method is to analyse changes in intracellular ß-catenin levels by western blots following *Wnt* expression or exposure to Wnt conditioned medium. Yet, only Wnts with potent ß-catenin/canonical signalling activities such as Wnt3a trigger noticeable changes in ß-catenin levels (Mikels and Nusse, 2006). Also, this method often detects a number of interactions with little specificity. For example, Wg was found to increase ß-catenin levels in cells transfected with mFz-3, -4, -5, -8 and dFz2 whilst Wnt5a was not able to induce ß-catenin stabilisation in any of the above cases (Takada *et al.*, 2005). Yet, other reports had reported that Wg was unable to bind mFz3 (Bhanot *et al.*, 1996) (see table I. 1). Clearly, more refined approaches to understand Wnt/Fz signalling specificity are needed.

The best established, most reliable and precise way to quantify the cellular response to canonical Wnt signalling is the Topflash assay, which measures the transcriptional response to Wnts (Molenaar *et al.*, 1996). It does so by the transfection of cell lines with a construct carrying the firefly *luciferase* gene under the control of the seven TCF/LEF promoter sequences (Xu *et al.*, 2004). Activation of the canonical pathway activates transcription of the *luciferase* reporter, which can be monitored by the luminescence emitted when cells are exposed to a luciferin substrate. The light emitted is proportional to the amount of luciferase protein present, and thus enables a precise quantification of the transcriptional response to Wnt signalling. An internal control is included which enables the normalisation of the amounts of cells present and for transfection efficiency. This internal control is a *luciferase* gene from another organism, *Renilla*, which is expressed under a constitutively active promoter. This luciferase reporter requires a different substrate than the *firefly* luciferase and therefore it is possible to separate the two light-emitting reactions (de Wet *et al.*, 1985; Jones *et al.*, 1979; Wood *et al.*, 1984). This experimental system is widely used to study how the transfection of various Wnt signalling pathway components and Wnt-conditionned media affect ß-catenin-mediated transcription (Mikels and Nusse, 2006; Xu *et al.*, 2004). It is summarised in figure I. 9.

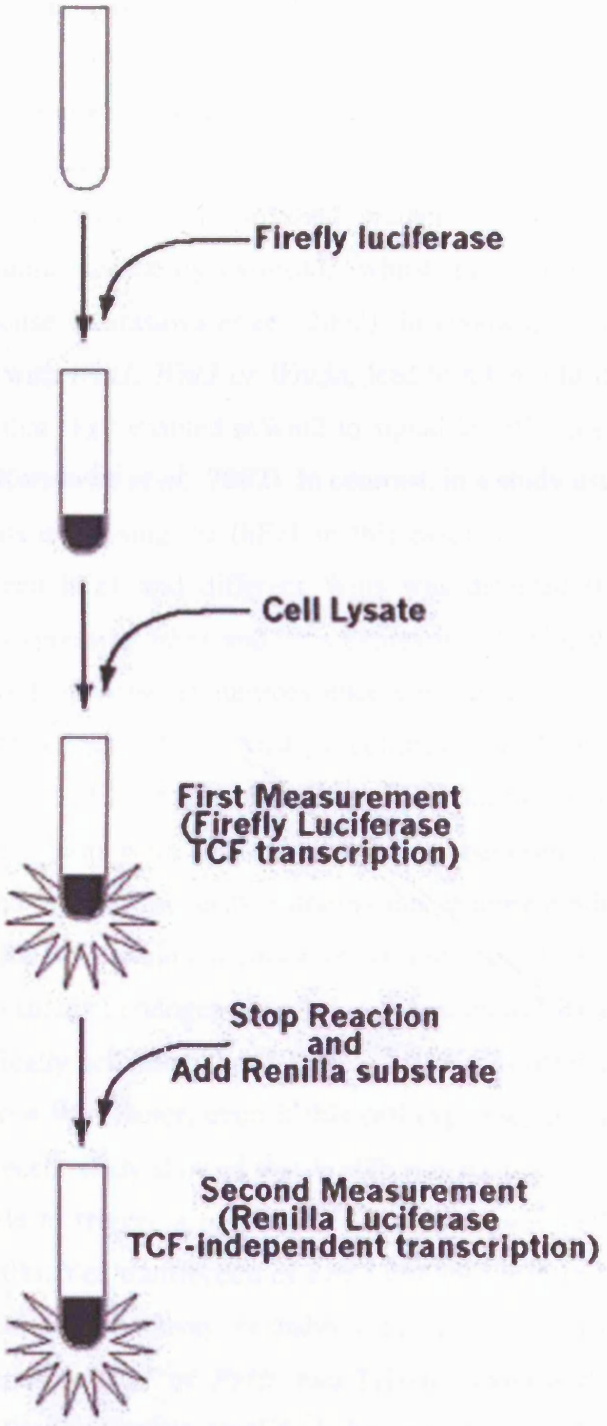


Figure I. 9. Schematic representation of the Topflash assay

Several studies have also used the Topflash assay to examine potential synergism between Wnt and Fz. Topflash assays addressing Wnt/fz interactions have either used co-expression of *Wnt* and *Fz* or co-cultures where cells expressing *Wnt* are mixed with other expressing *Fz*. Topflash assays using neat conditioned media have so far not been reported. In the case of rFz9 and mWnt-2, transfection of *rFz9* in 293 cells increased TCF-mediated luminescence by two-fold, whilst transfection of *mWnt2* alone did not trigger any response (Karasawa *et al.*, 2002). In contrast, co-transfection of *rFz9* with *mWnt2,* but not with *Wnt1, Wnt3 or Wnt3a,* lead to a ten-fold increase in luminescence, thus suggesting that rFz9 enabled mWnt2 to signal in 293 cells and therefore acted as a Wnt2 receptor (Karasawa *et al.*, 2002). In contrast, in a study using a paracrine signalling assay, where cells expressing *Fz* (hFz1 in this case) were co-cultured with cells expressing *Wnts*, synergism between hFz1 and different Wnts was detected (Gazit *et al.*, 1999). Co-cultures of cells expressing *hFz1* and cells expressing Wnt3a, Wnt3 and Wnt1 showed a robust (20-50 fold) increase in luminescence compared to co-cultures of control cells with *hFz1*-transfected cells. In contrast, co-cultures of cells expressing *hFz1* with cells expressing *Wnt7a, Wnt7b, Wnt5a, Wnt5b* or *Wnt4* did not induce a significant change. Interestingly, the authors were able to show binding between hFz1 and Wnt3a as well as between hFz1 and Wnt5a, thus demonstrating that Wnt/Fz binding may not be sufficient to activate signalling in certain circumstances (Gazit *et al.*, 1999).

The abundance of endogenous co-receptors such as LRP5/6 and the ability of Wnt factors to specifically activate either LRP5 or LRP6 may often determine whether a cell might respond to a Wnt factor, even if this cell expresses the appropriate *Fz* receptors. For example, a recent study showed that Wnt7b was unable to signal in 293 or 3T3 cells, whilst it was able to trigger a response in smooth muscle cell lines PAC-1 and A7r5 (Wang *et al.*, 2005). Yet, transfection of *LRP5* but not *LRP6* in 293 cells, enabled Wnt7b to activate the canonical pathway, probably using *Fz1* that is expressed by 293 cells. In fact, co-transfection of *Fz1* or *Fz10*, two Frizzled expressed in smooth muscles and shown to bind Wnt7b, further amplified the response in cells expressing Wnt7b and LRP5 (Wang *et al.*, 2005). These studies highlight the precise molecular requirements for cells to respond to Wnt factors. Clearly, LRP5/6 co-receptors must be taken into consideration when studying Wnt signalling through the canonical Wnt pathway.

I. 3. 5. 3. Non-Wnt Frizzled ligands

New players are coming into the landscape of Wnt signalling on a regular basis. In addition to novel receptor such as Ryk, new ligands have been found to activate Frizzled. Mutation in *Fz4* have been associated with a hereditary disease affecting the retinal vasculature (FEVR) (Robitaille *et al.*, 2002). *Fz4-/-* mice have a similar phenotype to mice with Norrie Disease carrying the mutation in the *Ndc* gene (Berger, 1998). This prompted Xu and colleagues to examine the co-operation of the *Ndp* gene product Norrin with *Fz4*. In 293 cells, Norrin was able to activate ß-catenin/TCF mediated transcription in cells co-transfected with *mFz4* and *LRP5* or *LRP6*. Norrin was found to specifically bind the CRD domain of Fz4. Additionally, Norrin associates with the ECM and signals locally around Norrin-expressing cells (Perez-Vilar and Hill, 1997; Xu *et al.*, 2004). Therefore, Norrin seems to function in a manner analogous to Wnt ligand, perhaps more specifically in the context of vascularisation processes. Once again, these data illustrate that new players affecting Wnt/Fz signalling are still emerging and that the complexity of Fz signalling is increasing rapidly.

1. 4. Thesis overview

Wnt signalling affects developing neurons in a number of ways. Wnt7a causes the remodelling of mossy fibre and granule cell growth cones, axonal thickening and accumulation of synaptic proteins (Hall *et al.*, 2000; Lucas and Salinas, 1997). Wnt3 regulates the arborisation to NT-3 dependent DRG neurons as they reach their targets in the spinal cord (Krylova *et al.*, 2002). Both of Wnt7a and Wnt3 effects activate the canonical Wnt pathway, because their effects on neurons can be mimicked by chemical inhibitors of GSK-3ß. In contrast, Wnt7b stimulates the dendritic arborisation of hippocampal neurons through a non-canonical pathway involving Rac and Jnk (Rosso *et al.*, 2005). How can the two closely related Wnt7a and Wnt7b signal to different pathways? How does Wnt3 trigger neuronal branching via the canonical pathway and Wnt7b dendritic branching through a non-canonical pathway? How does Wnt7a signal to the cytoskeleton? How do synaptic proteins accumulate at the remodelled growth cone in

response to Wnt7a? Many questions like these remain in order to understand the effects of Wnt in the developing nervous system.

I. 4. 1. Thesis aims

The aim of this thesis was to uncover the receptors mediating the responses induced by Wnt7a and Wnt7b. Understanding the link between the extracellular signals and the intracellular events will be of great value to design further experiments aiming to underpin the molecular events that regulate neuronal behaviour. Ultimately, understanding the molecular requirements for the establishment of specific neural circuits could contribute to the development of regenerative therapies after neural injury and could also provide insights into neuronal processes such as memory storage and cognition.

First, we examined the expression patterns of *frizzled* receptors by *in situ* hybridisation and compared them to the expression patterns of *Wnt*. Several Frizzled, such as *Fz3* and *Fz7* are highly expressed in specific areas of the post-natal mammalian brain at the times when Wnts play an important role. Based on these expression patterns, we carried-out binding assays to assess the physical interactions between the Wnt and Fz expressed in overlapping patterns. A cell surface binding assay was used as well as immunoprecipitation experiments. Finally, we evaluated the ability of the Wnt/Fz pairs detected in the binding assays to signal through the canonical pathway. The role of LRP6 was also investigated and confirmed that it was necessary for the transduction of Wnt7a signals through Fz3. In contrast, Wnt7a was found to be able to signal through Fz7 without requiring LRP6 co-expression.

Chapter II

Materials and Methods

II. Materials and methods

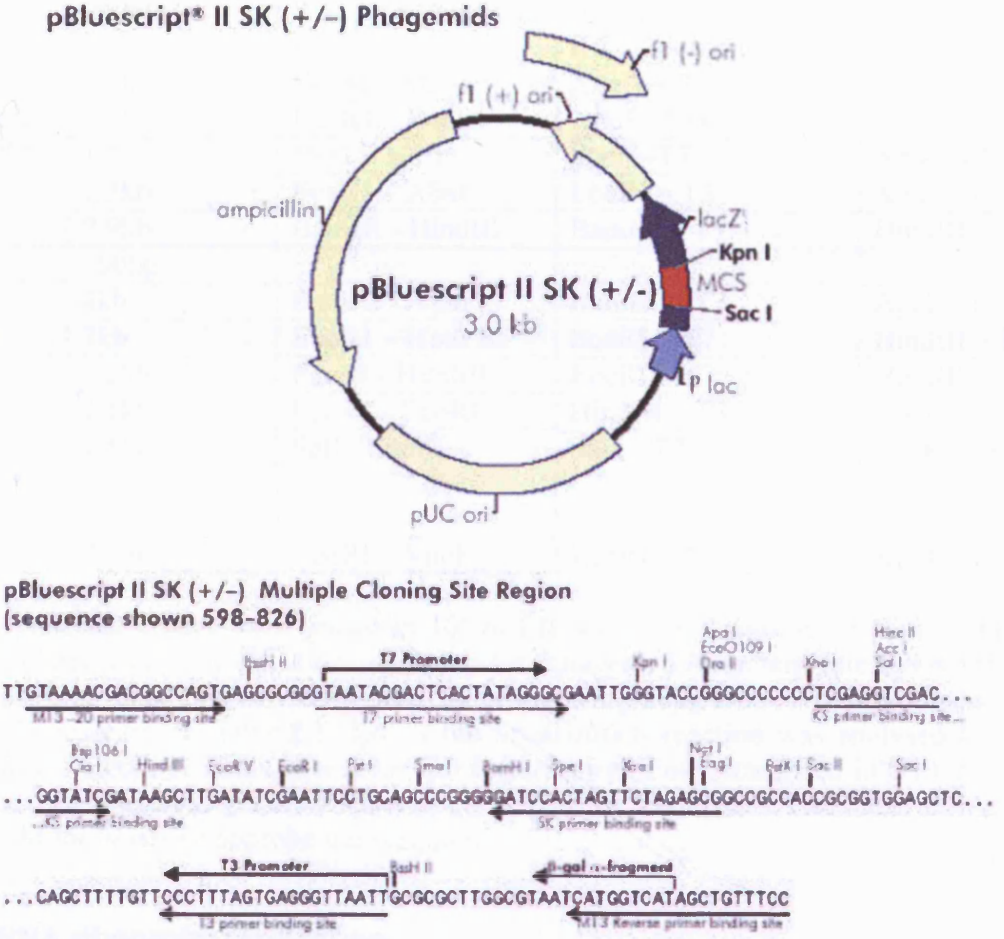
All chemicals were purchased from Sigma-Aldrich (referred to as Sigma) unless otherwise stated. All restriction enzymes and polymerases were purchased from Promega.

II. 1. in situ hybridisation

All aqueous reagents were treated with 0,05% DEPC for at least 2hrs to de-activate RNases and subsequently sterilised by autoclaving unless otherwise stated.

II. 1. 1. Template DNA for riboprobes

Most riboprobes used for *in situ* hybridisation were prepared from plasmids containing full length cDNAs cloned into pBS SK. Figure I. 1 is a schematic diagram of pBS SK. Table II.1.1 summarises the plasmids used. In some cases, *Fz* cDNA were excised from pRK5 and recloned into pBS SK. To do this, 2µg of *Fz-pRK5* DNA were digested using a combination of Restriction Enzymes (R.E) flanking the Fz insert and absent from the *Fz* internal sequence (see table I.1). In parallel, 1µg of the target host plasmid, pBS SK, was digested with the same restriction enzymes. The fragments were then isolated by 1% agarose (Promega) gel electrophoresis, and extracted from the agarose using the QIAquick gel extraction kit (Qiagen). A 3:1 ratio of insert cDNA : plasmid DNA were then ligated in a 10µl reaction catalysed by 1µl T4 DNA ligase (promega). A negative control consisting of the target plasmid alone was also ligated. 1µl of the ligation reaction were used to transform XL-1 blue competent bacterial cells (Stratagene). Selected colonies were grown in 5ml LB broth + 10µg/ml ampicillin overnight. 3ml of these cultures were used to extract plasmid DNA using the QIAspin miniprep kit (Qiagen). Plasmids were verified by a series of restriction digests.



The pBS SK + was used for the productions of riboprobes presented in this thesis.

Fz cDNA were cloned into the multiple cloning site region (red). Riboprobes were transcribed by the bacteriophage RNA polymerases T3 and T7, which recognise their promoter sequences flanking the MCS. The additional features of pBS SK used were it s ability to be replicated in bacteria (F1 ori) and the *ampicillin*-resistance gene that enabled the selection of transformed bacterial colonies.
Source: Stratagene website

Figure II. 1. Schematic diagram of pBS SK

Table II. 1. Summary of riboprobes used for in situ hybridisation

Gene	Insert size	R.E. insertions sites (5' – 3')	Antisense probe R.E. – polymerase	Sense probe
mWnt7a	3.2kb	EcoRI - Xho	Xba 1 – T7	Xho 1 – T3
mWnt5a	2.5kb	EcoR1 – EcoRI	XhoI – SP6	BamH1 – T7
mWnt7b	1kb	Not1 – Not1	SacI – T7	Xba – T3
mFz3	2.3kb	EcoR1 – XbaI	EcoRI – T3	XbaI – T7
mFz4	2.9kb	BamHI - HindIII	BamHI – T7	HindIII – T3
mFz5	500bp			
mFz6	4kb	BamHI - ApaI	BamHI – T7	ApaI – T3
mFz7	3kb	EcoR1 – Hind III	EcoRI – T7	HindIII – T3
mFz8	2.2kb	EcoRI - HindIII	EcoRI – T7	HindIII – T3
mWnt11	2.1kb	EcoRI – EcoRI	HindIII – T3	XbaI – T7
mWnt3 (pGEM3 Z)	1.55kb	SalI - EcoRI	SalI – T7	EcoRI – SP6
mWnt6	2,95kb	EcoRI – XhoI	EcoRI – T7	KpnI – T3

Selected clones were grown in 100ml LB and large quantities of plasmid DNA were isolated using the QIAfilter midiprep kit (Qiagen). Linear template DNA for the antisense and sense probes were generated by digesting 20µg DNA over-night using the enzymes described in table II.1. 1µl of the linearization reaction was analysed by 1% agarose gel electrophoresis to test for full linearisation. Fully linearised DNA was then isolated by 1% agarose gel electrophoresis in RNase free conditions. The linear DNA was then used for *in vitro* riboprobe transcription.

II. 2. RNA riboprobe production

All the reactions described in this section and the next were done using ultra pure RNase-free H_2O. "Cold" riboprobes were first generated in order to test the effiency of the *in vitro* transcription reaction. The conditions for cold probe reactions were the same as those indicated in table II. 1., except that 35S-UTP was omitted and a 10mM (ATP, UTP, CTP, GTP) stock was used as a source of nucleotide. The reaction was carried for 60-90 min and analysed by 1% agarose electrophoresis.

Radiolabelled probes were for *in situ* hybridisation. 35S-labelled RNA was transcribed in 2hr reactions at 37°C. Table II. 2 describes the contents a reaction yielding radioactive riboprobes for the hybridisation onto one slide containing 4 sections.

Table II. 2. *In vitro* transcription reaction

Linear DNA template	1.5µg	
5x Transcription buffer	4µl	Promega
10mM (ATP, GTP, CTP)	0.75µl	Promega
RNAsin	0.55µl	Promega
10mM DTT	1.12µl	Sigma
10mM ^{35}S-UTP (0,74MBq/µl)	0,6µl	Amersham
RNA polymerase	1.3µl	Promega
H_2O	csp 20µl	Sigma

For the generation of riboprobes for more than two or three slides, the amount of ^{35}S-UTP was increased to 1.2 or 1.8µl, respectively. If more than three slides were tested, separate reactions were run.

After this transcription step, the template DNA is hydrolysed by adding 73µl H_20, 10µl DNase buffer and 2µl Dnase (Promega) and incubating at 37°C for 30min. The RNA was then precipitated. First, 1µl of 10mg/ml yeast RNA (Sigma) was added. 10µl 3M NaAco and 300µl EtOH were added and precipitated for one hour at -80°C. The RNA was then centrifuged at 13000g for 15min. The supernatant was discarded and the RNA washed twice, with 80% EtOH followed by centrifugation. After two washes, the supernatant was careful collected and discarded, whilst the RNA pellet left to air-dry. The RNA was then resuspended in 20µl 10mM DTT. 1µl of the radioactive RNA is then added to 5ml PCS (Amersham) for scintillation counting.

II. 1. 3. Scintillation counting and riboprobe dilutions

1µl of ^{35}S-RNA was counted in a Beckman LS6500 scintillation counter. The ^{35}S-RNA was diluted in order to apply 200-400µl of hybridisation buffer containing $0.1x10^5$CPM of ^{35}S-RNA to each slide.

To clarify the calculations made, here is an example of a typical experiment where 2 slides were probed with probe A and three slides were probed with probe B. If the reading of 1µl of probe A gave 900 000 CPM and the reading of probe B was 2 000 000 CPM, this indicates we have a total of 900 000 x 19 (volume left) = 17 100 000 CPM and 2 000 000 x 19 = 38 000 000 CPM for probe A and probe B, respectively. Therefore, by diluting 8,8µl of probe A into 800µl of hybridisation buffer (see below) and 6µl of probe B into 1200µl, we obtain two solutions containing 10000 CPM/µl ($0,1x10^5$CPM/µl). In this manner, the same volume of hybridisation mix of equal radioactivity was applied to each slide.

II. 1. 4. Brain Section preparation

Mouse brains were quickly isolated and fixed overnight in 4% PFA. The brains were washed and dehydrated before being embedded in wax. All steps described here

were of 30min duration and occurred at RT with gentle agitation unless otherwise stated. Brains were washed in two 30min steps in PBS to remove residual amounts of PFA, before being dehydrated through a series of increasing alcohols (15%, 30%, 50%, 70%, 90% and 100%). The 100% ethanol was repeated three times and the brains were left overnight in 100% ethanol to ensure complete dehydration. The following day, brains were transferred into toluene. The brains were then transferred to Xylene at 60°C. All the following steps are carried out in an oven at 60°C without agitation. The brains are transferred to a 50/50% Xylene/Wax solution and then three 100% wax solutions. Finally, the brains are embedded in wax and the mould left to cool and solidify overnight at RT before sectioning or transferred to 4°C for storage. 12µm sections were cut using a Leitz 1512 microtome, collected on poly-L-lysine pre-coated slides (VWR) and stored at 4°C for no more than 20 days.

Before hybridisation, sections were de-waxed through two 10min washes in Xylene. The sections were then re-hydrated by going through a series of alcohols (100%, 100%, 100%, 90%, 70%, 50%, 30%) for 2min each, and then left twice for 10min in PBS.

II. 1. 5. Hybridisation protocol

Hydrated sections are first fixed in 4% PFA for 10min and subsequently washed twice in PBS for 10min. Sections are incubated with 20µg/ml proteinase K (Sigma) in PBS-5mM EDTA at 37°C, to facilitate probe penetration into the tissue. Sections are then washed in 0,2% glycine-PBS for 5min and twice in PBS for 5min. Sections are then re-fixed in 4% PFA-PBS and subsequently immersed in PBS. Sections are then transferred to a solution 0,25% Acetic anhydride in which was added 0,1M TEA (Fisher scientific), and left for 10min. Sections are washed twice in PBS for 5min before dehydration through a series of 2min incubation in 30%, 50%, 70%, 80%, 95%, 100% ethanol. Sections are air-dried before applying hybridisation buffer.

Sections underwent a pre-hybridisation step with 300µl of hybridisation buffer at 60°C for 2hrs (see table II. 3 for the buffer composition). For hybridisation reactions, sections are covered with parafilm (Pechiney plastic).

Table II. 3. Hybridisation buffer (10ml)

50% Formamide	5000µl	Sigma
500µg/ml Baker's yeast RNA	500µl (10mg/ml stock)	Sigma
0,05M DTT	500µl (1M stock)	Sigma
2X SSC	1000µl (20X stock)	See solutions and buffers
1X Denhart Solution	200µl	See solutions and buffers
10% Dextran sulfate	2000µl (50% stock)	Sigma
5mM EDTA	100µl (0,5M stock)	Sigma
csp H_20	2500µl	

Before diluting the riboprobes in hybridisation buffer as described in I. 3, probes are denatured at 80°C for 5min. Hybridisation is carried-out overnight in a humidity

chamber at 72°C, by adding 200-400µl of hybridisation buffer containing $0.1x10^5$CPM ^{35}S-RNA and covering slides with parafilm

The following morning, sections are extensively washed. The first wash was done in a 20mM ß-mercaptoethanol 5X SSC solution for 30min at 72°C. The second was done in 20mM ß-mercaptoethanol 2X SSC/50% formamide for 45min at 72°C. Sections are washed twice in 5mM EDTA 20mM ß-mercaptoethanol PBS solution for 10min at 37°C and another two times in 5mM EDTA-PBS for 10min at 37°C. Unspecifically bound RNA is removed by a 30min treatment of 20µl/ml RNaseA (Roche) PBS. Sections are then washed for 15min in 0.35M NaCl 5mM EDTA PBS at 37°C. Two washed in 2X SSC at 72°C are then carried out. A final wash in 0.1X SSC at 72°C was done. Sections are then de-hydrated in a series of alcohol (50%, 70%, 95% and 100%) and air-dried.

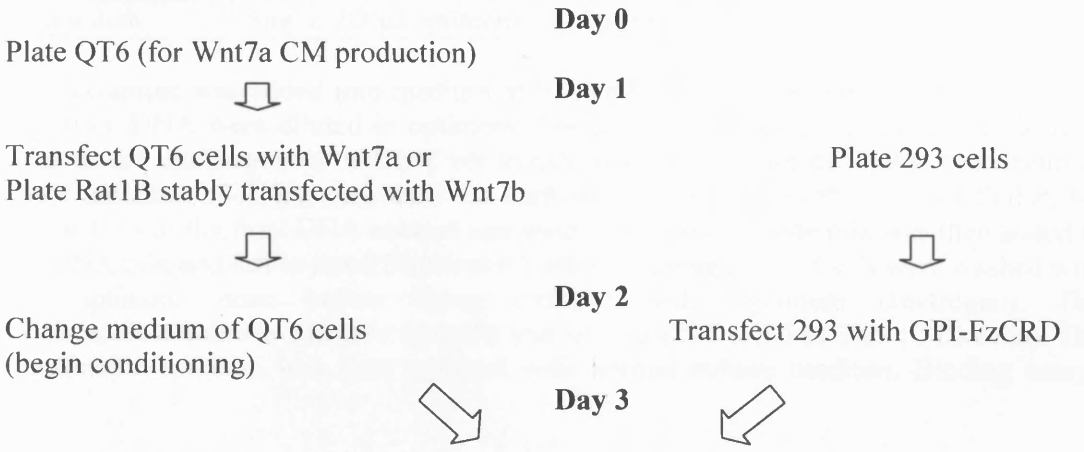
Once dry the sections are placed in a light-proof cassette and put in contact with a XAR radiographic film (Fuji) for 24-36hrs. This film provided the first indication of the success of the experiment.

In a dark room, sections are then dipped in K5 photographic emulsion (Ilford) at 52°C and the samples left to dry overnight. The reaction is left to develop 14-25 days at 4°C in a sealed container. On the day of the developing, samples are transferred to RT and left several hours to equilibrate to RT. Samples are then immersed in D19 developper (kodak) 2min, in fixative (30% sodium thiosulphate) for 1min and then in three water solution for 10min. After development, sections are stained in 0.02% toluidine blue. Sections are then de-hydrated in a series of alcohol, dipped in Xylene and mounted using XAM (VWR). Samples were analysed using the dark field microscopy in a Zeiss Axoplan II microscope. Images were captured a 5X objective using Open Lab and modified using Adobe photoshop.

II. 2. Binding assays

The binding assay requires the synchronised production of Wnt-conditionned medium and the generation of FzCRD -transfected cells (Hsieh *et al.*, 1999). A Schematic diagram of the time-scale of procedures is depicted in figure II. 2.

Figure II. 2. Schematic diagram of Wnt-conditioned medium production and cell surface binding assay



Binding Assay

Use the CM for the binding assay on 293 cells expressing GPI-FzCRD

II. 2. 1. Wnt-conditionned media production

Wnt7b-HA condition ed medium was produced from Rat1B cells stably transfected with Wnt7b-HA-pCS2. 10^6 Rat1B cells were plated in 60mm plates on day 1. Culture medium consisted of DMEM (Invitrogen) containing 5% Serum and 20U/ml Penicillin/Streptomycin. On day 2, the culture medium was replaced with N2 medium containing 1% FC serum. N2 medium was used because it is the media of choice in the laboratory to produce conditioned media for neurons. On day 3, the Wnt7b-conditionned was collected and centrifuged at 1500G for 3min to remove cells in suspension, before being used for binding assays.

Wnt7a-HA conditionned medium was produced from QT6 transiently transfected with Wnt7a-HA-pCS2. $7x10^5$ QT6 cells were plated on 60mm plates on day 0. QT6 culture medium consisted of DMEM (Invitrogen) supplemented with 5% FCS, 2% CS and 20U/ml Penicillin/Streptomycin. On day 1, cells were transfected with 5µg Wnt7a-HA-pCS2 (see next section). On day 2, the culture medium was replaced with N2 medium containing 1% FCS. On day 3, the Wnt7b-conditionned was collected and centrifuged at 1500G for 3min to remove cells in suspension, before being used for binding assays. An aliquot of 90µl of CM taken and mixed with 30µl loading sample buffer (LSB) for subsequent analysis of the level of Wnt7a/b-HA present in the CM by western blotting (see SDS PAGE and Western blotting II.2.5).

II. 2. 2. Transfection

Cell transfections were done using lipofectamine (Invitrogen) and Optimem (Invitrogen) medium. Transfections were done at day 1 for Wnt7a-HA CM production in QT6 and on day 2 for GPI-myc-FzCRD transfection in 293 cells. Table II. 4 highlights the amounts of reagents used. All transfers of solutions containing lipofectamine were done in a drop-wise manner.

Table II. 4. Amounts of reagents used for cell transfections

Culture dish	DNA mix	Lipofectamine mix	Optimem
4/24 well plates	1µg in 50µl optimem	2µl in 50µl optimem	200µl
60mm dish	5µg in 200µl optimem	20µl in 200µl optimem	1000µl

Lipofectamine was added into medium at RT, and left to stand 5min at RT after gentle agitation. DNA were diluted in optimem. The amounts of DNA indicated in table II. 3 refer to the total amounts of DNA per transfection. In the case of double transfections, equal amounts of DNA were used for each plasmid, except in the case of EGFP, for which 10% of the final DNA content was used. The lipofectamine mix was then added to the DNA mix and left to stand 30min at RT after gentle agitation. Cells were washed with RT optimem once before being covered with Optimem (Invtrogen). The DNA/lipofecatmine is added to the cells and left 1hr (293 cells) or 3hrs (QT6 cells). The transfection medium was then replaced with normal culture medium. Binding assays

were carried out 24 hours after transfection, whilst for conditioned media production, conditioning occurred over-night starting 24hr after transfection.

II. 2. 3. Cell surface binding assay

On day 1, $6x10^5$ 293 cells were plated in 60mm dishes containing ten 10mm coverslips. This enabled comparable cell density and transfection efficiency in a large number of coverslips. On day 2, cells were briefly washed in RT PBS and exposed to Wnt-conditioned medium for one hour at room temperature, with occasional gentle agitation. For PI-PLC treatment, cells were treated 45min with 2U/ml PI-PLC (molecular probes) in PBS and rinsed twice in PBS before addition of the CM. After exposure, the Wnt-conditioned medium was removed and the coverslips washed three times with RT DMEM and twice with RT PBS. Cells were then fixed with 3% PFA for 20min. PFA was washed by three 10min PBS washes. The coverslips were blocked for 45min with 5%BSA-PBS. Coverlslips were then incubated with primary antibodies diluted in 1%BSA-PBS. Most coverslips were probed with anti-HA antibody (for Wnt-HA binding) and anti-myc (for GPI-myc-FzCRD expression). Controls for the staining procedure consisted of coverslips stained with either a single primary antibody mentioned above, or not exposed to primary antibodies. See table II. 5 for antibody sources and dilutions

Table II. 5. Antibodies used in cell-surface binding assays

Antibody	Dilution	Supplier
Rat anti-HA	1:400	Roche
Mouse anti-myc	1:3000	Sigma
Donkey anti-rat Biotinylated secondary antibodies	1:400	Amersham
Alexa fluor 488- or 594-conjugated Goat anti-mouse secondary antibodies	1:800	Molecular probes (Invitrogen)

Primary antibodies were washed three times for 5min in PBS. Secondary antibodies are then added for 45min in 1% BSA-PBS. As a positive control for the HRP, cells exposed to mouse anti-myc primary antibody are treated with anti-mouse biotinylated antibodies. Secondary antibodies are washed three times in 0,02% Tween-PBS and once with PBS. In order to remove endogenous peroxydase activity from the cells, coverslips are dipped for 8 min in 5% H_2O_2-Methanol, and subsequently re-hydrated in two 10min incubation in PBS. The Avidin Biotinylated enzyme Complex (ABC reagent, Vector labs) is then added for 30min according to the manufacturer's instruction. In this step, avidin-HRP complex binds the biotinylated secondary antibodies. The ABC solution is then washed in two 30min incubations of 30min.

The HRP reaction is developped by dipping the coverslips in a 0,01% H_2O_2-H_2O solution 0,1% DAB. The DAB stock solution is a 0,2% DAB 0,1M Tris pH 7,2. Extreme care is taken when manipulating DAB. The reaction is left to develop for 15-20 min in the dark, until a precipitate is visible by light microscopy in positive controls for the HA epitope or negative controls un-exposed to biotinylated antibodies begin to darken. The

reaction is terminated by transferring the coverslips to a 0,1% Azide-PBS solution for 10 min. The coverslips are finally rinsed in PBS to remove the azide, and mounted using fluoromount (Southern biotechnologies).

I. 2. 4. Immunoprecipitation protocol

Immunoprecipitation was carried out in QT6 cells transfected with *Wnt7a-HA* or co-transfected with *Wnt7a-HA* and *myc-Fz7CRD-IgG* or *myc-Fz8CRD-IgG* (Hsieh *et al.*, 1999). For each condition, two 60 mm dishes were transfected with 15µg DNA and 15µl lipofectamine as described in I.2.2. Untransfected cells were also subjected to immunoprecipitation as a negative control. Protein A-agarose (Sigma) was used to directly precipitate myc-FzCRD-IgG without the addition of a primary antibody.

All procedures were performed on ice or in the cold room. 24 h after transfection, cells were collected by scraping in 100 µl RIPA buffer (see solutions and buffers) and homogenised by passing through a needle. The suspension was spun at 5000 g for 3 min to pellet-down un-solubilised membrane and nuclear components. The supernatant was collected. A 5µl aliquot was taken for subsequent Western blot analysis and labelled as "cytosol" corresponding to the starting material of the experiment. The protein content of each sample was determined using the Dc protein (Lowry) assay (Bio-Rad) following manufacturers instructions and using Microsoft Excel to determine the equation of the calibration curve. Protein A-agarose beads were first washed in PBS three times and equilibrated in RIPA buffer before use with the cell extracts. 15 µl of protein A-agarose beads were added to 200µg of protein from the cell lysates, in incubated over-night with agitation. The samples were centrifuged a 5000G for 3min. The supernatant was collected and labelled "supernatant", corresponding to un-precipitated material. The pellet was then washed 5 times with 100µl RIPA buffer. After the last wash, the pellet is resuspended in SDS loading buffer and boiled for 5min. This step denatured all proteins still attached to the beads, and thus released the proteins bound to the myc-FzCRD-IgG.

I. 2. 5. SDS-PAGE and Western Blotting

Bio-rad miniprotean II systems were used for SDS PAGE and Western blotting. For analysis of Wnt7a/b (MW=44kDa), myc-FzCRD-IgG (MW=50kDa), ß-catenin (MW=92kDa) and ß-actin (41kDa), 10% acrylamide gels were used. For analysis of Rac (MW=25kDa), 15% acrylamide gels were used. See solutions and buffer for buffer compositions.

After electrophoresis, proteins were transferred to nitrocellulose membranes. The antibodies used to probe these membranes are summarsied in table II. 6.

Table II. 6. Antibodies used in western blotting

Antibody	Dilution	Source
Rat anti-HA	1:500	Roche
Mouse anti-myc	1:2000	Sigma
Mouse anti-ß-catenin	1 :500	BD Bioscience
Mouse anti ß-actin	1 :2000	Sigma

Rabbit anti rac	1 :500	Santa Cruz
HRP-conjugated secondary antibodies	1 :500	Amersham

The HRP signal from the membrane was detected using the ECL western blotting detection kit (Amersham), exposed to photographic film and developed using a Compaq Xograph Compact developer. In some cases, the band intensity was quantified using NIH image.

II. 3. Wnt signalling assays

Activation of Wnt signalling in cell lines was measured using three methods: quantification of cytosolic ß-catenin levels, Topflash assays and Rac assays.

II. 3. 1. Cytosolic ß-catenin levels quantification

The cytosolic levels of ß-catenin were analysed in 293 and QT6. $3.5x10^5$ cells were plated in 6 well plates/35mm dishes. When the cells were transfected, the cells were collected 48hours after transfection. When Wnt-conditioned medium was applied, cells were incubated for 6h in the presence of Wnt-CM at 37°C. Each condition was tested at least in duplicate. Cells were collected directly in SDS-LSB, analysed by SDS PAGE and western blotting. ß-catenin content was analysed by doing the radio of the intensity of the ß-catenin band over the intensity of the ß-actin (loading control) band. To account for variation in sample loading and protein transfer, each sample was loaded in triplicate. To normalise the data obtained from different experiments, the average ß-catenin/ß-actin radio for cells exposed to control CM was assigned as 1. The ß-catenin/ß-actin ratio of other conditions were thus divided by the control ß-catenin/ß-actin radio. After this normalisation step, a given condition obtaining a normalised value of 1.2 indicated a 20% increase in ß-catenin.

II. 3. 2. Topflash assays

TCF-mediated transcription was analysed using the Topflash reporter system, in which the expression of the Firefly luciferase gene is controled by the TCF/lef promoter (DasGupta *et al.*, 2005). $7x10^4$ 293 cells stably transfected with the super topflash reporter (Xu *et al.*, 2004) (Topflash cells) were seeded in 24-well plates (10mm dishes). In every condition tested, cells were transfected with 0.1µg pUAS-ruc-gfp (Yu and Szalay, 2002), which served as an internal control. This renilla luciferase internal control enabled to account for cell numbers and transfection efficiency of each sample (see below). All conditions were done in triplicate for each experiment, except when otherwise stated.

Assays were done using the Promega Dual-luciferase kit following the manufacturer's instructions. Briefly, cells were rinsed with PBS and lysed for 15min at RT in 100µl Passive lysis buffer. Cells are collected and homogenesised by pipetting and transferred on ice. In a tube enabling luminescence reading, 20µl of the cell lysates is added to 100µl of LARII substrate (Promega). This triggers a light reaction of the firefly luciferase which is controlled by the TCF promoter. Samples were read for 10 seconds in

a single-tube 20/20 luminometer (Promega). 100µl of "stop and glow" (Promega) reagent is then added and the samples read for another 10sec. This reaction quenches the luminescence from the firefly luciferase and provides a substrate for the internal control, the renilla luciferase to generate a light reaction. Relative Light Units (RLU), the read out for this experiment, consist of the firefly (TCF-dependent) luminescence divided by the renilla (internal control for cell numbers and transfection efficiency) luminescence. In order to normalise data between experiments, the average RLU for the untreated control samples is given the value of 1. The RLU from all other conditions are then normalised to the control. In this way, the RLU data presented represent fold-activation compared to untreated samples.

II. 3. 3. Rac assay

Activation of a non-canonical pathway leading to the activation of Rac was tested by a pull-down assay using GST-PBD (Pak/cdc42 Binding Domain) (Upstate). The PBD specifically binds the active form of Rac. Active Rac is thus isolated through a precipitation reaction where the GST-PBD replace the protein A-agarose. The conditions for this assay are identical to the Immunoprecipitation protocol (II. 2. 4). The precipitate is then analysed by immunoblotting. The amount of active Rac was compared to the level of total Rac present before the precipitation procedure.

II. 4. Solution and Buffers

II. 4. 1. Common buffers

PBS (Phosphate-Buffered Saline)
For 1l of 10X stock solution:
80g NaCl
2g KCl
2g KH_2PO_4
11.5 Na_2HPO_4
dilute in 800ml dH_20 and adjust pH to 7.4
csp 1l dH_2O

TBST (Tris-Buffer Saline 0.1% Tween)
For 1l of 10x stock solution:
80g NaCl
2g KCl
30g Tris base
dilute in 800ml and adjust pH to 7.4
csp 1l dH_2O

I. 4. 2. DNA/RNA Electrophesis

All gels were run 1X TAE Buffer. 1% Agarose (promega)-TEA gels were used.

TAE buffer (Tris-acetate/EDTA electrophoresis buffer)
For 1l of 50X stock solution:
242 Tris base
54.7ml glacial acetic acid
100ml 0.5M EDTA (pH 8.0)
csp 1000ml dH_2O

SSC buffer (Sodium Chloride Sodium Citrate Solution)
For 1l of 20X stock solution
175g NaCl
88.2g Sodium Citrate
csp 1l dH_2O

I. 4. 3. Cell culture

All cell lines were grown in DMEM (Invitrogen) and supplemented with 5% Foetal Calf Serum and 20 U/ml Penicillin/Streptomycin, unless otherwise stated. The cell line stably transfected with Wnt7b-HA-pCS were selected with 500µg/ml G418.

BIO (2'*Z*,3'*E*)-6-Bromoindirubin-3'-oxime
Cells were treated for 6hours or overnight with 2 µM BIO, a direct and specific inhibitor of GSK3ß, diluted in culture medium (Meijer *et al.*, 2003). Equal volumes of the vehicule DMSO was used as controls

BME stock
For 100ml
86ml ddH_2O
10ml Earl's Basal Salt solution (Invitrogen)
1ml BME amino acid (Invitrogen)
1ml BME vitamins (Invitrogen)
2ml 1M $NaHCO_3$
pH to 7.4

N2 + 1% FCS (used from conditioned medium production)
For 50ml
48.5ml DMEM
1ml B27 supplement (Invitrogen)
0.5ml N2 supplement (Invitrogen)
0.5ml FCS
14.5mg Glutamine (Sigma)
5.5mg Sodium Pyruvate (Sigma)
50mg Ovalbumine (TC grade) (Sigma)

SFM (Serum Free Medium)
500mg BSA (Sigma)
46.5ml BME stock

2.4ml 10% glucose
500µl ITS supplement (5µg/ml final concentration)
200µl P/S (20U/ml final concentration)
500µl L-Glutamine
Dissolve, pH to 7.4 and filter sterilise.

Paraformaldehyde (PFA) fixative
3-4% PFA was added to warm PBS and dissolved with gently heating until being completely dissolved.

RIPA buffer
50mM Tris pH 6.8
NaCl 150mM
1% Triton
1% NP40
0.5% Deoxycholate
1% SDS
10µg/ml Leupeptin
10µg/ml Pepstatin
10µg/ml Aprotinin A

I. 4. 4. SDS-PAGE

Sample Loading Buffer (5X concentrated)
250mM Tris (pH 6.8)
500mM DTT
10% SDS
0.5 Bromophenol Blue
50%Glycerol

Stacking Gel (5% Acrylamide) (prepare 10ml for 2 minigels)
6.8ml H_2O
1.7ml 30% Acrylamide (Sigma)
1.25ml Tris (pH 6.8)
0.1ml 10% SDS
for polymerisation add
0.1ml 10% Ammonium Persulfate (APS)
10µl TEMED

Running Gel (prepare 15ml for 2 minigels)
10% Acrylamide
5.9ml H_2O
5ml 30% Acrylamide (Sigma)
3.8ml Tris (pH 8.8)
0.15ml 10% SDS
for polymerisation add

0.15ml 10% Ammonium Persulfate (APS)
15µl TEMED

15% Acrylamide
3.4ml H_2O
7.5ml 30% Acrylamide (Sigma)
3.8ml Tris (pH 8.8)
0.15ml 10% SDS
for polymerisation add
0.15ml 10% Ammonium Persulfate (APS)
15µl TEMED

Western Blot transfer buffer (for 1 l 10X concentrate)
29g Glycine
58g Tris base
3.7g SDS
Add 800ml dH_2O
pH to 8.3
csp 1000ml dH_20

to make a working solution (1 l)
100ml 10X buffer
200ml methanol
700 dH_2O

Chapter III

Expression of Wnt and Fz genes in the post-natal brain

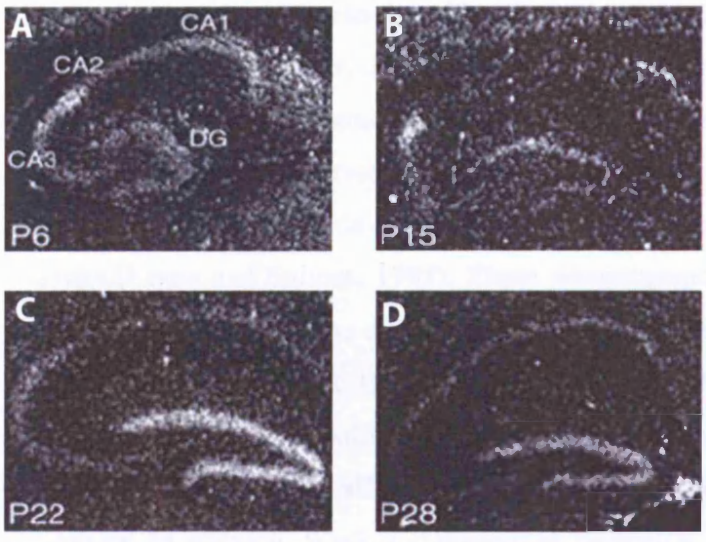
III. Expression of Wnt and Fz genes in the post-natal brain

III. 1. Introduction

The developmental events leading to the formation of vertebrate organisms rely on complex signalling events. A handful of families of signalling molecules are central to inter-cellular communication. Alongside TGF-ß, FGF and HH, the Wnt gene family has been shown to be central to many aspects of CNS development (reviewed by (Ciani and Salinas, 2005; Hall *et al.*, 2000; Lee *et al.*, 2000; Parr *et al.*, 1993; Rosso *et al.*, 2005). What molecular interactions regulate Wnt signalling in the CNS? Our laboratory is particularly interested in the role of Wnt factors in the development of the hippocampus and the cerebellum.

Many Wnt, Fz and Wnt antagonists are expressed during the development of the hippocampus (Grove *et al.*, 1998; Lee *et al.*, 2000). During embryogenesis, Wnt3a is expressed in cortical hem, and has been shown to control the proliferation of hippocampal precursors (Lee *et al.*, 2000). Much later, in the post-natal hippocampus, Wnt7b is markedly expressed by the CA (*Cornu ammonis*) and DG (dendate gyrus) regions of the hippocampus (Figure III.1), at times when pyramidal neurons are extending their dendrites (Pokorny and Yamamoto, 1981; Rosso *et al.*, 2005). Wnt7b was recently shown to act via Dvl and a non-canonical signalling pathway to activate Rac and JNK and this pathway stimulates dendritic arborisation (Rosso *et al.*, 2005). What receptors mediate these processes? Only by establishing which receptors are expressed in the hippocampus at the time of dendritic development can these questions be addressed. At the time our study began, there was no information regarding the patterns of *Frizzled* expression in the post-natal mammalian brain. Therefore, establishing the domains of *Fz* expression at times when late aspects of neurodevelopment such as dendritogenesis and synaptogenesis are occurring was our first objective.

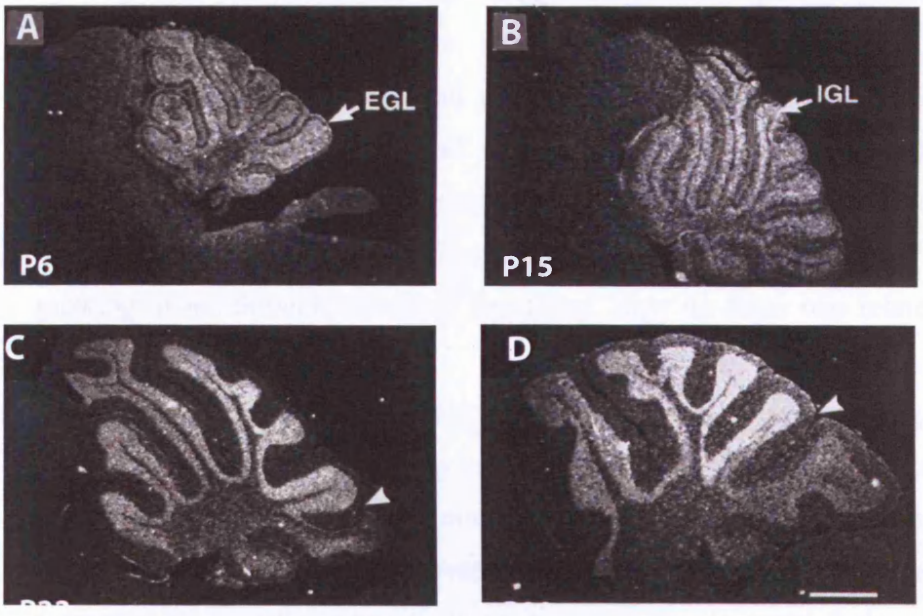
Several Wnts are expressed during cerebellar development and have been shown to regulate the behaviour of growing axons. *Wnt7a* is expressed by cerebellar granule cells throughout post-natal development (Figure III. 2) (Lucas and Salinas, 1997). *Wnt7a* expression is particularly important at times when mossy fibres arrive from the Pons to



III. 1. Wnt7b is dynamically expressed in the post-natal hippocampus

Sagitall mouse sections through the hippocampus at P6, P15, P22 and P26 processed for *in situ* hybridisation for *Wnt7b*

A-D:. *Wnt7b* is dynamic over post-natal maturation of the hippocampus. At P6, Wnt7b is mainly expressed in CA1 and CA3 of the hippocampus, where Pyramidal neuron cell bodies are located. *Wnt7b* is also found at lower levels in CA2 and in the DG, where hippocampal granule cells are found (A). At P15, *Wnt7b* expression not longer detected in the CA2 but is maintained in the CA1, CA3 and DG (B). At P22, Wnt7b expression is increased in the DG and Wnt7b remains expressed at low levels in the CA regions (C). In the young adult, at P28, Wnt7b continues to be expressed in the DG whilst expression in CA regions becomes hardly dectable.
Taken from Rosso *et al*, 2005.



III. 2. Wnt7a is expressed by cerebellar granule cells

Sagitall mouse cerebellar sections at P6, P15, P22 and P26 processed for *in situ* hybridisation for *Wnt7a*.

A-D: *Wnt7a* is expressed bu cerebellar granule cells throughout post-natal development. At P6, *Wnt7a* is expressed in both the EGL, where dividing granule cells are located, and in the IGL, where mature granule cell bodies are found (A). At P15, *Wnt7a* expression increases in the IGL but is no longer detected in the EGL (B). At P22, *Wnt7a* expression is the high in the IGL, but regional differences *Wnt7a* expression are detected (white arrowhead) (C). In the adult, at P28, expression remains high in the IGL although strong regional differences are detected, with notable downregulation in the vestibulo-cerebellum (white arrowhead) (D).
Scale bar = 0,65mm.
Taken from Lucas *et al.* 1997

make synapses with granule cell. Wnt7a signals to mossy fibre axon to begin the formation of synapses by inducing axonal thickening and growth cone enlargement on one hand, and the accumulation of synaptic proteins such as Synapsin-1 on the other (Hall et al., 2000). Interestingly, when granule cells grown in vitro come into contact with an exogenous source of Wnt7a, they also accumulate synapsin-1 and thicken their axons (Lucas and Salinas, 1997). These synaptogenic changes occurring in neurons are likely to be caused by the canonical Wnt pathway, because pharmacological inhibition of GSK-3 mimics the effects of Wnt7a (Lucas and Salinas, 1997). Yet, the receptors transducing Wnt7a signalling remain unknown. Also, it remains unclear whether the same signalling cascade affects the cytoskeleton and leads to the accumulation of synaptic proteins. In addition, Wnt3 is expressed by cerebellar Purkinge cells and its expression is modulated by their pre-synaptic partners, the granule cells (Salinas et al., 1994). What are the receptors mediating Wnt signalling in the cerebellum? Which Frizzled receptors are expressed in the Pons and cerebellum? Addressing these questions could clarify the mechanisms by which Wnts signal in neurons. Identifying specific Wnt / Fz combinations would provide new insights into how specific pathways and cellular responses are triggered in neurons.

The potential involvement of Wnt signalling in synaptic plasticity and axonal regeneration was also investigated. Are Wnts and their receptor Frizzled still expressed in the adult brain? Can their expression pattern suggest new areas of research? Additionally, Wnt7b stimulates dendritogenesis via a non-canonical pathway whilst Wnt7a signals to growing axons through canonical signalling. How do these two related Wnts signal to these different pathways?

To address which receptors were responsible for Wnt signalling in neurons of the post-natal mouse, *Wnt* and *Fz* gene expression patterns were first studied in the adult (post-natal days 30-40 (P30-40) mouse, and subsequently during post-natal development. Indeed it was interesting to investigate whether the signals that regulate the initial formation of synaptic connections continued to be expressed during adulthood. *Wnt7b* and *Fz3* were both strongly expressed in the adult hippocampus. *Wnt7a*, *Fz7* and *Fz3* were expressed in adult cerebellum. Furthermore, we investigated how these genes were expressed during post-natal development and found the above were strongly and

dynamically expressed in these regions. Additionally, we discovered that *Fz3* was expressed in the Pontine Nuclei, the nuclei where the cell bodies of mossy fibres are located. These patterns of expression enabled us to carry out biochemical and functional studies to address the interactions between Wnt and Fz expressed in these brain areas, which are the subject of the next chapters.

III. 2. Results

III. 2. 1. Overview of Wnt and Frizzled gene expression in the adult

Expression patterns were first analysed in the adult. Indeed, the role for Wnt signalling in the adult has so far not been demonstrated and therefore it was important to investigate whether *Wnt* and *Fz* genes were expressed in the adult. 5 *Wnt* genes (*Wnt5a, Wnt6, Wnt7a, Wnt7b and Wnt11*) out of the 19 Wnt genes found in the mouse genome were screened. Also, 4 *Frizzled* (*Fz3, Fz4, Fz6, Fz7*) out of the 10 known *Frizzled* genes were tested. Some of these genes were selected because of their involvement in earlier aspects of CNS development (e.g. Wnt7a, Wnt7b, Fz3, Fz4). (Caricasole *et al.*, 2003; Liu *et al.*, 1999; Rosso *et al.*, 2005; Wang *et al.*, 2001; Wang *et al.*, 2002). Analysing the expression patterns of all *Wnt* and *Fz* genes is an enormous task, and therefore we focused our work on genes strongly expressed in the hippocampus and cerebellum.

Radioactive *in situ* hybridisation was the method chosen to investigate the expression of *Wnt* and *Fz* genes in the mouse brain. The experience of lab members at successfully using this method to detect very low levels of Wnt expression in the CNS was the main reason why radioactive *in situ* was chosen over non-radioactive methods. Using this protocol, two sets of data were generated for each experiment. First, the sections exposed to radioactive probes were exposed to X-ray film. The autoradiographs provide an overview of gene expression throughout the brain, but could not clearly reveal small regions of gene expression or areas where expression was low. Observing the slides once they have been photographically processed enables detailed analysis of gene expression using dark-field microscopy. Dark field images were taken using a 5X

magnifying lens, and therefore most high resolution images limit themselves to specific brain regions.

The autoradiographs obtained for Wnt genes provided good insight into which genes were expressed in the adult (Figure III. **3**). Yet, at this resolution it is sometimes not possible to specifically assign which brain regions are expressing a given gene. Interestingly, the two Wnt involved in post-natal development of the CNS, *Wnt7a* and *Wnt7b*, were the two Wnt with the most striking gene expression patterns (Fig. III. 3). *Wnt7a* was strongly expressed in the adult cerebellum and some expression in the olfactory bulb was also detected. *Wnt7b* may be faintly expressed in the cerebellum as well, but the most striking areas of expression were the neocortex, the olfactory bulb and the hippocampus. Although it cannot be confirmed at this resolution, *Wnt7b* expression was detected in the DG area of the hippocampus. *Wnt6* was expressed in the cerebellum and *Wnt5a* may also be expressed at low levels in this area. On the other hand, *Wnt11* which displayed specific expression patterns in embryonic (E15.5) specimens used as positive controls was not expressed in the adult brain.

The autoradiographs displaying an overview of Fz gene expression revealed even more striking gene expression patterns (Figure III. 4). *Fz3 was* strongly and ubiquitously expressed in the adult mouse brain. *Fz3* expression in the hippocampus; the cerebellum and the olfactory bulb was strong. In the cortex, distinct layers expressed *Fz3*. Additionally, *Fz3* was expressed throughout the forebrain regions encompassing the visual cortex the anterior boundary of the caudate nucleus. Although it is difficult to assess expression in small areas at this level, *Fz3* may be expressed in the pontine nucleus. *Fz7* was the second *Frizzled* with a striking expression which at first seems limited to the cerebellum. Yet, more central sections displaying the olfactory bulb indicate *Fz7* may also be expressed in this area. *Fz4* were not detected in the adult mouse brain.

All the sections presented here we subsequently dipped in photographic emulsion and analysed at higher magnification using dark field microscopy. Expression of *Wnt* and *Fz* in the cerebellum and the hippocampus was of particular interest. The most striking expression patterns detected in this study are shown in figure III. 5. *Wnt* and *Fz* were

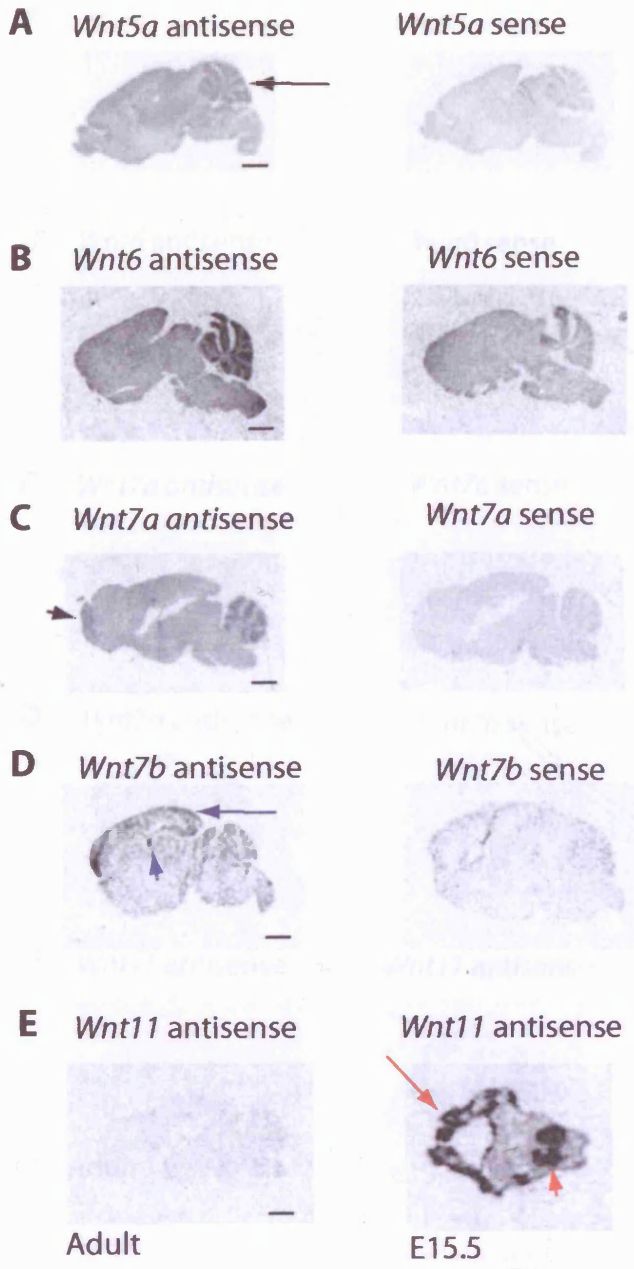


Figure III. 3. Overview of *Wnt* genes expression in the adult mouse

Autoradiographs of adult mouse sagittal section processed for *in situ* hybridisation for *Wnt* genes. Left panels display the signal obtained for antisense probes. Right panels display the signal obrained to sense probes.

A. *Wnt5a was* expressed at low levels in the cerebellum (black arrow) and the cortex.
B. Wnt6 was exclusively detected in the cerebellum.
C. *Wnt7a* was expressed in the cerebellum and in the olfactory bulb (arrowhead).
D. *Wnt7b* was expressed in the DG of the hippocampus (blue arrowhead), in the occipital lobe of the cortex (blue arrow) and in the olfactory region.
E. *Wnt11* was not detected in the adult mouse. A positive control using transverse sections of a E15.5 mouse embryo demonstrated this probe detected *Wnt11* in the limbs (red arrow) and the developping CNS (red arrowhead).
Scale bar = 2mm

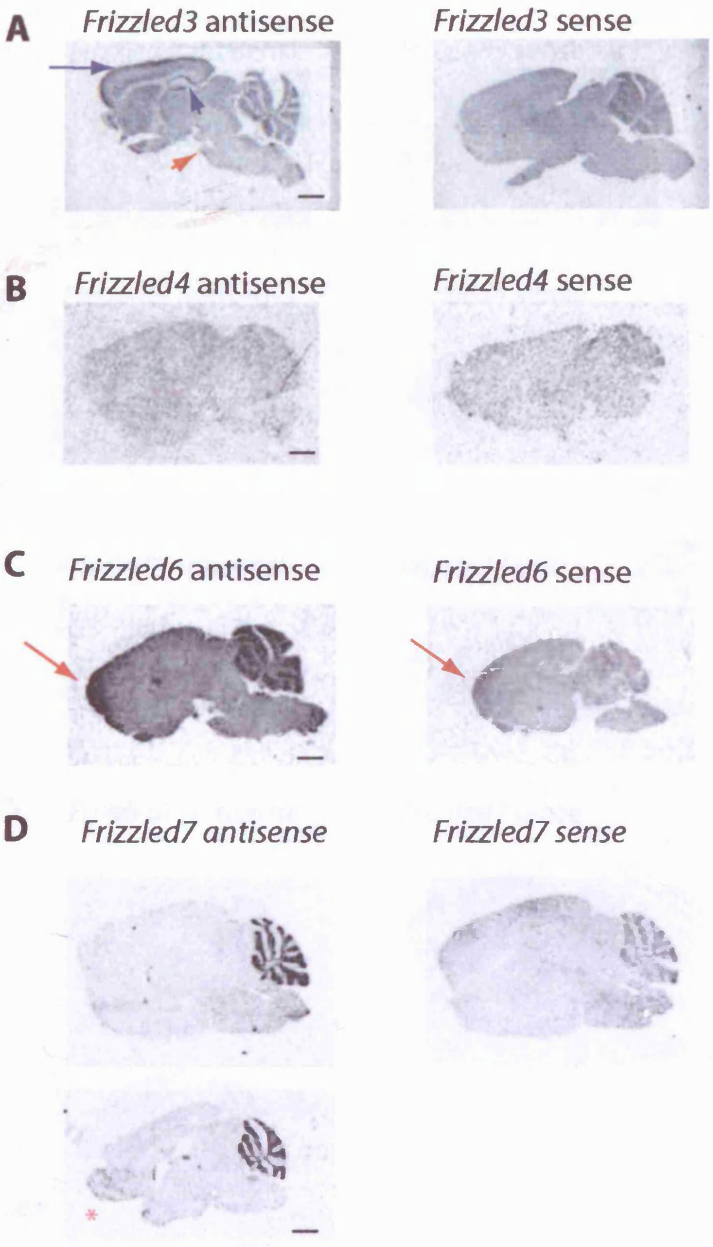


Figure III. 4. Overview of *Frizzled* gene expression in the adult mouse brain

Autoradiograph of adult mouse sagittal sections processed for *in situ* hybridisation *Fz* genes. Left panels display the signal obtained for antisense probes. Right panels display the signal obtained to sense probes.

A. *Fz3* was highly expressed in the cortex (blue arrow), the hippocampus (blue arrowhead), the cerebellum, the olfactory system, the visual cortex and the Pontine nuclei (red arrowhead).
B. *Fz4* did not appear to be expressed in the adult mouse brain.
C. *Fz6* was expressed in the cerebellum. The signal obtained in the frontal areas of the brains may not be specific as the sense probe also produced some signal in this area (red arrows).
D. *Fz7* was clearly and specifically expressed in the cerebellum. Also, some Fz7 is detected in the olfactory bulb 'red asterix).
Scale bar = 2 mm

A. Adult Cerebellum

B. Adult hippocampus

C. Sagittal brain overview and hippocampal regions

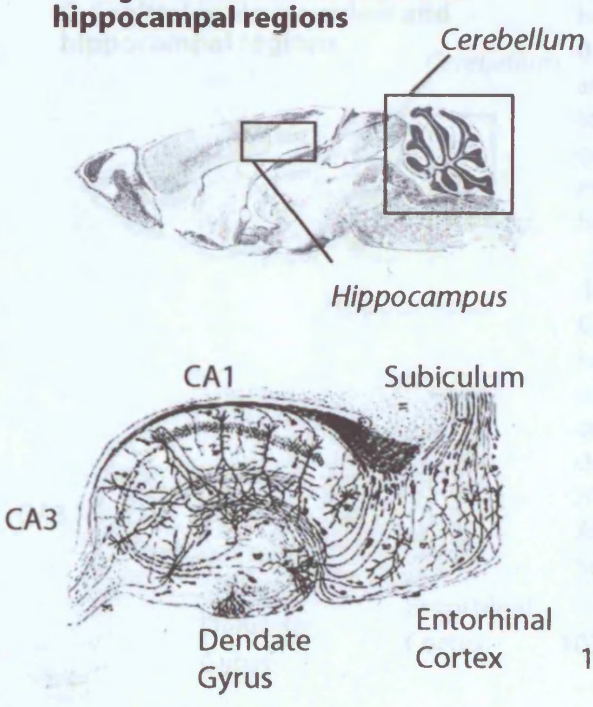


Figure III. 5. Comparative expression of *Wnt* and *Fz* in the adult

Sagittal brain sections of adult mouse processed for *in situ* hybridisation for *Wnt7a* and *Fz7*.

A: In the adult cerebellum, *Wnt7a* and *Fz7* were expressed in granule cells whose cells bodies are located in the IGL. *Fz7* expression is homogenous throughout the cerebellum, whilst *Wnt7a* expression is decreased in the vestibulocerebellum (white arrow).

B: In the hippocampus of the young adult (P22), *Fz3* and *Wnt7b* were expressed. *Fz3* is strongly expressed in both pyramidal neurons of the the CA regions and granule cells of the DG areas. In contrast, *Wnt7b* is mainly expressed in the DG which mainly contains hippocampal granule cells. Low levels of *Wnt7b* expression in the CA3 region were nonetheless detected.

C. Top panel: Sagittal view of the mouse brain, highlighting the regions depicted in A and B. Bottom panel: Early diagram representing the cellular organisation of the hippocampus (Ramon y Cajal drawing taken from medweb.bham.ac.uk)

Abbreviations: IGL: Internal Granule Layer, CA: Cornu Ammonis, DG: Dentate Gyrus

Scale Bars in A and B: 0,2mm

detected in the adult brain, thus suggesting Wnt signalling play are role in the mature brain. In particular, *Wnt7a* and *Fz7* appeared to be highly expressed by cerebellar granule cells (Figure III. 5. A). *Wnt7b* and *Fz3* were detected in the hippocampus (Figure III. 5. B). *Fz3* was particularly highly expressed in the CA1 region, where pyramidal cell bodies are located and in the dentate gyrus (DG), where hippocampal granule cells are located. *Wnt7b* expression appeared limited to the DG. This overview of Wnt and Fz expression patterns in the adult indicate that *Fz3* and *Wnt7b* are expressed in the hippocampus and *Fz7* and *Wnt7a* are expressed in the cerebellum.

III. 2. 2. Fz gene expression during post-natal development

III. 2. 2. 1. Fz3 is ubiquitously expressed in the post-natal brain

The finding that *Fz3* was strongly expressed in the adult led to a more in depth analysis of the spatio-temporal expression of *Fz3* during post-natal development. An overview of *Fz3* expression a P6, P10, P22 and in the adult (past P28) mouse brain is presented in Figure III. 6. At P6, the most striking areas of Fz3 expression were the cerebellum and some layers of the neocortex, but expression in the hippocampus was not clearly detected (Fig. III. 6. A). At P10, expression was maintained in the cerebellum, the neocortex, the hippocampus as well as the pontine nuclei, the source of mossy fibres that enter the cerebellum to form synapses with granule cells (Fig. III. 6. B). In the young adult, at P22, expression was strong and widespread. *Fz3* expression in the cerebellum and throughout the neocortex was strong. *Fz3* is also expressed in many frontal areas, including the olfactory bulb. Importantly, *Fz3* expression was also detected in the pontine nuclei (Fig. III. 6. C). In the adult, expression of *Fz3* was very similar to that at P22, except that expression in the cerebellum was significantly decreased (Fig. III.6.D). Fz3 was the most ubiquitously expressed Frizzled receptor that was analysed in this study.

Analysis of an adult section exposed to photographic emulsion enables a closer examination of the *Fz3* expression (Figure III. 7). *Fz3* expression in the adult hippocampus was striking. *Fz3* was most strongly expressed in the CA1 and DG regions, whilst expression in the CA3 region of the adult was weak (Fig. III. 7). In the frontal

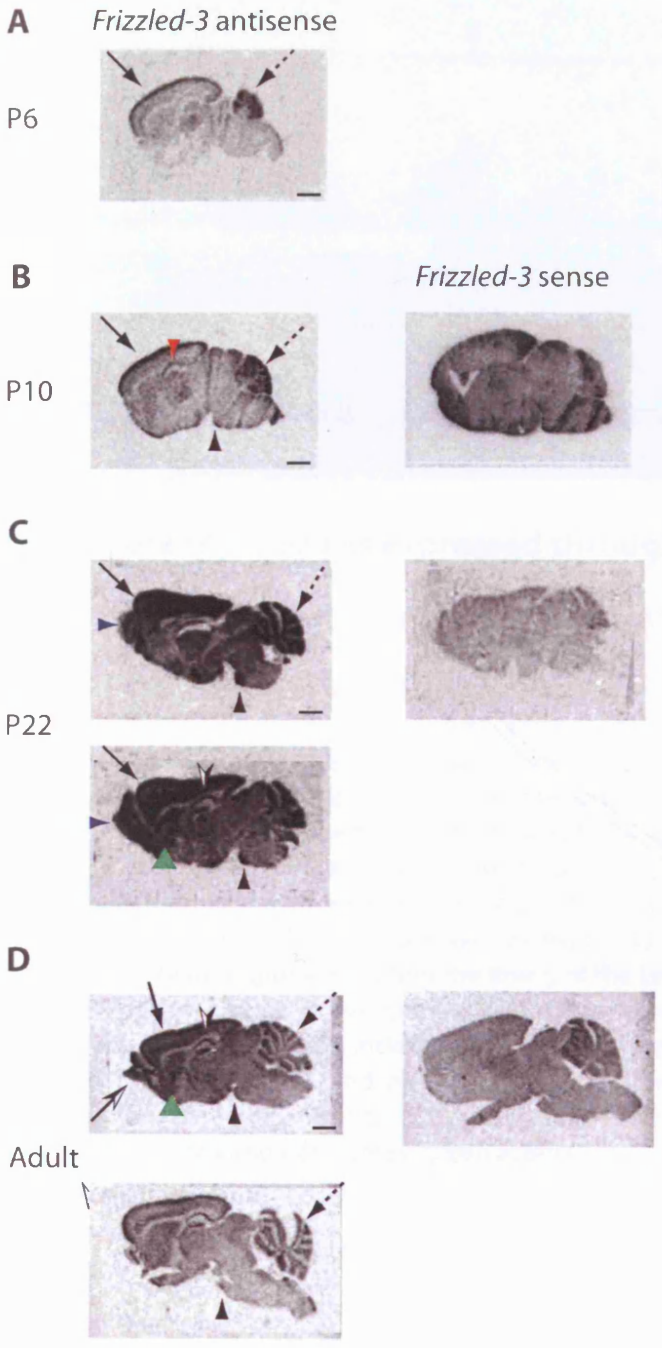


Figure III. 6. Overview of *Frizzled3* expression in the post-natal mouse brain

Autoradiographs of post-natal mouse brain processed for *in situ* hybridisaton for *Fz3*

A-D: Time-course of Fz3 expression in the post-natal brain. At P6, *Frizzled3* was expressed in the outer layers of the cortex (black arrow) and in the cerebellum (dashed arrow) (A). B. At P10, *Frizzled3* expression was high in the cortex and cerebellum. In addition, *Fz3* was clearly expressed in the hippocampus (red arrowhead) and in the Pons (black arrowhead) (B). At P22, expression of *Fz3* was widepsread. *Fz3* was expressed in the cortex, the hippocampus, the cerebellum, the Pons, as well as in the olfactory bulb (blue arrowhead). *Fz3* Expression was widespread in frontal areas including the visual cortex (green arrowhead) (C). In the adult, *Fz3* is expressed in the cerebellum, the hippocampus, the cortex, the olfactory bulb, the Pons and the visual cortex (D).
Scale bars = 2mm

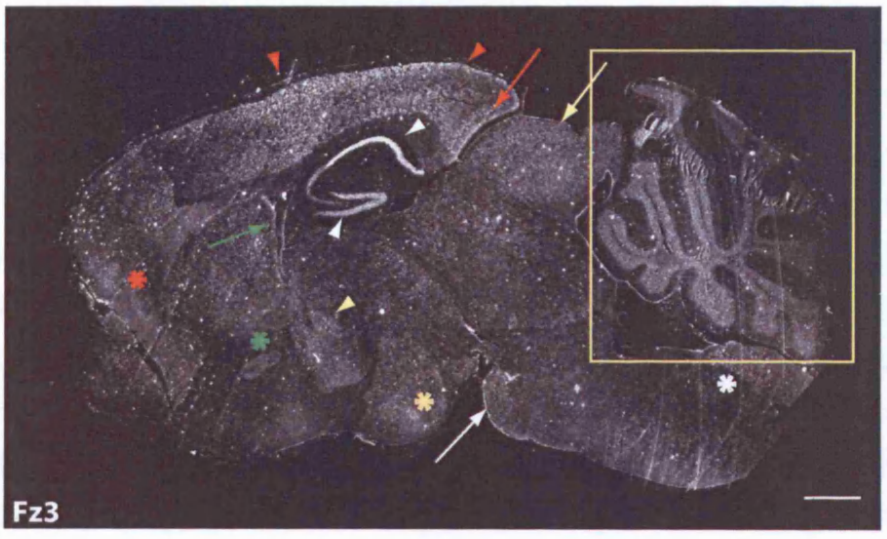


Figure III. 7. *Fz3* is expressed throughout the adult brain

Sagittal sections of adult mouse brains processed for insitu hybridisation for *Fz3*.

The three most distinctive areas of *Fz3* expression are the hippocampus (white arrow heads), the neocortex (red arrows and arrowheads) and the cerebellum (yellow box). In the hippocampus, *Fz3* expression is particularly high in the DG and CA1 regions of the hippocampus (white arrow heads). In the cortex, (parietal and occipital, red arrowheads) and the retrosplenial area (red arrow). In the cerebellum, Fz3 expression is heterogeneous, with Fz3 being more highly expressed in frontal areas than in dorsal areas. Interestingly, Fz3 was expressed in the pontine nuclei, albeit at low levels (white arrow). *Fz3* expression was also detected in numerous frontal brain regions, including the lining of the lateral ventricules (green arrow), the colliculum (yellow arrow), the thalamic reticular nucleus (yellow arrowhead). Other areas of *Fz3* expression include as parts of the dorsal medulla (white asterix), nuclei of the hypothalamic and premammillar areas (yellow asterix), parts of the frontal cortex / olfactory region (red asterix) and parts of the caudate nucleus/nucleus accumb ens and optic areas (green asterix).

Scale bar = 1mm

neocortex, expression was also very pronounced. Different layers of the parietal and occipital cortex expressed *Fz3* (red arrowheads, Fig. III. 7), and expression in the retrosplenial area was also very marked (red arrow). *Fz3* expression in the IGL of the cerebellum was detected. In addition, a number of brain regions express low levels of *Fz3* expression over large areas such as in the colliculum (yellow arrow). Yet, there were also smaller areas which specifically expressed *Fz3*, such as the Pontine nuclei (white arrow) and the reticular thalamic nucleus (yellow arrowhead). Having only sagittal sections at our disposal, it was difficult to assign some smaller areas of Fz3 expression, such as nuclei around the hypothalamic and pre-mammilary regions as well as in the frontal parts of the brain (asterix). Clearly, Fz3 is strongly and ubiquitously expressed in the adult brain and therefore it is likely Wnt signalling has functions in the adult CNS. Which other *Wnt* are expressed in similar patterns as *Fz3*? How does Fz3 expression change during post-natal development? What other Fz are expressed in the same regions? Addressing these questions could shed light on which specific Wnt/Fz are involved in known events but also suggest new areas of research.

III. 2. 2. 2. Fz3 is strongly expressed in the hippocampus

Closer examination of *Fz3* expression in the hippocampus during post-natal development revealed a stable pattern of expression (Figure III. 8). Expression of *Fz3* was maintained in the DG and CA1 region throughout postnatal development, from P6 to P22. At P6, Fz3 expression was clearly higher in the DG than in CA regions, but this difference was less striking at later stages (Fig. III. 8. A). *Fz3* expression was also detected at P6 in the CA3 region, but seems to be reduced in this region at P10 and P15 (Compare III.9.A with B and C). In the young adult, at P22, *Fz3* expression was very strong in the DG and CA1, and can also be detected in the CA3. In comparison, during post-natal development, *Wnt7b* expression is dynamic (Figure III. 1) (Rosso *et al.*, 2005). *Wnt7b* expression is high in the CA3 region at P6 but low in other hippocampal regions. During post-natal development, the location of high Wnt7b expression shifts to being the DG and is maintained in the CA1 region (Rosso *et al.*, 2005). Therefore, in the adult where *Fz3* is strongly expressed in the CA3, it is possible that signalling between pyramidal cells of the CA3 region and afferent axons of the DG or target dendrites of the CA1 could implicated Fz3 and Wnt7b (see discussion). The other *Frizzled* genes tested in

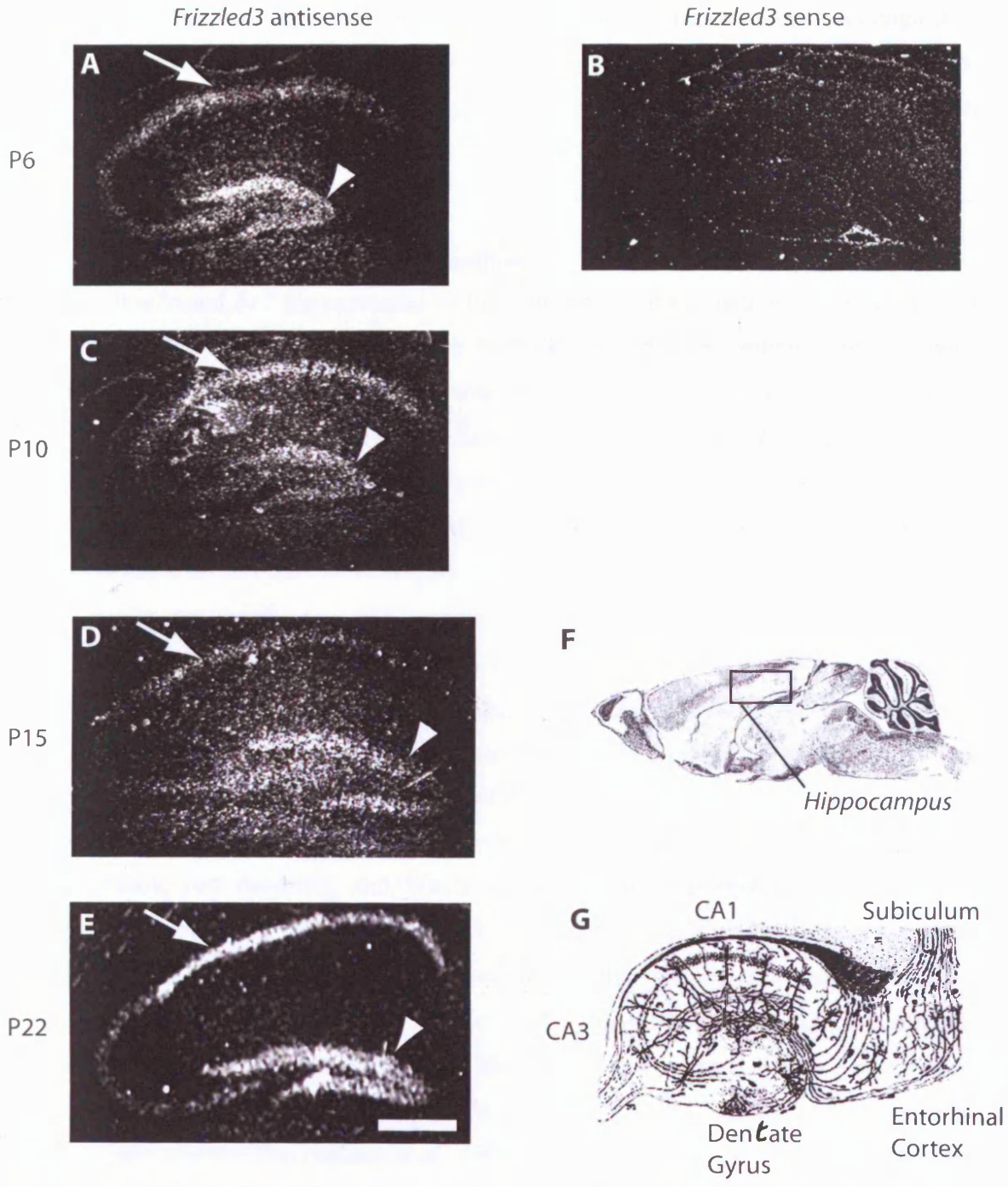


Figure III. 8. *Frizzled3* is expressed in the post-natal hippocampus

Sagittal hippocampal sections of post-natal mice hippocampus processed for in situ hybridisation for *Fz3*

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A-E: *Fz3* is expressed by cells of the hippocampal pyramidal layer at P6 (**A**) and expression is maintained throughout postnatal development (**C** and **D**). Expression is highest in the Dentate Gyrus (arrowheads) and CA1 regions, (arrows) but *Fz3* is also expressed in the CA3. In the young adult (**E**) *Fz3* expression remains high.

F: Overall view of a sagittal brain section. The box represents the localisation of the hippocampus.

G: Schematic drawing of the hippocampal network by Ramon y Cajal

Scale bar= 0,1mm

this study *(Fz4, Fz6, Fz7)* were not detected in the hippocampus. Although it is likely that other *Wnt* and *Fz* genes are expressed in these areas, these expression patterns presented here suggest that the dendritogenic effect of Wnt7b on hippocampal neurons could be mediated by Fz3 (Rosso *et al.*, 2005).

III. 2. 3. Fz expression in the cerebellum

Wnt7a and *Fz7* are expressed by the same neuronal population in the cerebellum (Figure III. 5. A). The Internal granule layer (IGL) of the cerebellum is packed with granule cell bodies and also contains a small number of Golgi cells. It is therefore likely that cerebellar granule cells were the cells responsible for the intense signal obtained for *Wnt7a* and *Fz7* expression, although expression by glial cells remains a possibility (Fig. III. 5. A). *Fz3* was also expressed in the IGL of the adult cerebellum but its expression was markedly lower than *Fz7* (Compare Figure III. . A and E, also see figure III. 6 and 10). *Wnt7a* expression was higher in some lobes than others. *Wnt7a* was expressed at lower levels, in the vestibulocerebellum, an observation already reported (Lucas and Salinas, 1997). *Fz7* was also down-regulated in the vestibulocerebellum but this difference in gene expression was less marked than that observed for *Wnt7a*. Is this down-regulation observed at earlier post-natal stages?

Most synapses formed in the IGL consist of mossy fibre terminals making contact with granule cell dendrites, and Wnt7a signalling has been shown to influence the formation of the mossy fibre terminal (Hall *et al.*, 2000). Yet, Wnt signalling is known to regulate itself through autocrine loops and the fact that granule cells themselves may respond to Wnt7a *in vitro* suggest that Fz7 could potentially function as a receptor for Wnt7a (Lucas and Salinas, 1997). Also, since Wnt3 is expressed by the post-synaptic targets of granule cells, the Purkinje cells, it is also possible that Fz7 is implicated in Wnt3 signal transduction (Salinas *et al.*, 1994). The finding that *Fz7* was expressed in the IGL of the adult cerebellum is the first report of a Frizzled being expressed in the adult cerebellum and constitutes a first step in our study aiming to clarify the mechanisms of Wnt signalling in the cerebellum. How does *Fz7* expression change during the post-natal events occurring in the cerebellum?

III. 2. 3. 1. Fz7 is strongly expressed by cerebellar granule cells

Cell migration in the cerebellum is a well understood event. Migration of granule cells from the EGL to the IGL occurs during post-natal development. It was therefore possible to confirm that granule cells were indeed responsible for the *Fz7* expression detected in the adult IGL. *Fz7* expression in the cerebellum was analysed at P6, P10, P15 and P22 (Figure III. 9). At P6, a strong signal was detected in the EGL of most cerebellar lobes. Interestingly, three lobules of the vestibulocerebellum expressed lower levels of *Fz7*. Signal within the IGL was only slightly above background. This indicates that as granule cells are proliferating in the EGL, they are expressing Fz7.

In contrast, at P10, *Fz7* was found in both the EGL and the IGL. This corresponds to a time when granule cells are migrating from the EGL to the IGL (Altman, 1972). Therefore *Fz7* is expressed in both the dividing granule cells of the EGL and post-mitotic granule cells in their final position in the IGL. *Fz7* expression was maintained at P15, once all granule cells have finished their migration. *Fz7* may thus be playing different roles for granule cells at different times during their maturation. In the dividing granule cells of the EGL, cell-cycle control is important and the cell morphology changes dramatically (Hatten, 1999). In contrast, in the IGL, contacts with incoming mossy fibres are initiated, the granule cells mature and develop their dendritic arbors (Hamori and Somogyi, 1983) (Larramendin, 1969). *Fz7* expression in the IGL was still robust at P22 and in the adult (Fig. III. 5). The down-regulation of *Fz7* in the vestibulocerebellum detected at P6 holds true at P15 and P22 (arrowheads in Fig. III. 9. E). It is interesting the highlight that *Wnt7a* is similarly down-regulated in that region (Lucas and Salinas, 1997). Therefore, it is possible that *Wnt7a* and *Fz7* may be signalling in the IGL in an autocrine fashion. Clearly, Fz7 and Wnt7a must play an important role in the development of granule cells, and Fz7 is a candidate receptor for Wnt7a signals triggering pre-synpatic maturation (Hall *et al.*, 2000). Many questions are raised by these expression patterns. What functions plays Wnt signalling in the mature cerebellum? Is Fz7 the main receptor triggering the canonical response known to affect cerebellar neurons? Are other *Fz* receptors contributing to the process?

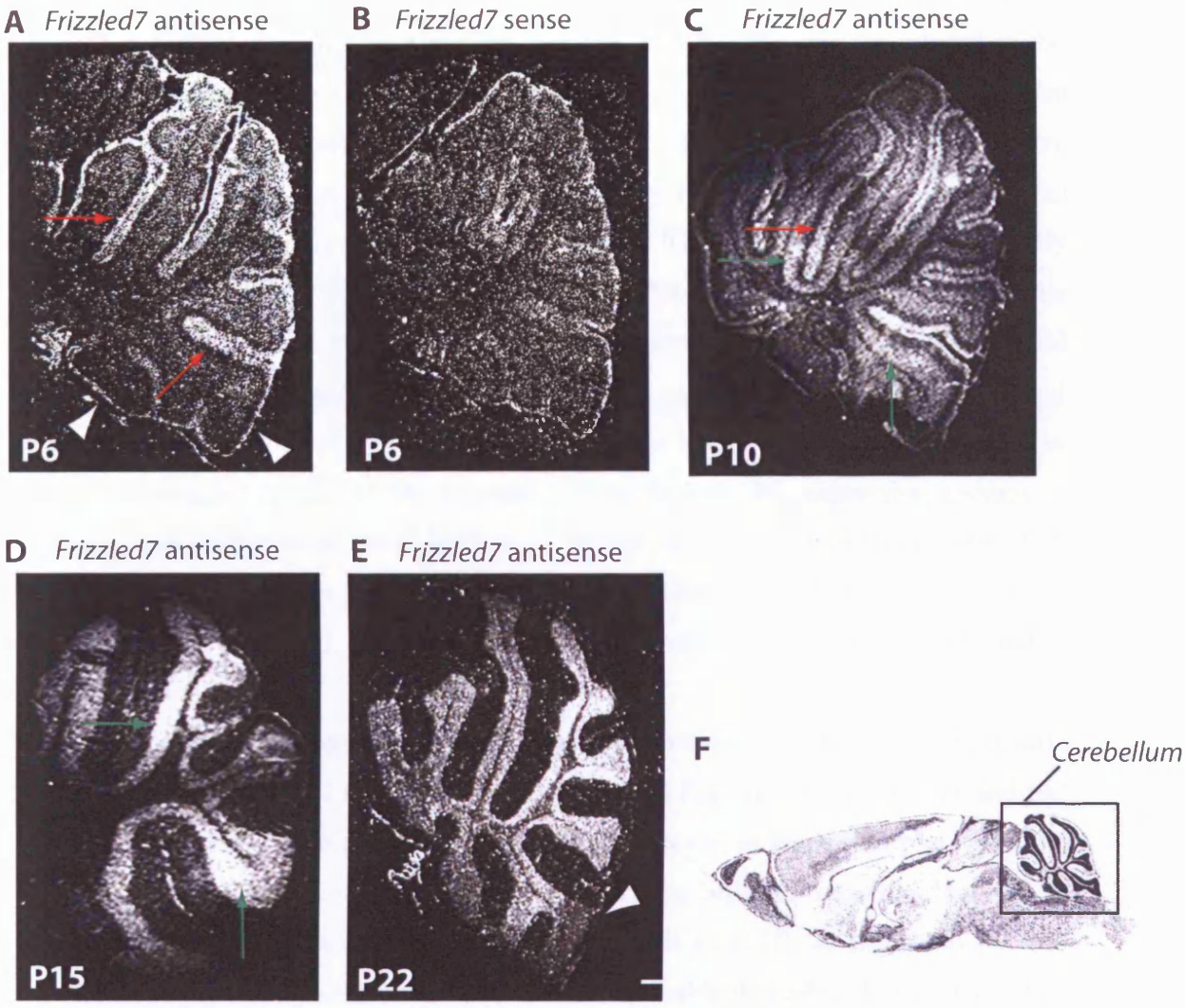

Figure III. 9. Frizzled-7 is expressed by cerebellar granule cells

Sagittal cerebellar sections processed for *in situ* hybridisation for *Frizzled-7* during post-natal development.

A-E: Fz7 was expressed by cerebellar granule cells during post-natal development. At P6, *Fz7* expression was limited to the EGL (red arrows), where granule cells precursors are proliferating. Fz7 expression was markedly lower in the vestibulocerebellum (white arrowheads). The sense probe (**B**) complementary to the *Fz7* antisense indicates the background signal obtained through this procedure. At P10, *Fz7* expression reached from the EGL into the IGL (green arrows) (C). This corresponds to the migration of post-mitotic granule cells from the EGL to the IGL. At P15, Fz7 expression was limited to the IGL, the final location of granule cells. (D). At P22, as mice reach adult hood, *Fz7* expression was maintained in the granule cells of the IGL. *Fz7* expression was reduced in some lobes of the vestibulocerebellum (white arrow head)
F: Positionning of the cerebellum within a sagittal mouse section
Abbreviations: EGL: External Granule Layer, IGL: Internal Granule Layer.
Scale bar = 0,4mm

III. 2. 3. 2. Fz3 is expressed in the cerebellum and the pontine nuclei

Unlike *Fz7* which was nearly exclusively expressed in the cerebellum, *Fz3* was strongly expressed in many brain regions (see Fig. III. 7). Of particular interest in the context of Wnt signalling and neuronal behaviour, *Fz3* was expressed in the cerebellum (Figure III. 10) and the pontine nuclei (Figure III. 11). *Fz3* displays an expression pattern which was similar to that of *Fz7* in the cerebellum over the post-natal period. *Fz3* expression was detected in the EGL at P6, in both the IGL and the EGL at P10 and only in the IGL in the young adult (P22) (Fig. III. 10). Therefore it appears the cerebellar granule cells express *Fz3*. Nonetheless there is an important difference between *Fz7* and *Fz3* expression in the cerebellum. *Fz7* was strongly expressed at P6 in the EGL and remains strongly expressed by the granule cells in the IGL in the adult (Fig. III. 9). In contrast, although *Fz3* was highly expressed in the EGL at P6, expression appears to decrease once cells have migrated in the IGL. Indeed, at P10, *Fz3* expression in the IGL was lower than expression of *Fz7* at the same stage. Therefore Fz3 must play a role in proliferating granule cells but does not play a central role once granule cell bodies translocate to the IGL.

In the adult, although some *Fz3* expression remains in the IGL, *Fz3* expression was clearly lower than *Fz7* (compare Fig. III. 9. E with Fig. III. 10. C). Are Fz3 and Fz7 redundant receptors performing overlapping functions? Or on the contrary are Fz3 and Fz7 responding to different Wnt ligands and activating different signalling pathways? *Wnt7a* is strongly expressed by cerebellar granule cells (Fig. III. 2) and could perhaps signal autocrinely through *Fz3* and/or *Fz7*. Yet, it is possible that other Wnt are signalling to granule cells. What other *Wnt* are expressed in the cerebellum? In particular, granule cell axons encounter the dendrites of Purkinge cells in the molecular layer. If Wnt signalling is to play a part in retrograde signalling as it does at the granule cell – mossy fibre synapse, which *Wnt* is expressed by Purkinge cells? Finally, which *Frizzled* receptors are expressed by mossy fibres, whose cell bodies are located in the Pontine nuclei? Shedding light on these questions could provide interesting insights into Wnt signalling in the cerebellum.

One of the most important questions regarding Wnt-induced synaptogenic signalling in the cerebellum is which receptors are mediating Wnt7a signalling in mossy

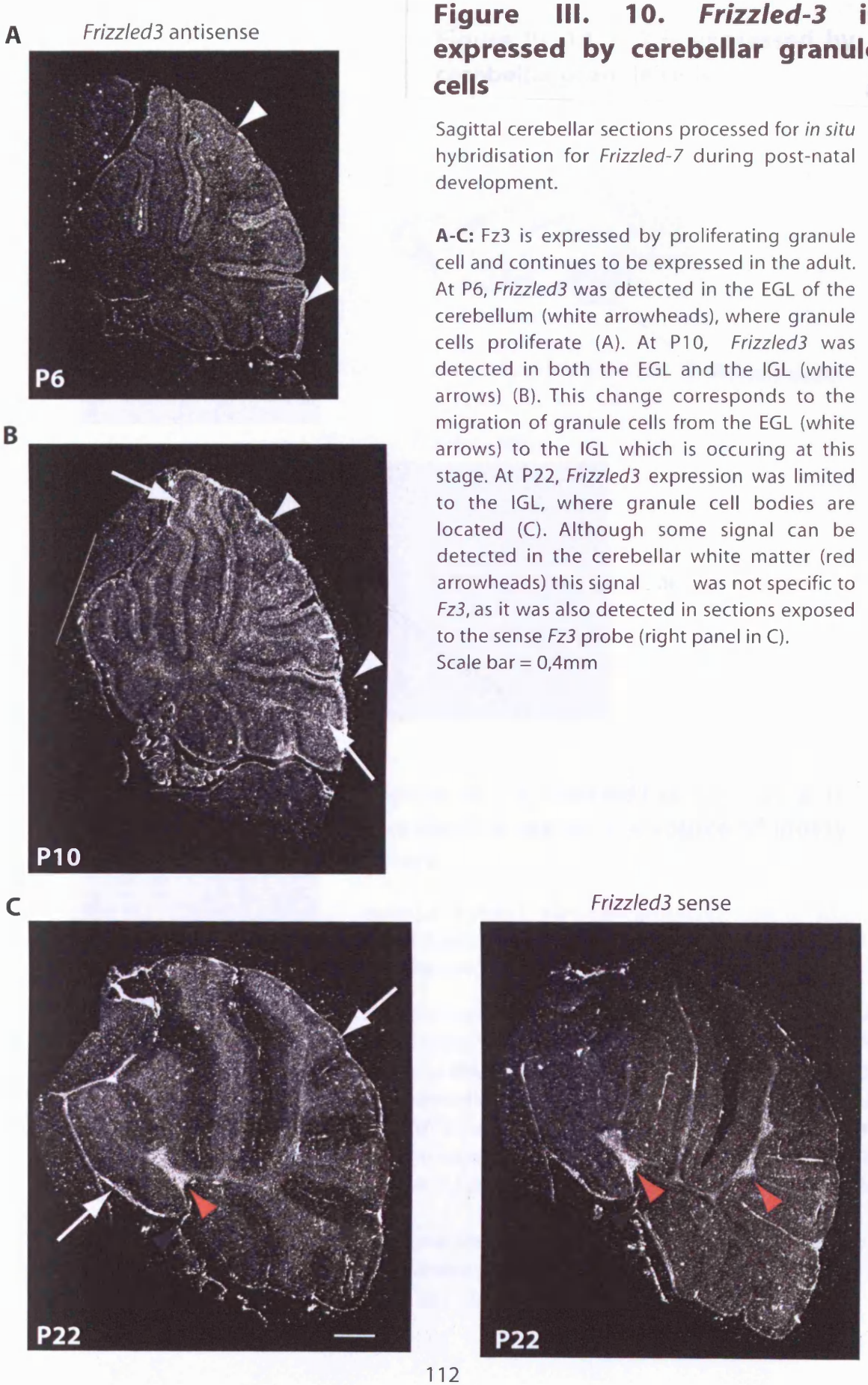

Figure III. 10. *Frizzled-3* is expressed by cerebellar granule cells

Sagittal cerebellar sections processed for *in situ* hybridisation for *Frizzled-7* during post-natal development.

A-C: Fz3 is expressed by proliferating granule cell and continues to be expressed in the adult. At P6, *Frizzled3* was detected in the EGL of the cerebellum (white arrowheads), where granule cells proliferate (A). At P10, *Frizzled3* was detected in both the EGL and the IGL (white arrows) (B). This change corresponds to the migration of granule cells from the EGL (white arrows) to the IGL which is occuring at this stage. At P22, *Frizzled3* expression was limited to the IGL, where granule cell bodies are located (C). Although some signal can be detected in the cerebellar white matter (red arrowheads) this signal was not specific to *Fz3*, as it was also detected in sections exposed to the sense *Fz3* probe (right panel in C).
Scale bar = 0,4mm

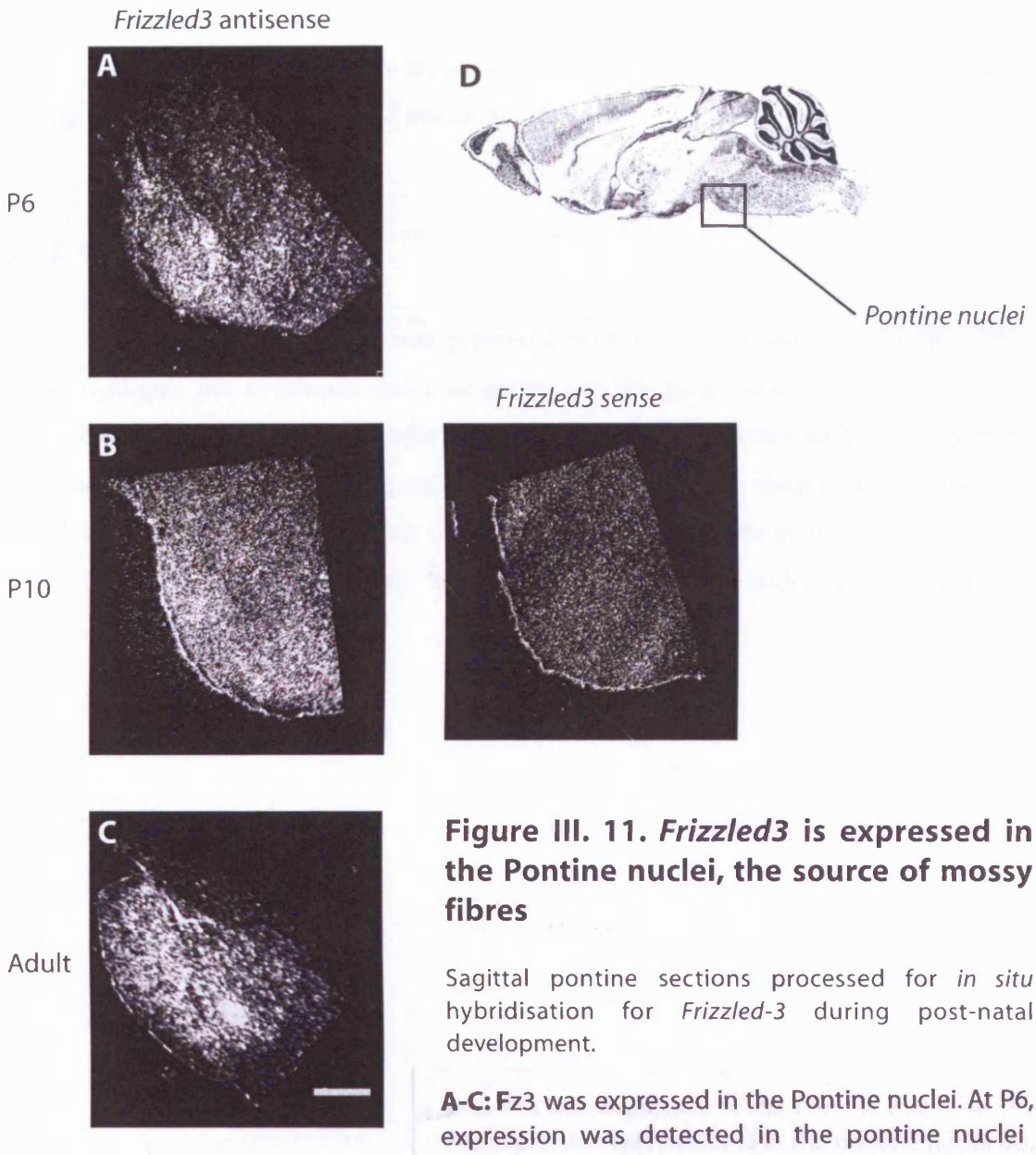

Figure III. 11. *Frizzled3* is expressed in the Pontine nuclei, the source of mossy fibres

Sagittal pontine sections processed for *in situ* hybridisation for *Frizzled-3* during post-natal development.

A-C: Fz3 was expressed in the Pontine nuclei. At P6, *Fz3* expression was detected in the pontine nuclei (A). There lie the cell bodies of mossy fibre which extend to the cerebellum. Expression of *Fz3* was maintained at P10 (B) whereas no signal was detected in adjascent sections probed with sense probes (right panel in B). In the adult, Fz3 remained expressed in the pontine nuclei (C).

D. Sagital view of the brain depicting the localisation of the Pontine nucleus

Scale Bar = 0,2mm

fibres *in vivo*? The mossy fibres that make contact with cerebellar granule cells have their cell bodies located in the Pontine nucleus. The only Frizzled receptor that was found to be expressed in the Pontine nuclei in this study was *Fz3* (Fig III. 11). *Fz3* was expressed in the Pontine nuclei, expression was detected at P6, P10 and in the adult. This finding was of great significance as it puts the spotlight on *Fz3* as a candidate receptor for Wnt7a during cerebellar development and maturation.

III. 2. 4. Summary Table

The gene expression patterns presented here are summarised in table III. 1. This table highlights the developmental time-points and the genes studied. Although many time-points and genes were not studied, this table indicates that in the adult, expression of *Wnt* and *Fz* is widespread. In particular, *Fz3* was the most ubiquitously found *Fz*. Previous reports of gene expression analysis by *in situ* hybridisation are also mentioned, and illustrate the other *Wnt* genes, Wn*t-3* for example, is also widely expressed in the adult brain (Salinas *et al.*, 1994).

Table III. 1. Summary of Wnt and Fz expression

Gene	**P6**	**P10**	**P15**	**P22**	**Adult**
Fz3	Cb, Ctx, (Hip)	Cb, Ctx, Hip, PN	Cb, Ctx, Hip, PN, OB	Cb, Ctx, Hip, PN, OB	(Cb), Ctx, Hip, PN, OB, Col, RTN
Fz4	N.D.	N.D.	N.D.	N.D.	N.D.
Fz6	N.D.	N.D.	N.D.	N.D.	N.D.
Fz7	Cb, (Hip CA only)	Cb, (Hip CA only)	Cb	Cb	(Hip CA3), Fim, PC, Cb
Wnt3					*Hip DG*[2], *Col*[2], *PN*[2], Cb PC, *ION*[2]
Wnt7a	Cb GC	Cb GC	Cb GC	Cb GC	Cb GC
Wnt7b	*Hip*[1]	*Hip*[1]	*Hip*[1]	*Hip*[1]	Hip, Ctx
Wnt11					N.D.

This table highlights the domains of expression of the *Wnt* and *Fz* genes tested in this study. It also mentions expressions patterns of *Wnt7b* and *Wnt3* published in previous *in situ* hybridisation studies.

Abbreviations used: Cerebellum : Cb, Cortex : Ctx, Hippocampus : Hip, Pontine Nuclei : PN, Olfactory bulb : OB, Reticular Thalamic Nucleus : RTN, Colliculum :Col, Hippocampal fimbria : Fim, Plexus Choroidus : PC, Granule Cell : GC, Purkinge Cell :GC, Inferior Olivary Nucleus : ION
N.D. : Not detected in this study
Parentheses indicate expression was detected at low levels in the present study.
Italic fonts indicate the data was generated by *in situ* hybridisation studies published before the present study.

References :

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2. Salinas, P. C., Fletcher, C., Copeland, N. G., Jenkins, N. A., and Nusse, R. (1994). Maintenance of Wnt-3 expression in Purkinje cells of the mouse cerebellum depends on interactions with granule cells. Development *120*, 1277-1286.

III. 3. Discussion

The expression pattern of *Wnts* and *Fzs* presented here suggest that Wnt signalling plays an important part in the post-natal brain. Even in the adult brain, some *Wnt* and *Fz* genes remain strongly expressed. What is the function of Wnt signalling in the adult brain? Although Wnt factors have to-date not been involved in events occurring in the adult brain, Wnt signalling affects various aspect of neuronal behaviour during development. Wnt signalling affects the neuronal cytoskeleton by affecting microtubules dynamics (Ciani *et al.*, 2004). More specifically, Wnt3 is known to stimulate the arborisation of a NT3-responsive spinal neurons (Krylova *et al.*, 2002). In the hippocampus, Wnt7b stimulates dendritogenesis (Rosso *et al.*, 2005). In the cerebellum, Wnt7a acts as a synaptogenic factor in the cerebellum, stimulating the formation of the pre-synaptic glomeruli of the mossy fibres (Hall *et al.*, 2000). The gene expression data presented here highlighted *Frizzled* receptors which may be implicated in these processes.

Neurons continue to be generated in the adult, and the physiology of neurons is modified during synaptic plasticity. Therefore events similar to developmental events also occur in the adult. Neurogenesis occurs in the adult hippocampus, where progenitor cells are found (Eriksson *et al.*, 1998). In consequence, axon path-finding, dendritogenesis and synaptogenesis must also occur in the adult. In addition, synaptic plasticity has points in common with developmental events. Both morphological changes of dendrites and changes in the neurotransmitter release properties of the pre-synaptic terminal occur in the context of plasticity (Magee and Johnston, 2005; Malenka and Bear, 2004). Since several *Wnt* and *Fz* genes are expressed in areas where these events occur, it is possible that Wnt signalling is involved in regulating these events. Yet, in order to be able to fully hypothesise on the potential role of specific Wnt and Fz in these processes, the gene expression patterns of all Wnt and Fz genes were investigated.

III. 3. 1. Wnt and Fz gene expression in the post-natal brain

At the time this study was initiated, only a handful of gene expression studies for *Wnt* and *Fz* in the adult mammals had been published (Sagara *et al.*, 1998; Wang *et al.*,

1996). Early studies of Fz gene expression in the adult we carried-out by northern blotting (Sagara *et al.*, 1998), RNA protection and *in situ* hybridisation (Wang *et al.*, 1996). Sagara and colleagues reported the cloning of human *Fz1*, *Fz2* and *Fz7* and studied the expression of these genes in adult tissues by Northern blotting. Although all three genes we detected in the fetal brain, *Fz7* was the only Fz gene to significantly expressed in the adult human brain. Wang and colleagues reported the cloning of mouse m*Fz3, mFz4, mFz5, mFz6, mFz7* and *mFz8*. RNase protection experiments showed the *mFz3* was nearly exclusively expressed in the brain and was the most highly expressed *Fz* gene found in the brain. *Fz7* and *Fz4* were also found in the adult mouse brain and were found ubiquitously in many different adult tissues (Wang *et al.*, 1996). *mFz2* and *mFz6* were found a low levels in the adult brain and *mFz5* was not detected there. *In situ* hybridisation in embryonic, post-natal day 1 and adult sections confirmed that Fz3 was strongly expressed throughout the CNS throughout development and in the adult, whilst expression of Fz4 in the adult is limited to the adult choroids plexus (Wang *et al.*, 1996). These data confirm our findings that *Fz3* and *Fz7* are the main Frizzled receptors expressed in the adult brain.

A detailed description of *Wnt* and *Fz* expression patterns in the post-natal cerebral cortex was published in 2004 (Shimogori *et al.*, 2004). This study revealed that a great number of components of the Wnt signalling pathway were expressed in the adult brain and throughout post-natal development. *Fz3* was confirmed to be the most highly and most ubiquitously Frizzled gene expressed in the post-natal brain. *Fz3* was found in the same areas as those presented in table III.1. In addition, the authors describe several structures within the olfactory areas expressing *Fz3* and *Fz7*. The authors report that in the adult cortex, *Fz1, Fz2, Fz8, Fz9, Fz10* were expressed at low levels and ubiquitously in the neocortex. In constrast, *Fz3* expression is higher and is detected in specific layers of the neocortex *(Shimogori et al., 2004)*. Interestingly, *Fz7* was found to be strongly expressed throughout hippocampal CA areas at P0 and subsequently decreases to barely detectable levels in the adult CA3 region, as reported here. Importantly, Shimogori and colleagues report a strong *Fz1* expression in the adult hippocampal DG region. *Fz2*, *Fz9* and to a lower extent Fz8, were also detected in the hippocampus (Shimogori *et al.*, 2004). *Wnt7b* was the only *Wnt* reported to be expressed in the CA3 region in the adult,

whilst no Wnt is detected in other CA regions. In contrast, the DG area expresses *Wnt5a, Wnt7a, Wnt7b* and *Wnt8*. It is striking that many *Wnt* and *Fz* genes are expressed in the hilus of the DG, an area where new granule neurons are generated (Eriksson *et al.*, 1998). The large number of *Wnt* and *Fz* gene expressed make it impossible to assign possible receptor-ligand interactions at this stage. Yet, these expression patterns suggest that Wnt signalling could play different roles in the adult. Indeed, it is possible that processes occurring in the adult are similar to developmental processes, and thus that Wnt may regulate neuronal differentiation, axon growth and formation of new synapses in the adult hippocampus. The study presented here highlighted that Fz3 and Wnt7b were highly expressed in the post-natal hippocampus, but clearly other *Wnt* and *Fz* also come into play in this area. Investigating the expression patterns of other components of the Wnt signal transduction machinery could provide insights into which signalling cascades may be activated in specific areas.

The LRP5/6 co-receptors and the TCF/lef family of transcription factors are critical for canonical Wnt and their expression is necessary for canonical signalling to take place (Pinson *et al.*, 2000; Riese *et al.*, 1997; Tamai *et al.*, 2000). Therefore, analysing the domains of expression of these genes can shed light on areas where canonical signalling can be activated. The Allen atlas has recently published expression patterns of *LRP5*, which is expressed in the hippocampus and cerebellar granule cells (www.brain-maps.org). This data completes the expression patterns presented here and highlight that LRP5 may be involved in Wnt signal transduction in these brain regions. It remain possible that *LRP6* has overlapping expression patterns but this remain so far unkown.

Shimogori and colleagues studied the expression of *TCF* transcription factors and found them to be expressed at low levels throughout the adult cerebellar cortex despite being well-detected at birth. Although the authors suggest that non-canonical signalling is likely to dominate in the adult, it remains highly likely that even expressed at low levels, TCF remain able to drive transcription of Wnt target genes. In fact, *TCF4* is expressed homogenously in the cortex as well as in the hippocampal DG. In addition, many *Wnt* and *Fz* are expressed in this region (Shimogori *et al.*, 2004). Therefore it is possible that canonical signalling is involved in the neurogenesis known to occur in the adult

hippocampal DG. Now that expression of *Wnt* and *Fz* has been described, can we suggest what may be the function for Wnt signalling in the post-natal brain?

III. 3. 2. Wnt and Fz gene function during post-natal development

By relating the expression patterns of *Wnt* and *Fz* with the established function of certain Wnt it is possible to speculate on the potential roles for Wnt signalling in the post-natal brain. Indeed, if a pair of interconnected neurons express a specific subset of *Wnt* and *Fz* genes, it is a possibility that these *Wnt* and *Fz* may function as a receptor-ligand pair. If neurons undergo Wnt-mediated changes, gene expression data of *Wnt* and *Fz* can shed light on the potential receptors mediating the process. Furthermore, since Wnt signalling is able to induce a number of different responses in neurons, we can also speculate on potential roles for Wnt signalling in the adult. What is the role of *Fz3*, the most strongly and specifically expressed receptor in the post-natal brain? Are *Fz* expressed in the same areas carrying-out the same redundant functions or do specific Fz receptors mediate specific responses? The gene expression patterns presented here provide a good starting point to investigate the role of Wnt signalling during the post-natal development of the brain, as well as associating particular *Wnts* and *Fz* receptors to specific neuronal processes.

III. 3. 2. 1. Wnt/Fz signalling of the hippocampus

Wnt factors play a crucial role in the development of the mammalian hippocampus. At the embryonic stage when the hippocampus is formed (E10.5- E12.5 in the mouse), a number of Wnt factors are expressed in the surrounding cortical hem (Lee *et al.*, 2000). In particular, the *Wnt3a* mutant shows a near complete absence of hippocampal formation. Although some hippocampal markers eventually appear around E18.5, there is a dramatic decrease in progenitor cell proliferation (Lee *et al.*, 2000). Wnt signalling is also important during post-natal development. Dendrites of hippocampal pyramidal neurons require dishevelled, a key mediator of Wnt signalling, to form normal dendritic arbors (Rosso *et al.*, 2005). *In vitro*, hippocampal neurons respond to *Wnt7b* which stimulates the growth and the branching of their dendrites. This dendritogenic activity of

Wnt7b is mediated via a non-canonical signalling pathway that involves the small GTPases Rac and JNK, but the receptors mediating Wnt7b signalling are unknown. How can the expression patterns presented here shed new light on the receptors mediating the effect of Wnt7b?

Both the timing and the location of Fz expression in the hippocampus highlight potential receptors mediating Wnt7b dendritogenic effect. *Wnt7b* is the main Wnt factor to be expressed post-natally by hippocampal pyramidal cells which localise in the CA regions, with Wnt5a being the other Wnt faintly expressed there (Shimogori *et al.*, 2004). Also, in the adult *Wnt7b* was strongly expressed in the DG where mature granule cells are localised. Wnt7b was shown *in vitro* to affect pyramidal cell, but its effect on hippocampal granule cell has not been investigated (Rosso *et al.*, 2005). Therefore cells of the CA regions must be equipped with the receptor machinery enabling them to activate the non-canonical pathway. Dendrites of pyramidal cells develop at early post-natal stages until adulthood (Pokorny and Yamamoto, 1981). The strong expression of *Fz3* in CA areas throughout post-natal development suggests it could act as a receptor for Wnt7b. This effect could be autocrine, but it is also possible that signalling between pyramidal cells of CA3 and CA1 regions or signalling between the granule cells of the DG and CA3 pyramidal neurons occurs. Nonetheless, the CA areas also express low levels of *Fz8* and *Fz7* post-natally, and *Fz7* was reported to be strongly expressed in all CA regions at birth (Shimogori *et al.*, 2004). Therefore, although *Fz3* appears a prevalent Wnt receptor in hippocampal pyramidal cells, *Fz7* and *Fz8* could potentially function as receptors for Wnt7b. What is the directionality of Wnt7b signalling in the hippocampus?

The expression patterns of *Wnt7b* suggest it could stimulate dendritic development in an anterograde manner. Indeed Wnt7b was shown to stimumate dendritic outgrowth and branching of hippocampla pyramidal neurons (Rosso *et al.*, 2005). *Wnt7b* expressed by granule cells of the DG could affect the dendrite of the pyramidal cells from the CA3 region. At earlier stages of post-natal development, Wnt7b expression in the CA3 region could stimulate arborisation of the target neurons of the CA1 region. Alternatively, it is possible that axons release Wnt7b to stimulate the growth and branching of dendrites of the same neuron in an autocrine loop. The sustained expression of *Fz3* throughout the hippocampus in the adult remains nonetheless elusive. Indeed, *Fz3*

could be implicated in other processes, for example by affecting dendritic modelling in the context of synaptic plasticity (Magee and Johnston, 2005).

In contrast to the CA area, many *Wnt* and *Fz* are expressed in the hippocampal DG? What is the function of Wnt signalling in hippocampal granule cells? The Dentate Gyrus can be subdivided in two areas: The hilus where neurogenesis was found to occur in several species (Eriksson *et al.*, 1998; Kuhn *et al.*, 1996) and the blades, where mature granule cells are found. Shimogori and colleague reported that three Wnt were expressed in the hilus (Wnt5a, Wnt7a and Wnt7b) whilst only Wnt7b is expressed in the blades in the young adult (Shimogori *et al.*, 2004). Another report also described that Wnt3 is expressed in the adult DG (Salinas and Nusse, 1992). Therefore it appears that these four Wnt (*Wnt3, Wnt5a, Wnt7a, Wnt7b*) and the Fz receptors expressed in the hilus (*Fz1, Fz2, Fz9*) could be implicated in neurogenesis known to occur in the adult hippocampus (Eriksson *et al.*, 1998; Ming and Song, 2005).

Wnt signals mediate a wide variety of processes during development, and thus could be implicated in different events occurring in the adult hippocampus. Wnt have been shown to have different effects on embryonic stem cells. Canonical Wnt signalling has been shown to maintain the pluripotency of ESC, but in another study Wnt3a acts in combination with BMP and SHH to trigger their differentiation (Murashov *et al.*, 2005; Sato *et al.*, 2004). These findings can be linked to the neurogenic events occurring in the hippocampal DG. It is thus possible that Wnt could regulate the proliferation or the fate of neuronal precursors in the adult hippocampus. In fact, *Wnt3* was shown to be expressed in the neurogenic "niche" of the hippocampus and Wnt signalling to be critical for the stimulation of neurogenesis (Lie *et al.*, 2005). Wnt are also implicated in patterning the developing nervous system and in many cell fate decisions throughout development (Logan and Nusse, 2004) therefore it also possible that Wnt assist the specification of the fate of differentiating neurons in the adult hippocampus. Finally, since Wnt/Fz signalling has also been implicated in neuronal outgrowth, guidance and branching, it is possible that *Wnt7b* and *Fz3* contribute to the development of newly generated granule cell axons. Although Wnt7b expression in the CA3 region was very weak, it cannot be excluded that Wnt7b expressed by pyramidal cells affects newly generated granule cell neurons in a retrograde signalling event. Most likely, a

combination of Wnt signals affect several individual steps in either maintaining the proliferation of neuronal precursors in the DG hilus and/or contributing to their differentiation into specific granule cell sub-types.

Fz7 and *Fz3* appear to be the most highly expressed *Fz* in the adult brain. Yet, they are expressed in distinct patterns, thus suggesting that these two receptors are likely to function in separate processes. For example, *Fz7* was found in the lining of the choroid plexus and thus could be implicated in regulating the production of cerebrosplenial fluid (Brown *et al.*, 2004). *Fz3* was detected in a laminar fashion in the neocortex and therefore may be required in processes specific to certain neuronal subclasses. Interestingly, *Fz3* was also expressed in the thalamic reticular nucleus and could therefore be implicated in modulating the thalamo-cortical pathway. Finally, the fact that *Fz3* and *Wnt7a* were expressed in the olfactory areas, where synaptic plasticity is on-going in the adult, once again highlights that Wnt signalling could be implicated in this process. Also, the fact that *Wnt7a* and *Fz3* are expressed in similar areas suggests that the two genes could function as a receptor/ligand pairs. Can Wnt7a bind Fz3 and activate signalling in cells expressing *Fz3?* This will be the focus of the next two chapters. Nonetheless, expression patterns in the cerebellar system can also reveal further clues regarding potential receptor-ligand pairs.

III. 3. 2. 2. Wnt signalling in the cerebellum

A number of signalling molecules regulate the formation of synapses. Secreted and cell-surface molecules are encountered by outgrowing axons in order to guide them towards their targets and form functional connections at the right place. Yet, only a subset of axons will stop growing and begin forming a synapse, whilst other axons can continue extending until they reach their target. The receptors expressed by outgrowing neurons, in particular those expressed at growth cones, are central to a cell's response to environmental cues. For example, *Wnt7a* is known to regulate the development of pre-synaptic terminals of the mossy fibres reaching the IGL of the cerebellum (Hall *et al.*, 2000). Incoming mossy fibre axons respond to granule cell-derived *Wnt7a*, but the specific receptors expressed by mossy fibres remained unknown to this date. *Fz3* was the only Frizzled receptor detected in the Pontine nuclei in this study and therefore

constitutes a likely receptor for Wnt7a. In addition, other *Wnt* and *Fz* genes are expressed by the cerebellum, such as *Fz7* and *Fz3* in granule cells, but their function remain unclear.

Establishing which specific receptors mediate the synaptogenic effects of Wnt7a would be a great step forward understanding how Wnt signals affect synaptic assembly. If the *in vitro* effects of Wnt7a conditioned medium could be mimicked by over-expression of certain *Fz* receptors, a whole range of experiments could be carried-out in order to clarify the molecular requirements for synaptogenic Wnt signalling. For example, studying the behaviour of a cell doubly-transfected with a Fz and a dominant-negative mutants of certain Wnt signalling pathway components could establish the molecular requirements for synaptogenic signalling. Therefore the discovery that *Fz3* was expressed in the Pontine nuclei, where the cell bodies of the mossy fibres are located, is a major step forward (Fig. III. 11.) It is most interesting that expression was detected at times of synaptogenesis in the cerebellum, from P6 to P15, but also in the adult. It is therefore possible that Wnt signalling continues to affect the function of the mature synapse. Yet, our study has not addressed the expression of all 10 mammalian Frizzled and no other studies reveal whether other Fz genes were expressed in the hindbrain. Therefore, although other receptors could be involved in the process, it is likely that Fz3 contributes to transducing Wnt7a synaptogenic signalling.

Although the best established role of Wnt signalling in cerebellar development is Wnt7a's role in the formation of the mossy fibres' glomerular rosette, it is likely that Wnt signalling also regulates other processes. Indeed, several *Fz* and *Wnt* are expressed by cerebellar cells. *Wnt7a*, *Fz7* and *Fz3* were all expressed by cerebellar granule cells during post-natal development. In fact, *Wnt7a*, *Fz7* and *Fz3* are already expressed by dividing granule cells in the EGL at P6 (Figs. III.13 and 14), and therefore could be involved in cell proliferation or differentiation at this stage. The fact cell cycle regulators such as cyclins are direct transcriptional targets of canonical Wnt signalling make this hypothesis likely.

At P15, granule cells have migrated to their final location; *Wnt7a*, *Fz7* and *Fz3* remain expressed but expression of Fz7 was higher than Wnt7a. Since granule cells are known to respond *in vitro* to Wnt7a, it is tempting to speculate that Wnt7a produced by granule cells may affect neighbouring granule cells and thus have autocrine effects in

addition to its paracrine effect on incoming mossy fibres. Perhaps Wnt7a also stimulates aspects of granule cell maturation such a axon extension or dendritic development. In this context, it is possible that both Fz3 and Fz7 participate in transducing Wnt7a signals.

Finally, the fact that expression of Fz3 decreases in the adult whilst Wnt7a and Fz7 remain highly expressed by granule cells indicates that Fz7 and Wnt7a could also function as a receptor-ligand pair at later stages, perhaps in the context of signalling between granule cells. It is possible Wnt signalling contributes to the plasticity within the cerebellar circuitry, for example by regulating the synaptic activity of granule cell axons in the molecular layer. Taken together, the gene expression patterns presented here highlight that both Fz3 and Fz7 could function as Wnt7a receptors, and that Wnt signalling may play a variety of functions in the post-natal cerebellum.

Cerebellar Purkinge cells express *Wnt3* (Salinas *et al.*, 1994) but its role in cerebellar development and function has not yet been examined. Cerebellar Purkinge cells have complex dendritic arbors in the molecular layer where they encounter a dense network of parallel fibres, the granule cell axons. It is therefore possible that the Wnt expressed by Purkinge cells contribute to the development and maturation of granule cell axons. In fact, Wnt3 is known to induce the terminal arborisation of axons of NT3-sensitive sensory neurons in the spinal cord (Krylova *et al.*, 2002). Parallel fibres do not branch-out like sensory neurons entering the spinal cord, but in contrast form *en passant* synapses with the Purkinge cell dendrites. It is possible retrograde Purkinge cell-derived Wnt3 signalling may contribute to the formation of the granule cell – Purkinge cell synapse. In this context, Fz3 and Fz7 could act as receptors for Wnt3. Many of the Wnt and Fz genes mentioned above were also expressed in the adult. Although little experimental evidence has been brought forward so far, it is tempting to speculate on the role of Wnt signalling in the adult.

III. 3. 3 Wnt/Fz signalling in the adult

Many synaptic events occurring at mature synapses are reminiscent of events leading to synapse formation. Indeed, the nervous system is in a constant state of adaptation and of learning. Therefore neural circuits must exhibit a form of flexibility, or plasticity, which enables them to modify the signals transmitted in response to previous

experience. Most of this plasticity occurs at the levels of synapses, which may either increase or decrease the strength of specific connections. Synaptic plasticity may occur at the levels of both the pre- and the post-synaptic terminals. On one hand, the amount of neurostransmitter release may be altered when the properties of pre-synaptic terminals are altered (Malenka and Bear, 2004). On the other hand, the intensity or the length of the response in the post-synaptic cells can also be modified. Many events occurring in the context of synaptic plasticity are still poorly understood, yet many share points in common with developmental events.

The changes occurring on the context of synaptic plasticity are often compared to developmental events leading to synapse formation. Indeed, both the neurotransmitter release properties and the morphology of the post-synaptic contact, the dendritic spine, may change in certain conditions (Magee and Johnston, 2005; Malenka and Bear, 2004). Since developing neurons are also required to acquire the capacity to release neurotransmitters in sufficient quantities and with precise timing, pre-synaptic plasticity may share points in common with the generation of functional pre-synaptic terminals. In fact, the latest publication from our laboratory supports the idea that Wnt signalling affect neurotransmitter release (Ahmad-Annuar *et al.*, 2006). Indeed, Wnt deficiency leads to a decrease in miniature excitatory postsynaptic currents (mEPSC), indicating a defect in neurotransmitter release. Therefore, Wnt have the potential to modulate pre-synaptic function and thus affect synaptic plasticity (Ahmad-Annuar *et al.*, 2006).

Similarly, changes occurring in post-synaptic dendrites, which often require neurotransmitter receptor accumulation or changes in dendritic spine morphology, can also be compared to a developmental event. For example, the cytoskeletal remodelling of axonal growth cones encountering their targets may share points in common with the remodelling and growth of dendritic spines thought to occur during memory storage (Bonhoeffer and Yuste, 2002). Although at this time, LTP and Long Term Depression are thought to be triggered by the intensity, the patterns and the timing of incoming stimuli, it is thought that developmental cues could play a role in synaptic plasticity. In fact, BDNF is emerging as the first developmental cue to play in role in plasticity. BDNF is implicated in several aspects of synaptic plasticity, in particular protein synthesis-dependent "late-phase" LTP (Bramham and Messaoudi, 2005). Therefore, it is possible

that the *Wnt* and *Fz* genes reported to be expressed in the adult brain could function as cues regulating the plasticity of both pre- and post-synaptic terminals.

III. 4. Conclusion

The gene expression data presented here highlighted the fact many *Wnt* and *Fz* are expressed post-natally. In particular, high levels of *Fz3* are found in many brain regions during development and in the adult. *Fz3* was found to be expressed in the Pontine nuclei, the source of mossy fibres and in the hippocamal CA regions, where pyramidal cells are located. Mossy fibres and Pyramidal cells respond to Wnt7a and Wnt7b, respectively, in processes mediated by distinct signalling pathways. Further experiments should therefore investigate whether Fz3 is able to bind and transduce Wnt7a and Wnt7b signals. Similarly, Fz7 is highly expressed by cerebellar granule cells and a role for Fz7 in Wnt7a signal transduction is also possible. In addition, the data presented here highlighted new regions where Wnt signalling could be important. Indeed, Wnt signalling may regulate the function of the cerebellum, the hippocampus, the neocortex and the olfactory bulb. In the following chapters of this thesis, biochemical and cell biology approaches were taken to investigate the signalling capacity of Wnt/Fz pairs suggested by the expression patterns presented here.

Chapter IV

Wnt/Fz Binding

IV. Wnt/Frizzled binding

IV. 1. Introduction

Throughout development Wnt signalling controls a variety of cellular events such as cell fate determination, cell migration and the formation of neuronal connections. In developing neurons, different Wnt factors have been shown to be involved in axonal path-finding, branching and remodelling as well as dendritic development and synaptogenesis (for review see (Ciani and Salinas, 2005)). In some instances, physical and genetic interactions between a Wnt and its receptor have been elucidated. This is the case for the role of Wnt signalling in axon guidance. At the *Drosophila* midline, derailed expressing neurons are guided to the anterior commissure away from dWnt5 expressing neurons of the posterior commissure (Yoshikawa *et al.*, 2003). In the mammalian spinal cord, Wnt4 and Fz3 function to guide commisural axons along the anterior-posterior axis of the spinal cord (Lyuksyutova *et al.*, 2003). Yet, in other circumstances such as dendritogenesis and synapse formation, the receptors transducing Wnt signals remain to be elucidated.

The seven-transmembrane Fz proteins were the first Wnt receptors to be identified. Until recently, this family of proteins remained the primary receptors for the Wnts (Logan and Nusse, 2004). However, a recent study suggests that in some instances Wnt may signal independently of Frizzled, using Ryk as a receptor (He, 2004). Frizzled receptors display two characteristics that demonstrate their importance in Wnt signalling: Frizzled molecules are able to bind Wnts, and can confer Wnt-responsiveness to transfected cells (Bhanot *et al.*, 1996). The CRD of Fz are responsible for binding to Wnts (Bhanot *et al.*, 1996; Hsieh *et al.*, 1999). Wnt binding to the surface of cells expressing FzCRD domains tethered to the cell surface by a GPI-anchor (GPI-FzCRD) is a common assay to study Wnt/Frizzled interactions (Bhanot *et al.*, 1996; Dann *et al.*, 2001; Hsieh *et al.*, 1999), and will be the main method used in this chapter. Yet, other regions of frizzled receptors, in particular the trans-membrane domains (Chen *et al.*, 2004) and co-receptor molecules such a LRP5/6 can also be involved in Wnt binding (Chen *et al.*, 2004; Tamai *et al.*, 2000). Therefore, although we can analyse the Wnt-binding

capacity of the CRD domains *in vitro*, it must be taken into consideration that binding *in vivo* may be more complex.

During post-natal development of the cerebellum, incoming mossy fibre axons develop large multi-synaptic structures as they approach their targets, the granule cells, in the granular layer. Wnt7a secreted by Granule Cells (GC) is responsible for these changes in axonal morphology (Hall *et al.*, 2000). Interestingly, when applied to GC cultures, Wnt7a has similar effects, thus showing Wnt7a can potentially act in both paracrine and autocrine fashion (Lucas and Salinas, 1997). In this paper, the specificity of Wnt7a synaptogenic effect on GC is highlighted: Neither Wnt1 nor the *Drosophila* Wg were able to mimic the effect of Wnt7a on GC, and Wnt7a was unable to trigger changes is cultured Dorsal Root Ganglia (DRG) neurons (Lucas and Salinas, 1997). A possible explanation for this specificity is that only MF and GC express the appropriate receptors to respond to Wnt7a. Although it is well established that these synaptogenic events occur through the inhibition of GSK-3ß, the receptor(s) triggering these responses remain unknown. As *Fz3* and *Fz7* were found to be expressed by the granule cells of the cerebellum, and *Fz3* by the Pontine nuclei (the source of MF), these frizzled proteins could potentially function as receptors that mediate Wnt7a effects. In order to verify these hypotheses, the binding capacity of candidate Wnt/Fz pairs must be studied.

Wnt7b is another factor that can trigger neurite branching, but it does so in dendrites rather than in axons, and utilises a non-canonical pathway involving Dvl, rac and JNK kinases (Rosso *et al.*, 2005). Hippocampal neurons responding to Wnt7b not only have more complex dendritic arbors, but also have longer dendrites. Wnt7b and Wnt3 therefore seem to stimulate neurite branching in very different ways. Nevertheless, the presence of other trophic factors could also explain why the outgrowth of neurons is affected differently in different systems. Because *Fz3* and *Wnt7b* are both strongly expressed in the hippocampus, Fz3 could act as a possible receptor for Wnt7b. The first step in order to verify this hypothesis was to investigate whether these two molecules could physically interact.

A fusion construct consisting of the extra-cellular domain of Frizzled was used to study Wnt/Fz binding. Indeed, binding of a ligand at the cell surface can sometimes be weak and difficult to detect using cell biology techniques. For this reason, a fusion

construct expressing the ligand-binding domain of Frizzled receptors, the CRD domain, at the cell surface was used to capture Wnt ligands (Hsieh *et al.*, 1999; Lyons *et al.*, 2004). The construct over-expressed at the cell surface consisted of the extra-cellular Cystein-Rich Domain (CRD) of Frizzled receptors fused to a myc epitope tag and a GlycoPhosphatidylInostiol (GPI) membrane anchor. These GPI-myc-FzCRD constructs enabled the accumulation of vast amounts of the ligand-binding region of specific Fz receptors at the cell surface, thereby facilitating the detection of the bound ligands (Hsieh *et al.*, 1999; Xu *et al.*, 2004). Also, these GPI-myc-FzCRD constructs are stable and easily targeted to the cell surface, and this may not always be the case with full length receptors. Finally, because they are functionally inert, binding of a Wnt-ligand to cells expressing GPI-FzCRD construct does not affect their internalization and thus makes it possible to detect Wnt-ligands bound at the cell surface. In contrast, binding of Wnt-ligand to full-length receptors triggers their internalization and would thereby decreases the sensitivity of the assay (Chen *et al.*, 2003). The data presented here were thus limited to the study of the interaction of the CRD domain of Fz with Wnt7a and Wnt7b.

IV. 2. Results

In order to assess whether the Fz and Wnt found to be expressed in the cerebellum and hippocampus could physically interact, a cell-surface binding assay was utilised. As Wnt7a and Wnt7b have not been purified, the optimisation of Wnt7a- and Wnt7b-conditioned media production procedure is mentioned. Secondly, we describe the cell-surface binding assays for Wnt7b and Wnt7a. These assays consisted of expressing the FzCRD domain at the surface of 293 cell lines and test their ability to retain Wnt ligands at the cell surface by immunostaining. In certain cases, co-immunoprecipitation of Wnt and FzCRD data are also presented.

IV. 2. 1. Wnt Conditioned Media production

Obtaining sufficient quantities of soluble, biologically active Wnt factors is one of the major hurdles that one needs to over-come in order to study the structural and

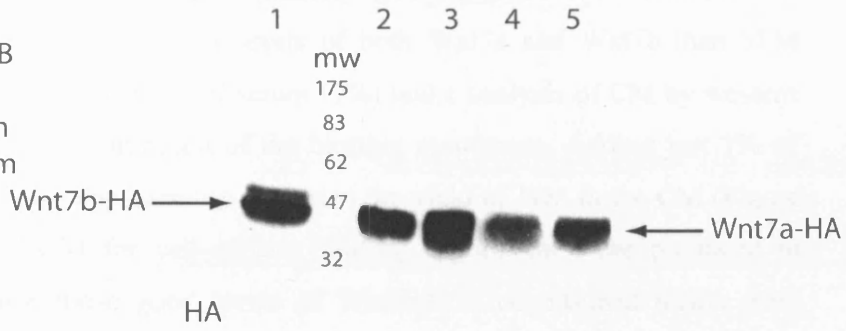
biochemical properties of Wnt factors. Indeed, Wnt are relatively insoluble, probably due to their palmitoylation and are notoriously labile (Bradley and Brown, 1990; Willert *et al.*, 2003). Although the Nusse laboratory has developed a purification protocol for Wnts that led to some Wnt proteins such as Wnt3a to become commercially available in their purified form (Willert *et al.*, 2003), most Wnt factors need to be produced in conditioned media of Wnt-transfected cells. Wnt7a and Wnt7b have not been purified. Thus they need to be produced in a conditioned medium (CM) by transfected cells for every experiment done. Therefore, optimising the protocol to produce sufficient amounts of soluble Wnt7a and Wnt7b for binding assay was an essential pre-requisite to this study.

Epitope-tagged Wnt7a and Wnt7b were produced in CM from different cell lines. Levels of Wnt in CM were assessed by western blotting (Figure IV. 1) for each cell-surface binding assay done. Wnt7b is easily produced by a Rat1B cell line stably transfected with the *Wnt7b-HA* construct (see materials and methods). On the other hand, the *Wnt7a-HA* construct needed to be transfected into QT6 cells for each experiment and therefore the yield of Wnt7a-HA CM production may be variable (Figure IV. 1). Panel A of figure IV. 1 is an example of how levels of Wnt7a-HA present in the CM varied between different experiments even though the same protocol was followed. Lanes 2, 3, 4, and 5 illustrate how Wnt7a transfected cells produce variable levels of Wnt7a CM despite using an identical protocol. In contrast, stably-transfected Rat 1B cells produce consistently high levels of Wnt7b CM (lane 1). Cell confluence and more importantly transfection efficiency are the main factors responsible for the variations in Wnt7a-HA CM production. Therefore, although a protocol can guarantee a certain amount of Wnt-CM produced, variation between experiments is unavoidable. Only binding assays where high levels of Wnt7a-HA were detected in the CM were taken into account. In addition, cell-surface binding experiments were repeated at least three times before drawing conclusions. Production of Wnt5a and Wnt1 CM from Rat 1B cells was also attempted but their presence was never detected in western blots (data not shown). Our attention was therefore focused on Wnt7a and Wnt7b, which are the two most highly expressed *Wnt* genes in the postnatal brain.

Considerable efforts were directed to improve the production of Wnt-conditioned media. The levels of Wnt production depend mainly on transfection efficiency and the

A. Wnt levels in conditioned media vary from one experiment to the next

Lanes:
1: Wnt7b CM from Rat1B cells
2, 3, 4, 5: Wnt7a CM from QT6 cells, produced from different transfections.



B. Wnt secretion is affected by the medium used for conditionning

1: Control SFM
2: Wnt7b SFM
3: Wnt7b N2+1%FCS
4: Wnt7a SFM
5: Wnt7a N2
6: Wnt7a N2+1%FCS
7: Control N2+1%FCS

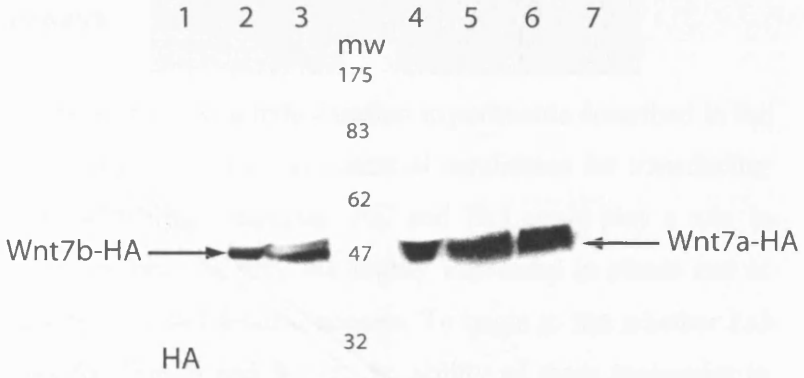


Figure IV. 1. Production of Wnt7a and Wnt7b conditioned media

Western blot analysis of Wnt7a-HA and Wnt7b-HA content in condition media. Wnt7b was produced from a Rat1B cell line stably transfected with *Wnt7b-HA*. In contrast, Wnt7a-HA was transfected 2 days before collecting the medium. Conditioning occured over-night.

A. Comparison of Wnt7a/b-HA levels in conditioned media produced in the same conditions. The amount of Wnt7a-HA present in the conditioned medium varied from one experiment to the next, mainly because of variations in cell density and transfection efficiency (compare lanes 2-5). Wnt7b produced from Rat1B cells stably transfected with Wnt7b produced more consistent levels of Wnt7b in the conditioned medium (lane 1).
B. Comparison of Wnt7a/b levels in condioned media produced in different conditions. The secretion of Wnt factors is improved when N2 medium is used instead of SFM (compare lanes 4 and 5). Supplementing this medium with 1% Fetal Calf Serum (FCS) further improves Wnt production (lanes 3 and 6). All samples were obtained from the same experiment.
Molecular Weight (mw) indicators are in kDa.
All blots were probed with anti-HA antibody.

medium that is applied to the cells for conditioning (Figure IV. 1, panel B). For conditioning, two serum-free media used from primary neuronal culture were used: "Serum Free Medium" (SFM) and "N2" medium (see materials and methods). N2 medium gave rise to significantly higher levels of both Wnt7a and Wnt7b than SFM (Figure IV. 1. B). Although high levels of serum (5%) make analysis of CM by western blot impossible due to protein saturation of the blotting membrane. Adding just 1% of serum to the N2 medium also considerably increased the yield of Wnt in the CM (Figure IV. 1. B). Consequently, CM for cell-surface binding experiment were produced in N2+1% serum. Only once these good levels of Wnt7a/b in conditioned media were obtained were cell-surface binding assay possible

IV. 2. 3. Wnt7b Binding assays

The gene expression pattern data of the *in situ* hybridisation experiments described in the previous chapter highlighted some Fz receptors as potential candidates for transducing Wnt signals in the cerebellum and the hippocampus. *Fz7* and *Fz3* could play a role in synaptogenesis and dendritogenesis because they are highly expressed in places and at times where Wnt affect synaptogenesis and dendritogenesis. To begin to test whether Fz3 and Fz7 could act as receptors for Wnt7b and Wn7a, the ability of these molecules to physically interact was investigated.

The original protocol that used Wnt-AP fusion proteins was modified into a staining protocol using HRP-conjugated secondary antibodies to detect membrane bound Wnt-HA to cells expressing Fz-CRD at their surface.[293] Cells expressing GPI-myc-mFz3CRD were exposed to control or Wnt7b-HA conditioned medium. Only cells exposed to Wnt7b CM display a strong HRP staining (arrows in IV. 2. A. 4). In contrast, cells expressing higher levels of the same construct but that were exposed to the control conditioned medium display very little background HRP staining (arrowheads in IV. 2. A. 2). Binding of Wnt7b to the CRD domain of mFz3 is specific because cells that were transfected with GPI-myc-mFz7CRD did not retain Wnt7b-HA at their surface (panel B), although both GPI-FzCRD proteins were expressed a similarly high levels on the cell surface. This experiment revealed that this staining protocol could be used to obtain

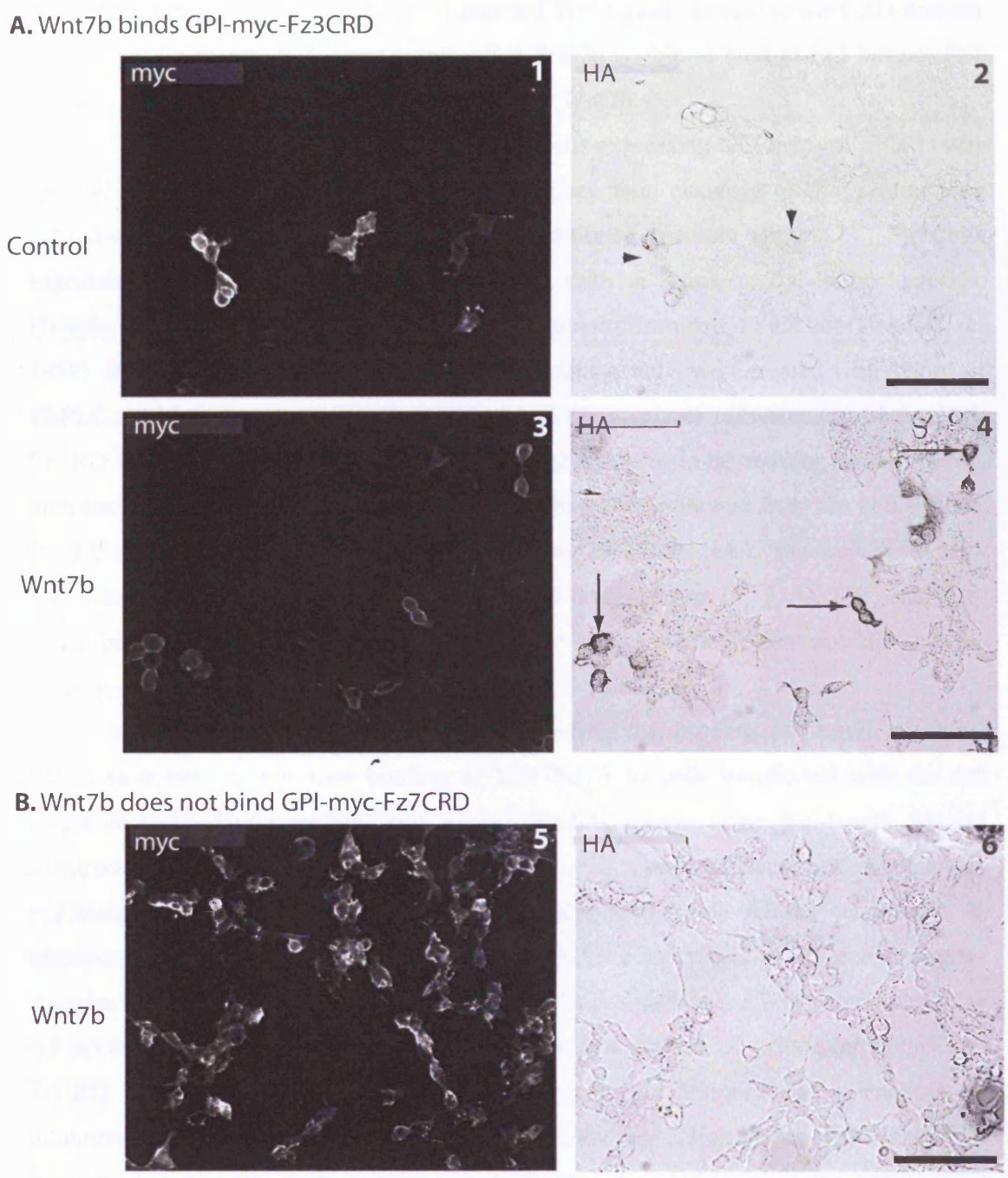


Figure IV. 2. Wnt7b binds to the surface of cells expressing Fz3CRD

Cell surface binding assay where Wnt7b CM or control CM was applied to cells expressing GPI-myc-FzCRD. The receptors were detected by immunofluorescence directed against the myc epitope (panels 1, 3, 5). The Wnt7b-HA binding the receptors were detected through an HRP reaction directed against the HA epitope which labelled positive cells with a dark precipitate (panels 2, 4, 6).

A: Wnt7b-HA applied to cells expressing GPI-myc-Fz3CRD (1-4) was retained at the cell surface of transfected cells (arrows in panel 1). In contrast, even cells expressing high levels of GPI-myc-Fz3 did not retain Wnt7b-HA (arrowheads in panel 2).

B: Wnt7b-HA was not retained at the surface of cells expressing GPI-myc-Fz7CRD (5-6) in the same experiment.

Scale bars are 50 µm.

qualitative data regarding the ability of secreted Wnt ligands to bind to the CRD domain of different Fz receptors. It demonstrates that Wnt7b is able to bind to Fz3 but not Fz7 and thus suggests that Fz3 could be a receptor for Wnt7b.

To ensure that the binding of Wnt7b to cells expressing GPI-myc-mFz3CRD was specific to the CRD domain of the construct, enzymatic cleavage of the protein was carried-out before adding the Wnt7b-HA conditioned medium (figure IV. 3). Cells expressing GPI-myc-mFz3CRD were treated with a Phosphatidyl-Inositol-specific PhosphoLipase C (PI-PLC) to release the CRD domain from its GPI anchor (Hsieh *et al.*, 1999). Before adding Wnt7b-HA conditioned medium, cells were treated with 2U/ml of PI-PLC. PI-PLC treatment considerably reduced the levels of myc staining of the GPI-FzCRD fusion protein (figure IV. 3. 3). Although some residual staining remained, this indicated that the majority of the myc-Fz3CRD had been removed from the cell surface. PI-PLC treatment completely abolished the binding of Wnt7b-HA to the transfected cells and reduced the HA-HRP staining to background levels (figure IV. 3. 4). The sensitivity of the binding to enzymatic cleavage of the CRD of the construct demonstrates that the binding of Wnt7b at the cell surface is specific to Fz3CRD.

Another important experiment demonstrating the binding of Wnt7b to mFz3 would have been to visualise binding of Wnt7b-HA to cells transfected with the full length receptor. Unfortunately, cell surface binding assays using full-length frizzled constructs were unsuccessful. The full-length Fz3 construct was not successfully expressed in cell lines and therefore these experiments could not be carried-out. In addition, binding of Wnts to Frizzled can trigger the endocytosis of the receptor/ligand complex (Chen *et al.*, 2003; Dubois *et al.*, 2001) and therefore the Wnt/Fz complex may not accumulate as much at the Wnt binding the cell surface of cells expressing GPI-FzCRD. Although interfering with endocytosis with low temperatures or endocytosis inhibitors could potentially enable these experiments, expression levels of full-length Fz were too low to carry these assays. This highlights the usefulness of the GPI-FzCRD constructs, which are stably expressed at high levels at the cell surface.

The ability of Wnt7b to bind the CRD domains of hFz5 was also studied (figure IV. 4). Cells expressing GPI-myc-hFz5CRD consistently retained Wnt7b-HA at their surface (figure IV. 4. A). In contrast, Wnt7b-HA was not detected at the surface of cells

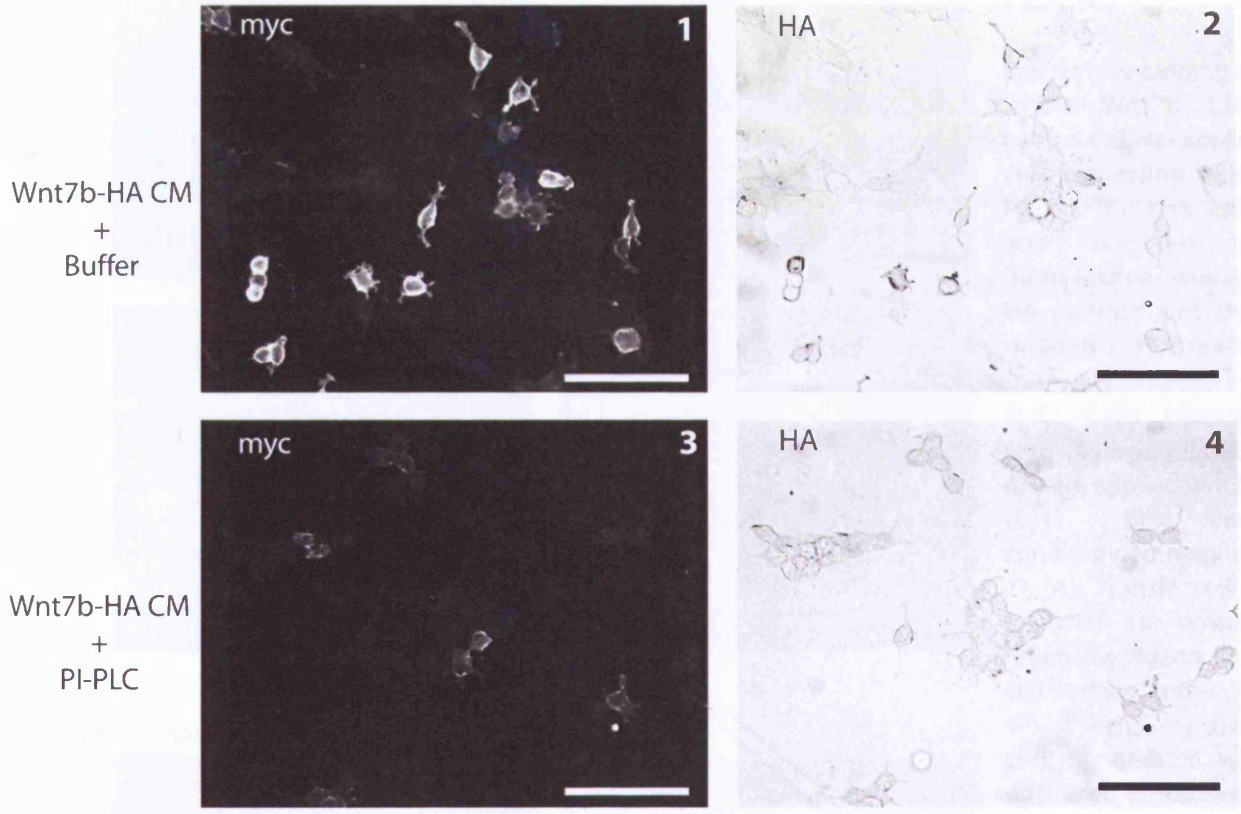


Figure IV. 3. PI-PLC treatment of GPI-myc-Fz3CRD expressing cells abolishes Wnt7b binding

Cells transfected with GPI-myc-Fz3CRD were treated with 2U/ml of PI-PLC (3-4) or a control solution (1-2) for 40 min before being applied Wnt7b conditioned medium and immunostained.

PI-PLC treatment significantly reduced the myc signal (3) and completely abolished the binding of Wnt7b-HA to the surface of transfected cells (4). This experiment illustrates that Wnt7b-HA binds specifically to the CRD domain of the GPI-myc-Fz3CRD construct. Scale bars are 50 µm.

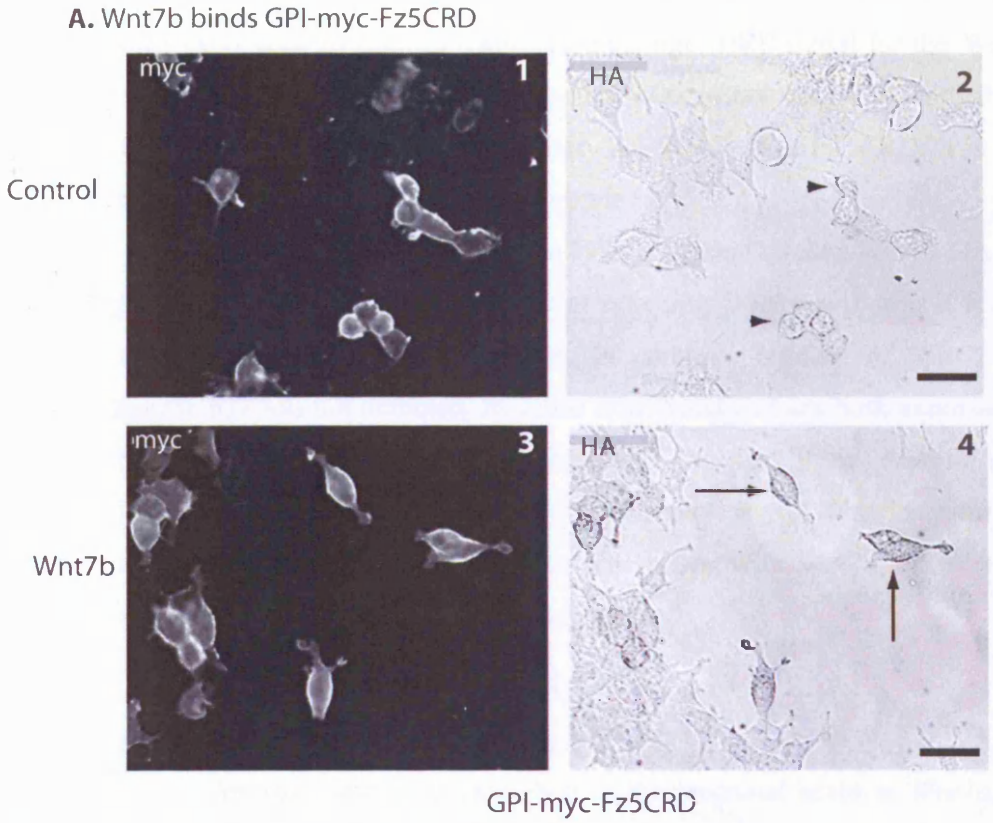


Figure IV. 4. Wnt7b binds Fz5CRD

Cell surface binding assay where Wnt7b CM or control CM was applied to cells expressing GPI-myc-FzCRD. The myc epitope was detected using fluorescence whilst the HA epitope was dected through an HRP reaction.

A-C: Cells transfected with GPI-myc-Fz5CRD (1-4) were applied Wnt7b-Ha (3-4) or control conditionned medium (1-2) (A). Transfected cells exposed to Wnt7b-HA retain the ligand at their cell surface (arrows in 4) whilst cells exposed to control medium remain unstained (arrowheads in 2). In contrast, the same Wnt7b CM did not bind GPI-myc-fz8CRD (panel 6) expressing cells and thus acted as a negative control (B). Wnt7b-HA was also retained at the surface of cells expressing GPI-myc-Fz3CRD (panel 8), but these cells appeared to have a lighter HRP staining, hinting the fact Wnt7b may be more efficiently bound by Fz5CRD than Fz3CRD.
Scale bars are 50µm.

expressing GPI-myc-hFz8CRD (Figure IV. 4. B). When the same Wnt7b-HA CM was applied to cells transfected with either GPI-myc-hFz5CRD or GPI-myc-mFz3CRD, the cells expressing hFz5CRD displayed a stronger HRP signal for the Wnt7b-HA (compare panels A and C figure IV. 4). Although we can not conclude from this experiment that Fz5CRD has a greater binding affinity for Wnt7b than Fz3CRD, it is still noteworthy to remark the difference in the signal obtained.

In summary, two of the three Frizzled tested bind to Wnt7b-HA at the cell surface. hFz5CRD appeared most efficient at retaining Wnt7b and mFz3CRD also displayed a strong interaction with the ligand. In contrast, binding of Wnt7b to mFz7CRD or mFz8CRD was not detected. Because *Wnt7b* and *Fz3* are both expressed in the post-natal hippocampus, these interactions highlight the fact these receptor/ligand pairs could interact functionally *in vivo*. In the next section, we describe similar experiments to assess the possible frizzled receptors interacting with Wnt7a, the Wnt gene expressed at strong levels in the post-natal cerebellum.

IV. 2. 4. Wnt7a Binding assays

Another Wnt gene expressed in the postnatal brain is *Wnt7a*, which is strongly expressed by the granule cells of the cerebellum (Lucas and Salinas, 1997). Wnt7a is a synaptogenic factor affecting both the mossy fibre terminals in the granular layer of the cerebellum *in vivo* as well as mossy fibre and granule cell in culture (Hall *et al.*, 2000; Lucas and Salinas, 1997). The receptors mediating these responses have not yet been identified. Studies described in the previous chapter indicated that the *Fz3* and *Fz7* receptors were both expressed in regions containing the cell body of neurons that are known to respond to Wnt7a. *Fz3* and *Fz7* are expressed by cerebellar granule cells and *Fz3* in the Pontine nuclei, the source of mossy fibres. In order to elucidate whether Fz3 and Fz7 could act as Wnt7a receptors, the binding capabilities of Wnt7a and these Frizzled were first investigated

Binding of Wnt7a to Fz3-CRD was easily resolved using the same binding assay as described above (Figure IV.5). Most cells expressing GPI-myc-Fz3CRD retained Wnt7a-HA at their surface, in the same manner they were able to retain Wnt7b-HA. Nonetheless, there is some heterogeneity in the binding. Binding was most visible around

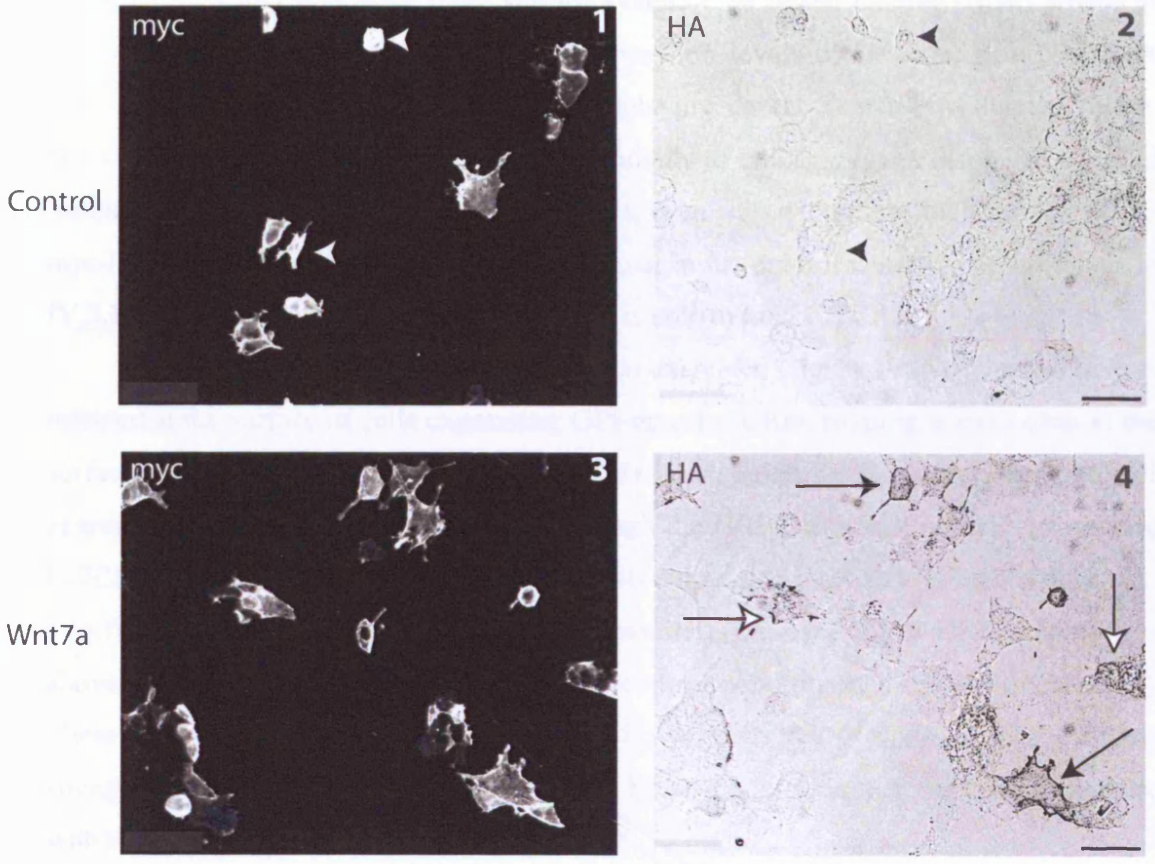


Figure IV. 5. Wnt7a binds Fz3CRD

Cells transfected with GPI-myc-Fz3CRD (1-4) were applied Wnt7a-Ha (3-4) or control conditioned medium (1-2). The myc epitope was detected using fluorescence whilst the HA epitope was detected through an HRP reaction.

Transfected cells exposed to Wnt7b-HA conditioned medium give rise a dark HRP staining indicating that Wnt7b-HA is retained at their cell surface (4). Staining was more pronounced in isolated cells (filled arrows) than in cells which were in aggregates (white arrows). When exposed to control conditioned medium even cells expressing high levels of GPI-myc-Fz3CRD remained unstained for HA. (arrow heads in a and b). This assay demonstrates that Wnt7a can bind Fz3CRD.
Scale bars at 50 µm.

the borders of cells in isolation or at the periphery of a cell aggregate, which tend to be more flattened and thus have more surface available for ligand binding (filled arrows in IV. 5. 4). Binding was co-related to the expression levels of GPI-myc-Fz3CRD, with cells expressing high levels of receptors displaying darker Wnt7a-HA staining (black arrow in Fig. IV. 5. 4). Cells which lie in the middle of cell aggregates displayed a fainter staining (white arrow in IV. 5. 4). Nonetheless, even cells expressing high levels of GPI-myc-Fz3CRD did not give rise to an HRP signal in the control condition (arrowheads in IV.5.1-2). These results indicated that Wnt7a is able to bind Fz3CRD.

Binding of Wnt7a to Fz7CRD was also examined (figure IV. 6). Wnt7a-HA was retained at the surface of cells expressing GPI-myc-Fz7CRD. Binding is most clear at the surface of cells expressing GPI-myc-Fz7CRD in isolation or at the periphery of cell aggregates (arrows in figure IV. 6. 4). Although the HRP precipitate on cells expressing Fz7CRD is not as dark as that observed on cells expressing Fz3CRD (compare figure IV. 5 to figure IV. 6, experiments were done in parallel), the level of HA-HRP staining was above that we observed on cells exposed to control conditioned medium (arrowheads). These data suggest that Wnt7a binds to Fz7. This suggests that Wnt7a binds Fz7 with less strength than it binds Fz3, but this can only be an assumption that we cannot measure with this experimental setup.

Binding of Wnt7a to Fz8 and Fz5 was also investigated in cell surface binding assay. Cells expressing GPI-myc-Fz5CRD never displayed an ability to bind to Wnt7a. On the other hand, although two experiments out of five hinted that Wnt7a may be retained to GPI-myc-Fz8CRD, we did not observe binding of Wnt7a to GPI-myc-Fz8CRD in experiments where we saw binding of Wnt7a to Fz7CRD and Fz3CRD. Therefore, neither Fz5 nor Fz8 are able to bind Wnt7a.

IV. 3. Discussion

It is well recognised in the field that the elucidation of the biochemical properties of Wnts has been hampered by the difficulty to obtain sufficient amounts of soluble, biologically active Wnt proteins (Hsieh, 2004). Yet, our laboratory persevered its effort to produce Wnt7a and Wnt7b, and good levels of these proteins in conditioned media produced by QT6 and Rat 1B cell lines, respectively, were obtained (figure IV. 1). High

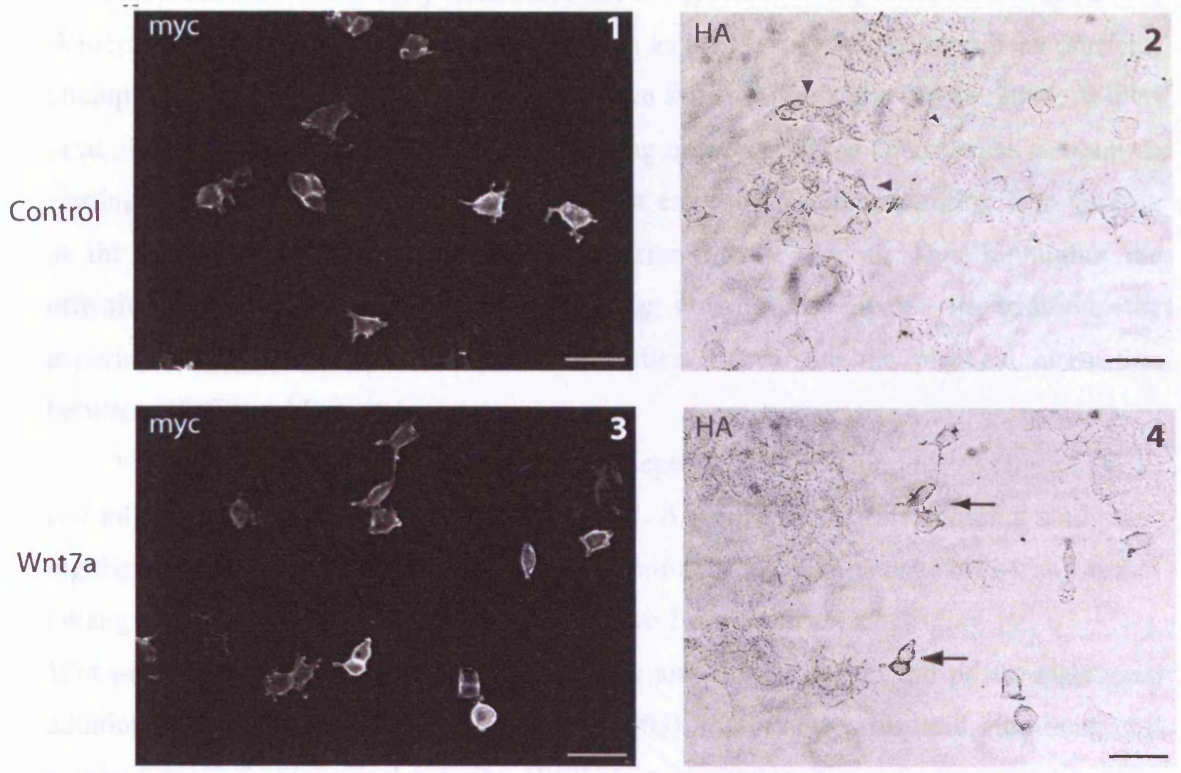


Figure IV. 6. Wnt7a binds Fz7CRD

Cells transfected with GPI-myc-Fz7CRD were applied Wnt7b-HA (3-4) or control condtioned medium (1-2). The myc epitope was detected using fluorescence whilst the HA epitope was dected through an HRP reaction. Binding is indicated by the dark precipitates at the cell surface of transfected cells.

Fz7CRD expressing cells retain Wnt7a at their surface (arrows in 4). A faint background staining may sometimes arise unspecifically (arrowheads in 2). These images suggest Wnt7a is able to bind Fz7CRD.
Scale bars are 50µm.

levels of Wnt in CM provided a source of ligands to study their interaction with immobilised receptors. In fact, most published reports about physical interactions between Wnt and their receptors use a source of Wnt which has been concentrated by size-exclusion chromatography (Bhanot *et al.*, 1996; Hsieh *et al.*, 1999; Mao *et al.*, 2001; Willert *et al.*, 2003). Although some Wnt such as Wnt3a and Wnt5a have been purified, attempts to purify other Wnt such as Wnt1 have failed (Mikels and Nusse, 2006; Willert *et al.*, 2003). The results presented here utilising un-concentrated conditioned medium as starting material are to our knowledge the first experiments demonstrating Wnt-binding at the cell surface without prior concentration of the ligand. This highlights the effectiveness of our protocol for producing conditioned media. In addition, the experiments presented here are the first studies to evaluate the physical interaction between Wnt7a and Frizzled receptors.

Wnt7a and Wnt7b bound to several receptors. Wnt7b bound mFz3 (figure IV. 2) and mFz5 (figure IV. 4) but did not bind mFz7. A similar cell surface binding study also highlighted that Wnt7b may bind mFz1 and mFz10 but cannot bind mFz4 and mFz7 (Wang *et al.*, 2005). Wnt7a binds mFz3 (figure IV. 5) and mFz7 (Figure IV. 6). Wnt proteins are highly hydrophobic proteins and this is due to the post-translational addition of palmitate residues (Willert *et al.*, 2003). Importantly, this lipid-modification is required for their biological activity (Willert *et al.*, 2003). These findings explain why studying the biochemical properties of Wnt can be difficult. Furthermore, it is thought that palmitoylation of Wnt increases their local concentration at membranes and therefore constitutes an important aspect of their mode of action.

Co-immunoprecipitation is another common method used to demonstrate protein-protein interactions. For example, the *Xenopus* secreted head-inducer Cerebrus was shown to co-immunoprecipitate with members of the *Nodal*, *BMP* and *Wnt* gene families (Piccolo *et al.*, 1999). Yet, in the case of serpentine transmembrane receptors, resolving receptor-ligand interactions with full length proteins can be difficult (Kristiansen, 2004). Indeed, the detergent conditions required to solubilise transmembrane proteins may often disrupt the receptor/ligand interactions. Although covalent cross-linking can sometimes resolve detergent-sensitive receptor/ligand interactions, we were unsuccessful at resolving Wnt/Fz binding with DTSSP (see material and methods), a commonly used reversible cross-

linker (Chan *et al.*, 2000). Precipitation attemps were also made using soluble forms of the Wnt-binding CRD domains of Frizzled fused to human IgG. These constructs consist of a human IgG heavy chain fused to the CRD domain of Frizzled (IgG-FzCRD) (Hsieh *et al.*, 1999). Because the IgG heavy chain can bind protein A-sepharose, it is possible to use the IgG-FzCRD fusion protein for pull-down assays or for solid phase binding assays. Yet, although we obtained encouraging results at studying the interaction of Fz7CRD-IgG and Wnt7a-HA, we were unable to demonstrate this interaction using this method. Attempts were also made with Fz3CRD-IgG, but these did not give satisfactory results due to poor expression and secretion of Fz3CRD-IgG.

Other methods have been developed to study the interaction of Wnt and their receptors. One approach to circumvent the problem of Wnt solubility, was to anchor Wnt at the cell surface and apply soluble ligand-binding domains of Frizzled (Leyns *et al.*, 1997; Rulifson *et al.*, 2000; Wu and Nusse, 2002), using the protocol that had identified sFRPs as Wnt-binding proteins (Leyns *et al.*, 1997). This "reverse binding" assay was made possible by fusing *Drosophila* Wnts to the C-terminal of a type II transmembrane protein (which have their C-terminus on the outside of the cell), Nrt. The ligand, FzCRD fused to Alkaline Phosphatase (FzCRD-AP), was soluble and possible to produce in large quantities. Using AP as an integral part of the ligand permits the quantification of the free and bound ligand, because each fusion protein has a specific enzyme activity, measured in OD/hr per mole. These reagents allow Wnt/Fz binding specificities to be studied and binding affinities to be measured. This technique was used to demonstrate that FzCRD proteins were able to bind several *Drosophila* Wnts (Wu and Nusse, 2002). The authors show that both Wingless (Wg) and dWnt2 are able to bind dFz, dFz2 and dFz3. A functional redundancy between dFz and dFz2 had already been demonstrated *in vivo* in *Drosophila*, where the double mutant *DFz,DFz2* knock-out is needed to phenocopy the *Wg* knock-out (Bhanot *et al.*, 1999). Yet, a previous study from the same group highlighted that dFz2 had a ten-time greater affinity for Wg than DFz. The K_d of Wg binding to dFz2 is $5.6x10^{-9}$ whilst the Kd for Wg-dFz1 binding is $4.6x10^{-8}$ (Rulifson *et al.*, 2000). Using dFz1-dFz2 chimeras, the higher affinity dFz2CRD was co-related with stronger activation of Wg signalling whilst cells expressing chimeras containing dFz1CRD showed weaker signalling activation. Therefore, although Wnts may bind

several Frizzled, *in vivo* "preferences" are likely to occur and the signalling capacity of each Wnt/Fz pair should be studied before drawing further conclusions.

In order to get an approximation of binding affinities through a solid-phase binding assay where either IgG (as a control) or FzCRD-IgG would be immobilised, concentrated Wnt7a-AP or Wnt7b-AP fusion proteins would be required. Studies from the Nathans laboratory are the only example where a soluble Wnt was used to quantify Wnt/Fz binding affinity (Hsieh *et al.*, 1999). XWnt8 was successfully expressed and purified as a XWnt8-AP fusion protein. XWnt8 bound mFz8CRD with a dissociation constant of 9nM (+/-2nM)(Hsieh *et al.*, 1999). The same assay was used to demonstrate that Norrin, a protein implicated in the development of the retinal vasculature, was able to bind mFz4CRD (Xu *et al.*, 2004). The publications from the Nusse and Nathans groups are the only ones in which the affinity of Wnt/Fz interactions. Most other studies describe Wnt/Fz binding in a qualitative manner, mainly because of the difficulty obtaining sufficient quantities of soluble Wnt ligands (Hsieh, 2004). It is not known whether Wnt7a-AP or Wnt7b-AP can be produced and concentrated as XWnt8-AP. Yet, if possible the conditioned medium protocol used here would enable the production of sufficient amounts of Wnt7a-AP or Wnt7b-AP to perform similar biochemical studies.

Solid-phase binding assays similar to those described in the publications of either Roel Nusse (Rulifson *et al.*, 2000) or Jeremy Nathans (Hsieh *et al.*, 1999), using membrane tethered Wnt7a or Wnt7b and FzCRD-AP fusions could be used to confirm the observation that Wnt7b binds to hFz5 preferentially to mFz3 and that Wnt7a has a higher affinity for Fz3 than Fz7 (Hsieh *et al.*, 1999; Wu and Nusse, 2002). Wnt7a binding Fz3 and Fz7, which are all highly expressed in the cerebellum, confirms that these receptors could act as Wnt7a receptors. Similarly, the fact that *Wnt7b* and *Fz3* are both expressed in the hippocampus and display a strong interaction is a good hint that these molecules may interact *in vivo*.

In vivo, composition of the extra-cellular matrix (ECM) may alter Wnt binding to cell surfaces. Indeed, Wnt signalling can be affected by changes in the ECM (Logan and Nusse, 2004) Wnts have been reported to be associated with the ECM (Bradley and Brown, 1990). Although most studies of cell surface binding of Wnt pre-treated cultures with heparinitinase in order to reduce background staining, this step was omitted in this

study because background staining was not a problem. Therefore, the assay developed here may be somewhat closer to the physiological situation than other experiments reported. Nevertheless, the ECM in the brain is certainly quite different to the one of cells cultured on glass *in vitro* and thus the binding affinity of Wnt and Fz could also be affected *in vivo* by the presence of brain-specific ECM components and other receptors expressed at the cell surface.

All the experiments described above nevertheless only take into consideration one domain of Frizzled receptors, the CRD. The CRD is highly conserved throughout the *Frizzled* gene family. The CRD is the core element for Wnt binding to Frizzled (Bhanot *et al.*, 1996). Its importance is highlighted by the fact that a family of secreted Wnt antagonist, the sFRP family, also contain this conserved CRD domain (Rattner *et al.*, 1997) and require the CRD to carry their function (Lin *et al.*, 1997; Moon *et al.*, 1997). Yet, recent evidence has emerged that the transmembrane domains of Frizzled may also be involved in ligand binding (Chen *et al.*, 2004) and that the presence of the LRP5/6 co-receptor may also affect binding (Tamai *et al.*, 2000). Indeed, LRP6 binds Wnt1 and a ternary complex of Fz8CRD/LRP6/Wnt1 can be isolated in over-expression studies. Naturally occurring complexes have not yet been isolated, but it is likely that some Wnt/Fz interactions are context dependent and could be different to the ones reported here and in the literature (Mao *et al.*, 2001; Wu and Nusse, 2002). One possibility is that the CRD may act to stabilise the Wnt ligand and enable other domains of the Wnt to make further contacts with other parts of the receptor or the receptor complex. In fact, a recent paper by Povelones and Nusse demonstrate that the function of the CRD is strictly to bind Wnt and enable the ligand to activate other parts of the receptor complex (Povelones and Nusse, 2005). With the recent availability of purified Wnts and the cloning of various Wnt co-receptors, it is likely that a clearer picture of the exact binding requirements for the activation of the different branches of the Wnt signalling pathway will emerge in the near future.

A recent study states that pathway specificity is determined by the ability for different Wnts to bind and therefore recruit LRP into the Wnt/Fz complex (Liu *et al.*, 2005). Experiments where the GPI-FzCRD construct could be co-transfected with LRP5/6 to evaluate the effect the receptor/co-receptor complex on Wnt binding would be

interesting. Perhaps the presence of LRP5/6 could strengthen certain Wnt/Fz interactions. Also, since LRP5/6 transfection alone can stimulate canonical signalling activity in transfected cells (Tamai *et al.*, 2000), it is possible that the presence of GPI-FzCRD could stimulate further this activity, by increasing the local concentration of Wnt at the membrane. In contrast, expression of the extracellular domain and the first transmembrane domain of the Frizzled in *Drosophila* acts as a dominant-negative receptor, leading to severe defects in wing development (Zhang and Carthew, 1998). Thus, expressing GPI-FzCRD may also have a domincant negative effect, by titrating out the Wnts from the functional Fz/LRP complexes. Therefore it is very difficult to predict the outcome of over-expression of FzCRD and LRP5/6. At this time it is thought that the intracellular regions of both Fz and LRP5/6 are necessary for signalling activation *in vivo (Tamai et al., 2000; Wong et al., 2003)* . Nonetheless, understanding the binding capabilities of individual proteins and specific domains of these proteins will clarify our understanding of the molecular mechanics of Wnt signalling. The findings presented here will enable further studies to understand in more depth how Wnt signalling is triggered in neurons.

IV. 4. Conclusion

In this chapter, the binding of Wnt7a and Wnt7b to the CRD domains of Fz was studied. Wnt7b was found to bind Fz3CRD and Fz5 CRD, whilst Wnt7a showed binding with Fz3CRD and Fz7CRD. These findings are encouraging because many of these genes were found to be expressed in overlapping brain regions. It is therefore possible that these receptor/ligand pairs function together *in vivo.* Yet, despite the clues obtained regarding the binding of Wnt7a and Wnt7b, it is not yet established whether these interactions have any biological significance. In fact , it has been reported for example that HFz1 can be immunoprecipitated with both Wnt3a and Wnt5a, but can only co-operate functionally with Wnt3a in an assay measuring canonical signalling (Gazit *et al.*, 1999). Therefore our binding studies need to be completed with assays evaluating the ability of the Wnt/Fz pairs to signal.

Chapter V

Wnt/Fz signalling

V. Wnt/Fz signalling

V. 1. Introduction

Establishing functional receptor/ligand pairs is particularly challenging in the context of Wnt signalling. Indeed, because of the three "branches" of the pathway, many different aspects of Wnt signalling can be studied. Cells can increase their intracellular levels of ß-catenin which may translocate to the nucleus to act as a transcriptional co-activator with TCF (Behrens *et al.*, 1996; Huber *et al.*, 1996). This is the best characterised consequence of Wnt signalling and is referred as the "canonical" or ß-catenin pathway. Therefore, measuring the intracellular levels of ß-catenin or monitoring the transcription of genes controlled by TCF are common methods to assess this branch of Wnt signalling. Nevertheless, reception of a Wnt signal can also trigger "non-canonical" pathways which modify intracellular calcium levels (Kuhl *et al.*, 2000; Slusarski *et al.*, 1997) or affect small molecule GTPases (Fanto and McNeill, 2004; Wong and Adler, 1993). Understanding the cell surface molecules that trigger these different pathways is one of the biggest challenges in the field of Wnt signalling at the moment

Understanding the receptor components that enable Wnt to regulate neuronal behaviour is critical to clarify the molecular basis of Wnt activity in neurons. Focusing on *Wnt7a* and activation the canonical pathway because of their implication in synaptogenesis, we have studied which Frizzled receptors were able to signal to the canonical pathway and how these receptors could respond to Wnt7a and Wnt7b. The contribution of LRP5/6 co-receptors was also investigated. We provide insights into which Fz are likely to play a role as receptors for Wnt7a.

V. 2. Results

In the previous chapters, it was demonstrated that *Wnt7a, Fz3* and *Fz7* are expressed in the cerebellum during development and in adulthood, and that Wnt7a is able to bind Fz3 and Fz7. In order to gain further insights into the roles of these molecules in the developing and adult brain, the ability of these molecules to functionally interact was

tested. Since the inhibition of GSK-3 in neurons has similar effects than Wnts in synaptogenesis and axonal remodelling, activation of the canonical / ß-catenin pathway by Wnts and Fz was studied in the first place (Hall *et al.*, 2002; Lucas and Salinas, 1997). In order to obtain quantitative data regarding the capacity of Wnt7a to activate different Fz receptors, assays quantifying the transcriptional response to canonical signalling were carried out. The focus of the study was Wnt7a because of its established role in synaptogenesis (Hall *et al.*, 2000) and the receptors Fz3 and Fz7 because of their expression in the cerebellar circuit and their ability to bind Wnt7a.

V. 2. 1. Activation of the canonical Wnt pathway by soluble factors

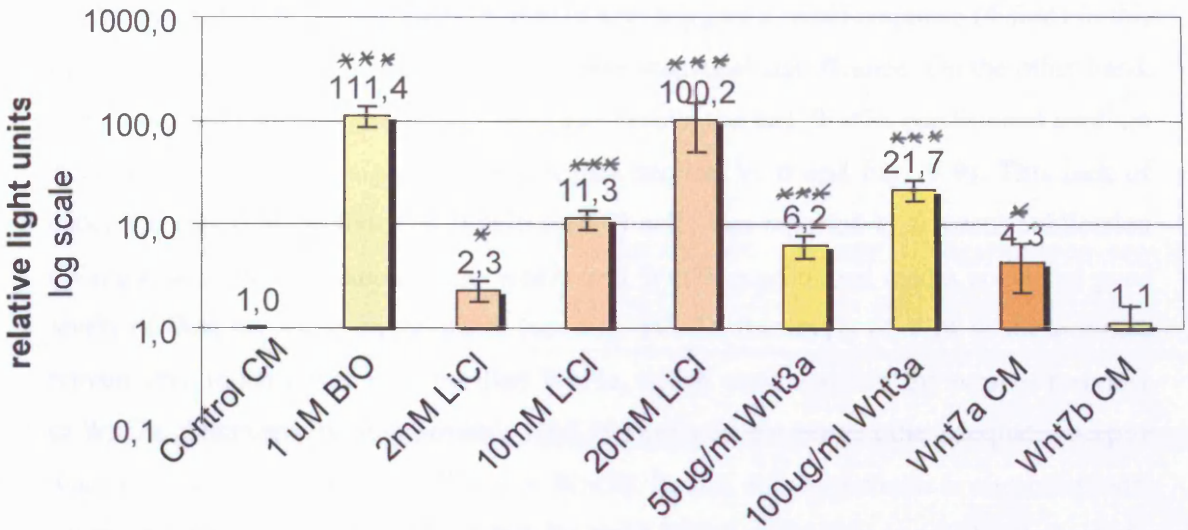
The main outcome of activation of the canonical Wnt pathway, is the stabilisation of ß-catenin. ß-catenin accumulates in the cytoplasm and in turn translocates to the nucleus where it co-operates with TCF to drive the transcription of target genes (Huber *et al.*, 1996). Therefore, three methods are commonly used to monitor activation of canonical signalling. Intracellular levels of ß-catenin can be measured by western blot. Translocation of ß-catenin to the nucleus can visualised by immunofluorescence. Finally, transcription from the TCF can be measured either by quantifying the mRNA of Wnt target genes or using reporter systems. The Topflash reporter in which luciferase genes are expressed under the control of TCF is the most common reporter used to study canonical signalling, and was the method used here (Molenaar *et al.*, 1996; Xu *et al.*, 2004).

The Topflash reporter system makes use of a cell line stably transfected with the Topflash reporter construct, in which the Firefly *luciferase* gene is expressed under the control of the TCF promoter (Molenaar *et al.*, 1996; Xu *et al.*, 2004). In addition, cells were transfected with small amounts of a *luciferase* gene from another organism, the *Renilla,* expressed under a constitutively active promoter, used as an internal control to account for changes in cell numbers present in the lysates as well as for transfection efficiency of the tested constructs. Light emitted from the samples if thus measured in two readings. A first reaction measures the luminescence caused by the firefly luciferase under the control of the TCF promoter (Topflash). This light-emitting reaction is then stopped, and following the addition of another substrate, luminescence from the control *Renilla*

luciferase is measured. The data is presented as Relative Light Unites (RLU) representing the ratio of the luminescence induced because of TCF-mediated transcription over the luminescence generated by the control *Renilla* luciferase. Therefore, RLU reflect activation of TCF-mediated transcription whilst accounting for cell numbers and viability, as well as transfection efficiency. Both Topflash assays and intracellular ß-catenin quantification were used to evaluate the roles of Frizzled receptors in Wnt7a and Wnt7b signal transduction.

The steady-state of ß-catenin is tightly regulated by its phosphorylation within a multi-protein complex composed the scaffolding proteins APC and Axin, GSK3ß and casin kinase Ia (see review (Logan and Nusse, 2004). In the absence of Wnt signals, GSK-3ß is active and ß-catenin is phosphorylated resulting in its degradation (Aberle *et al.*, 1997). In contrast, when this Wnt pathway is activated, GSK-3ß is inhibited and therefore ß-catenin accumulates inside the cell. Therefore, chemical inhibitors of GSK-3 mimic activation the Wnt pathway. LiCl was the earliest GSK-3 inhibitor used (Sinha *et al.*, 2005) but has become replaced with more specific inhibitors such as the one abbreviated BIO (see materials and methods)(Meijer *et al.*, 2003). A preliminary experiment was carried-out to evaluate the response of the 293 topflash cell line when exposed to these chemical GSK-3 inhibitors (Figure V. 1. A). In parallel, we compared the response induced by the GSK-3 inhibitors with the extracellular addition of either purified Wnt3a or conditioned media from cells expressing *Wnt7a* or *Wnt7b*. BIO (1 µM) was by far the strongest inducer of TCF transcription, with levels of luminescence 110-fold higher than those in control untreated cells (light yellow bar). High doses of LiCl (20 mM) also induced a very strong response of around 100 fold activation of transcription (light pink bars). Yet, these levels of activation probably do not represent physiological levels of activation, since application of purified Wnt3a, a Wnt classified as "canonical" that is known to trigger a response in HEK293 cells induced lower levels of activation (yellow bars) (Wong *et al.*, 1994). Indeed, application of 50 µg/ml or 100 µg/ml of Wnt3a gave rise to 6- or 21-fold increases, respectively, in TCF transcription compared to untreated controls. Application of 10 mM LiCl triggered a response of a similar order of magnitude (11-fold activation), and thus appears to be an experimental condition activating the canonical pathway to a similar extent than high amounts of Wnt. Lower

A. GSK3 inhibitors and Wnt3a activate TCF-mediated transcription in 293 cells



B. BIO causes a visible increase in β-catenin nuclear accumulation

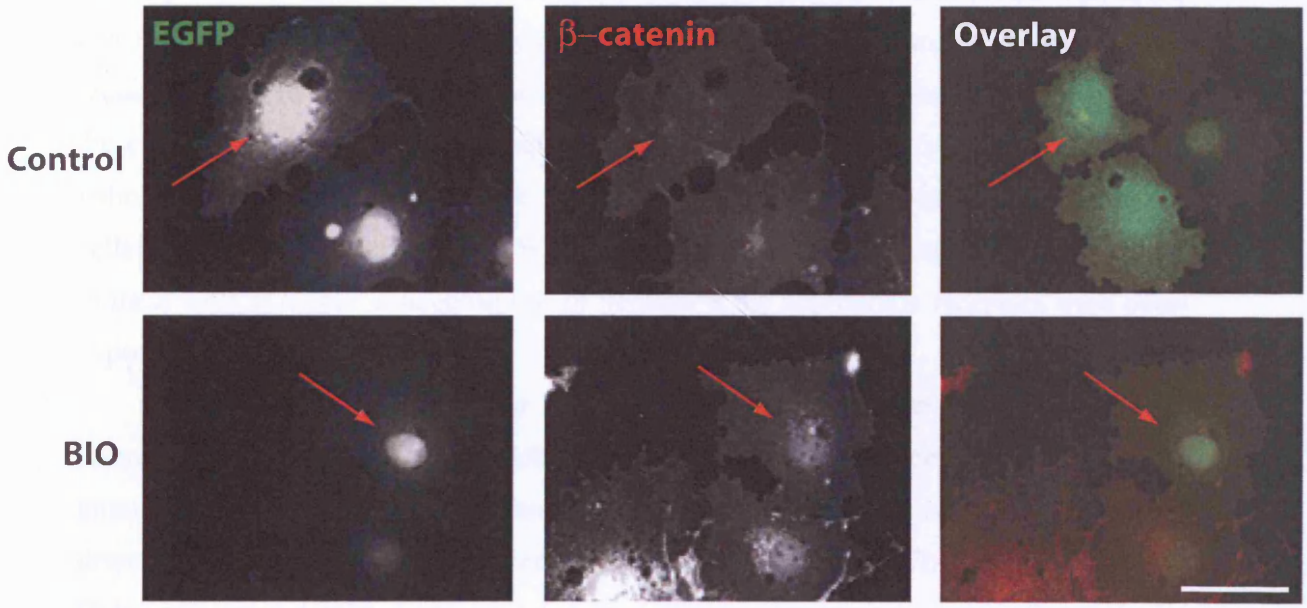


Figure V. 1. Activation of the canonical Wnt pathway by soluble effectors

A. 293 cells expressing the Topflash reporter gene were transfected with a *Renilla* luciferase control plasmid and exposed to soluble activators of the Wnt pathway for six hours. Fold increases in transcription from the TCF promoter are indicated on the chart.
Both 1 µM BIO and 20 mM LiCl led to very strong activation of canonical signalling, and Wnt3a also triggered significant responses. Wnt7a conditionned media only moderately activated transcription of the reporter gene. Wnt7b conditio ned medium always failed to trigger canonical signalling in these experiments. Error bars correspond to S.E.M. P values: *** P<0,001, *P<0,005

B. EGFP-transfected COS-7 cells were treated for 6 hours with the GSK-3 inhibitor BIO (10 µM) or a control. Cells were stained for β-catenin (red) and analysed by fluorescence microscopy. The transfected EGFP (green) can be used to visualise cells and their nuclei (arrows). Cells treated with BIO show varying degrees of β-catenin accumulation in their nuclei and the protein is also detected in the perinuclear region. In contrast, control treated cells display a low perinuclear β-catenin signal and no accumulation of β-catenin in their nuclei.
Scale Bar = 25µm

doses of LiCl (2 mM), on the other hand, only gave a weak 2-fold increase in TCF transcription. Finally, application of Wnt7a also triggers a small response (4-fold) in this cell lines, although this result failed to achieve statistical significance. On the other hand, Wnt7b did not induce signalling in 293 cells despite the fact Wnt7b-conditioned medium is active in other Wnt signalling assays (see section V. 6 and Fig. V.9). This lack of canonical signalling activity of Wnt7b on 293 cells was reported in a recent publication (Wang *et al.*, 2005). Although the Wnt7a and Wnt7b conditioned media contained good levels of Wnt for these experiments (see Fig. IV. 1), the levels of Wnt in these media remain very much lower than purified Wnt3a, which could explain the weaker response to Wnt7a. Alternatively, it is possible HEK293 cells do not express the adequate receptor machinery to fully respond to Wnt7a or Wnt7b. In fact, this hypothesis is consistent with several reports, which demonstrate that Fz and LRP5/6 expression in cell lines can make cell responsive to give Wnts. For example, 293 cells need to be co-transfected with LRP5 and Fz10 in order to respond to Wnt7b (Wang *et al.*, 2005). Also, LRP5 and Fz4 are required for Wnt5a to activate the canonical pathway (Mikels and Nusse, 2006). Nonetheless, these data highlighted that BIO could be used as a positive control for experiments, as an indicator of whether cells are able to respond optimally. Also, it was confirmed that the cells used were able to respond to Wnt3a and hinted that Wnt7a could potentially signal in these cells at higher concentrations or perhaps if the appropriate receptors were over-expressed.

Immunodetection of cellular ß-catenin can also be used to study canonical /ß-catenin signalling in cell lines. (Schneider *et al.*, 1996). COS7 cells were chosen for immunofluorescence studies because of their spread appearance and their easily detectable nuclei. Cells were treated with BIO, Wnt7a- or Wnt7b-conditioned-media. Only cells treated with 1µM BIO led to a visible increase of nuclear ß-catenin by immunofluorescence (Fig. I.1.B). In contrast, cells treated with Wnt7a or Wnt7b CM did not display significant changes in ß-catenin levels or localisation. This findings indicated that immunodetection of cellular ß-catenin was not useful to detect low levels of canonical signalling activation.

V. 2. 2. Not all frizzled receptors have the same capacity to activate canonical signalling

Having established possible receptors for Wnt7a and Wnt7b from the gene expression patterns and their binding capacities, the ability of Fz3, Fz7 and Fz8 to signal through the canonical pathway was investigated. It was anticipated that over-expression of the receptor for Wnt7a would either activate the canonical pathway or enable cells to respond to Wnt7a. The capacity of Fz7 to activate the canonical pathway has already been reported (Holmen *et al.*, 2002; Medina *et al.*, 2000) and was therefore the most likely candidate to transduce Wnt7a signals. Fz8 transduces canonical signals in zebrafish to specify patterning of the neuroectoderm (Kim *et al.*, 2002). Fz3 is also known to activate canonical signalling but has been more often implicated in planar cell polarity signalling (Holmen *et al.*, 2002; Umbhauer *et al.*, 2000). In addition, as LRP6 over-expression is known to activate the canonical pathway (Cong *et al.*, 2004; Holmen *et al.*, 2002; Tamai *et al.*, 2000) cells were also transfected with LRP6 in order to compare our findings with other published reports.

Transfection of Frizzled receptors was first carried-out to study the capacity of individual receptors to signal to the canonical Wnt pathway. Cell lysates were collected for Topflash assays and western blot analysis. Transfection of Fz7 leads to a significant (7-fold) increase in TCF-mediated transcription of the luciferase reporter gene compared to EGFP transfected cells (Figure V.2). Fz7 therefore has the intrinsic capacity to activate canonical signalling. Transfection of the LRP6 co-receptor increased TCF-mediated transcription to a similar extent (6-fold). This range of activation of the Topflash reporter gene following LRP6 transfection is comparable to the data reported in other publications (Holmen *et al.*, 2002). In contrast, transfection of Fz3 and Fz8 only gave rise to two-fold increases in TCF-mediated transcription (Figure V.2) and this effect did not reach statistical significance.

It was important to monitor whether the transfected proteins were successfully expressed in all experiments. Transfection efficiency was monitored by immunofluorescent staining of coverslips prepared in parallel to the samples for the Topflash assays. Western blot analysis of cell lysates was also attempted but the anti-Fz antibodies did not generate satisfactory result. The immunofluorescent stainings enabled the quantification of the cells in culture that expressed the receptors. An example of these

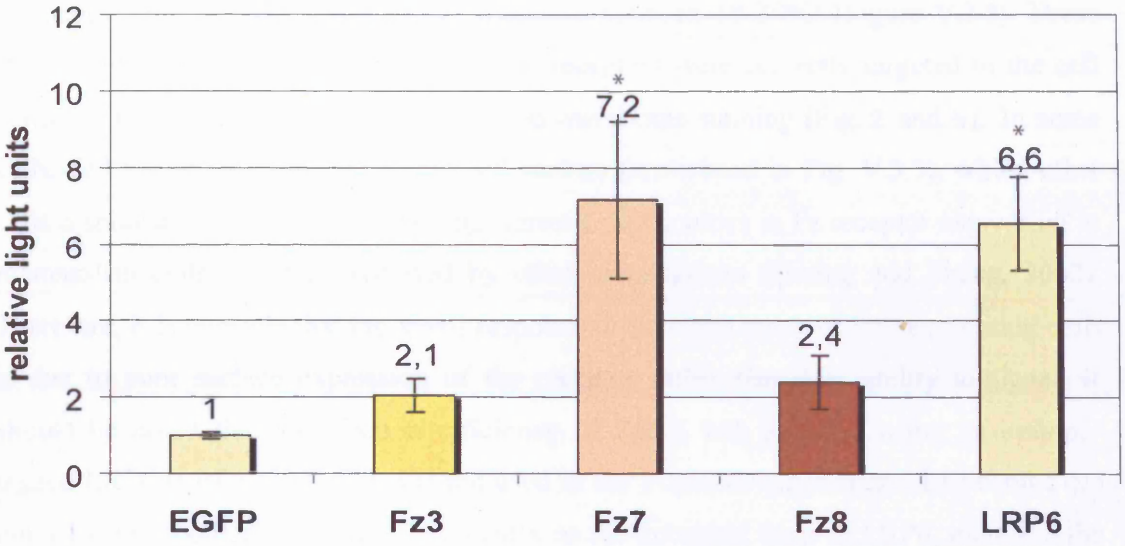


Figure V. 2. Effect of Receptor over-expression on canonical Wnt-signalling

293 cells expressing the Topflash reporter gene were transfected with *EGFP, Fz3, Fz7, Fz8* or *LRP6* alongside the *Renilla* luciferase control plasmid. Frizzled-7 displays an inherent ability to signal to the canonical pathway. Transfection of the co-receptor *LRP6* also stimulates Wnt signalling in these cells. On the other hand, transfection of *Frizzled3* or *Frizzled8* only weakly activates this signalling cascade. Error bars correspond to S.E.M of the triplicates of three independent experiments. ***P values: * P<0,005**

stainings is shown in Figure V.3. Both Fz7 and LRP6-myc were expressed with similar efficiencies (typically between 30 to 50%) (Figure V.3. **1**, **2** and **4**). Transfection efficiency for Fz3 was usually lower (typically between 10-30%) (Figure V.3.**3**). These staining also enabled me to verify whether the receptors were correctly targeted to the cell surface. Fz7 or LRP6-myc display a good membrane staining (Fig. **2** and **4**). In some cells, Fz3 is not well targeted to the cell surface (arrowhead in Fig. V.3.**3**), whilst other cells display a clear membrane staining (arrow). Difficulties in Fz receptor expression in mammalian cells has been reported by other investigators (Hering and Sheng, 2002). Therefore, it is possible that the small response in Topflash assay of Fz3-expressing cells is due to poor surface expression of the receptor rather than Fz3 ability to signal. It should be noted that transfection efficiency of LRP6 was assessed using an epitope-tagged LRP6 (LRP6-myc) that was not used in the Topflash experiments. LRP6-myc did not activate canonical signalling as potently as the untagged form of LRP6, therefore the untagged form of LRP6 was used in Topflash assays. Nonetheless, this LRP6-myc staining demonstrates that the 293 cells used were able to express LRP6 efficiently. It was not possible to detect Fz8 because no anti-Fz8 antibodies were available. Since *Fz8* was cloned by the same group as the other Fz receptors studied (Wang *et al.*, 1996), it can be assumed that the protein was expressed at least in the same fashion as Fz3. Taken together, these data confirm the hypothesis that *Fz7* is a good activator of canonical Wnt signalling, whilst *Fz3* and *Fz8* do not seem capable of activating this signalling cascade when transfected alone in this system.

V. 2. 3. Wnt7a synergises with Fz7

Having established that Fz3, Fz7 and Fz8 were functionally expressed, we applied Wnt7a conditioned medium to transfected cells in order to evaluate whether these receptors could mediate Wnt7a signalling (Figure V.4). Wnt7a CM applied to EGFP transfected cells led to a 4-fold increase in TCF-transcription compared to EGFP transfected cells treated with control CM. In the presence of Fz7, the response to Wnt7a CM was increased 23-fold compared to control conditions and 3-fold compared to Fz7 cells exposed to control CM. This synergism could be expected from the binding data presented in the previous chapter and Fz7 ability to signal to the canonical pathway

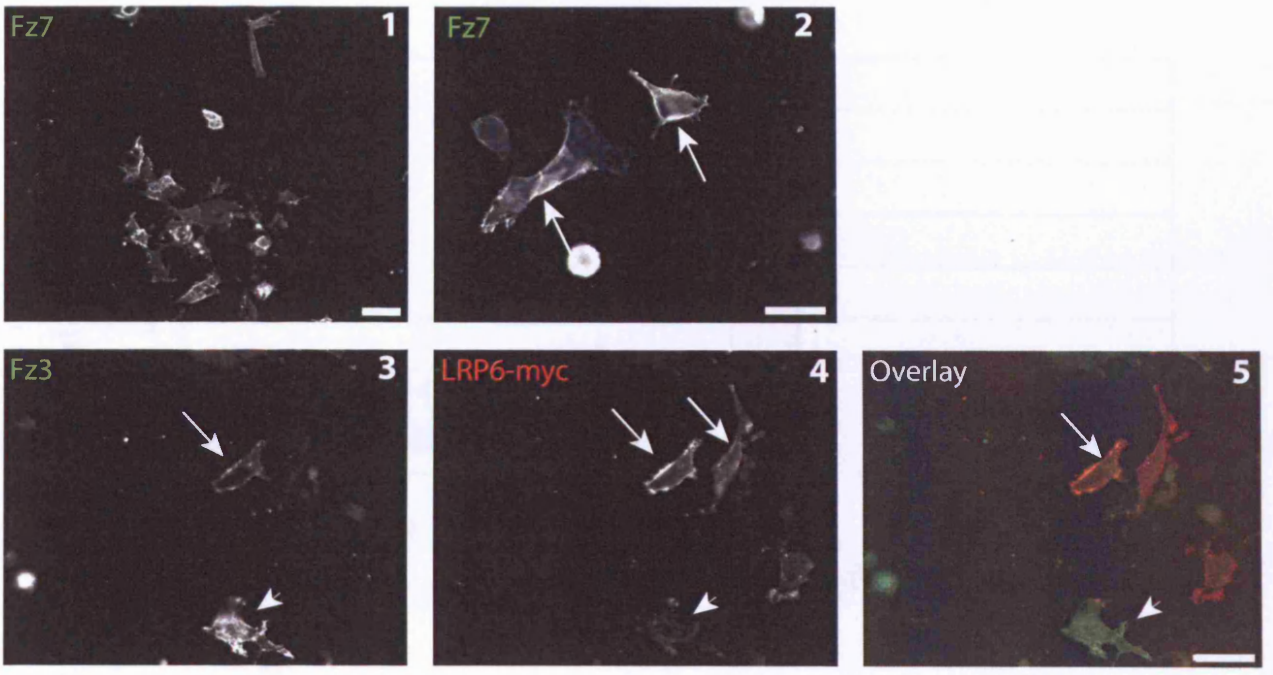


Figure V. 3. Expression of Fz receptors and LRP6

A fraction of the 293 cells used in Topflash experiments were plated and processed for immunostaining. The expression of different receptors was examined.

Fz7 is expressed in 30-50% of cells in culture (1) and is well targeted to the cell surface (arrows in 2). Fz3 is expressed in fewer cells (10-30% of cells in culture) than Fz7 and is not as efficiently targeted to the cell surface as Fz7 (arrowheads in 2 and 5). Yet, some cells express Fz3 at their surface (arrow in3 and 5). LRP6-myc is expressed in similar proportions to Fz7 (30-50%) and is detected at the cell surface (arrows in 4).
These data highlight that few cells expressed Fz3, whilst Fz7 and LRP6 appear to be more widely expressed.
All scale bars are 50µm.

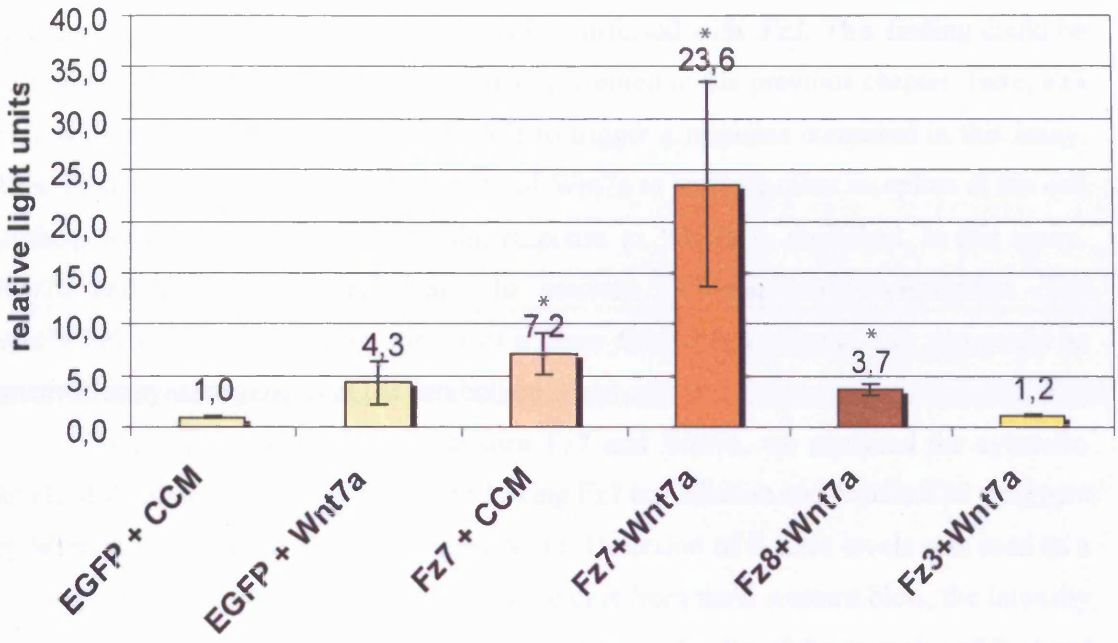


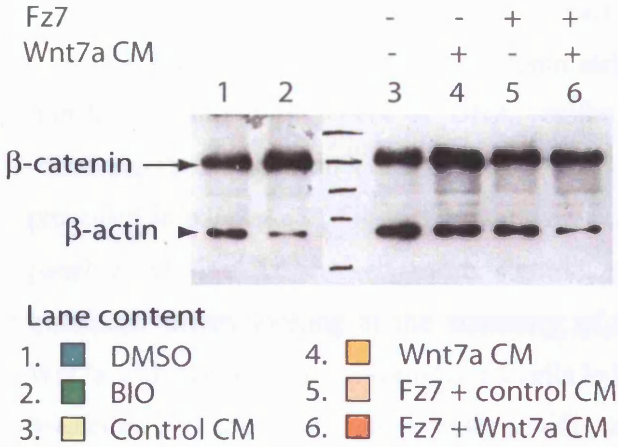
Figure V. 4. Fz7 synergises with Wnt7a

293 cells expressing the Topflash reporter gene were transfected with EGFP, Fz3, Fz7, Fz8 alongside the *Renilla* luciferase control plasmid and subsequently exposed to Wnt7a conditio ned medium. Cells expressing Fz7 are able to trigger a strong and significant response to Wnt7a conditio ned medium. The response of Fz7-expressing cells to Wnt7a was 23-fold higher than untreated cells, 5-fold higher than control cells exposed to Wnt7a and 3 -old higher than cells expressing Fz7 exposed to control medium. Cells expressing *Fz8* did not show an increased response to Wnt7a as compared to *EGFP* expressing cells. Interestingly, cells expressing *Fz3* seem to loose their ability to respond to Wnt7a, but these data did not achieve statistical significance. These data demonstrate that Fz7 may function as a receptor for Wnt7a to signal to the canonical pathway.
Error bars correspong to SEM obtained from triplicate readings of three independent experiments. P values: *<0,005.

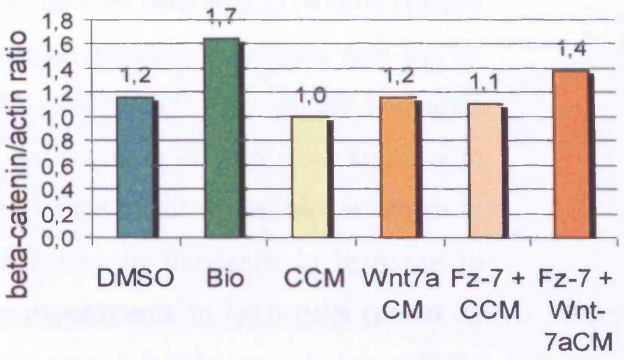
presented here. Expression of Fz8 and Fz3 was also tested. Fz8 was tested in a single experiment but it s expression did not confer alter the cellular response to Wnt7a. The response to Wnt7a seemed abolished in cell transfected with *Fz3*. This finding could be an indirect confirmation of the binding data presented in the previous chapter. Here, Fz3 may be binding Wnt7a without being able to trigger a response measured in this assay. This binding may reduce the availability of Wnt7a to activate other receptors at the cell surface, which would explain why the response to Wnt7a is abolished. In this assay, *Wnt7a* and *Fz7* act synergistically to activate TCF-mediated transcription. The Wnt7a/Fz7 pair may therefore consist of a *bona fide* receptor ligand pair that could be involved in synaptogenesis in the cerebellum.

To confirm the synergism between Fz7 and Wnt7a, we analysed the cytosolic levels of ß-catenin in two cells types following Fz7 transfection and Wnt7a CM treatment by Western blot (Figure V. 5 and Figure V. 6). Detection of ß-actin levels was used as a loading control. In order to obtain quantitative data from these western blots, the intensity of the bands was quantified with NIH image software. A ratio of the intensity of the band for ß-catenin over the ß-actin band was done. The ß-catenin/ß-actin ratio of untransfected cells treated with control CM was the baseline used to normalise the data. To account for inconsistencies in the western blotting procedure, samples were run three times in order to obtain a precise quantification of these two proteins in each cell lysate. It is widely accepted that this method can only detect large increases in ß-catenin levels. Assays were carried in 293 cells (Figure V. 5) and QT6 cells (Figure V. 6). In 293 cells, the increase caused by BIO is noticeable in the western blot presented in figure V.5, panel 1, and quantification of band intensity shows that ß-catenin levels are increased by 70% (panel 2 illustrates an example of the quantification data for the western blot presented in panel 1). This increase is smaller than the 110-fold increase in TCF-mediated transcription induced by BIO in the Topflash assay. Furthermore, it can be predicted that 10-20 fold increases such as those observed in the Topflash assay may not be detectable by western blotting. Nevertheless, in the western blot presented in panel 1 and its quantification in panel 2, cells exposed to Wnt7a CM show a 16% increase in ß-catenin levels and cell expressing Fz7 exposed to Wnt7a CM show an increase of 38% compared to untreated cells. The summary of two experiments in 293 cells is presented in panel 3 of figure V. 5. The trend

1. Effect of BIO, Wnt7a CM and Fz7 on β-catenin levels in 293 cells



2. Quantification of band intensity of western blot (1)



3. Effect of Wnt7a CM and *Fz7* over-expression in 293 cellls (summary of 2 experiments)

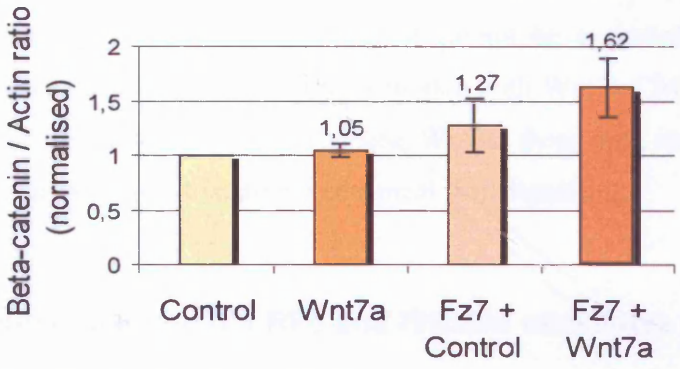


Figure V. 5. Wnt7a may co-operate with Fz7 to stabilise β-catenin in 293 cells

Western blots for cytosolic β-catenin (98kDa, arrow) and β-actin (loading control) (42kDa, arrowhead) (panel 1) following Fz7 transfection and Wnt7a exposure. Cells were transfected for 24h with Fz7 or untransfected (control), and then exposed to Wnt7a CM, the GSK-3 inhibitor BIO or control CM for 6h. The band intensities were measured and displayed as a β-catenin over actin ratio and normalised to the control.

293 cells treated with the GSK-3 inhibitor BIO (lane 2, in a) and treatment of Fz7-transfected cells with Wnt7a CM (lane 6) induce substantial increase in cytosolic β-catenin, whilst addition of Wnt7a to untransfected cells only leads to a small increase (lane 4) (1). Quantification of the bands of the western blot displayed in (1) is shown in panel 2. BIO treamtment increased β-catenin levels 70% whilst cells expressing Fz7 and exposed to Wnt7a increased β-catenin levels by 40%. To generate data of more significance, samples from two independent experiments were ran three times and the data pooled together after normalisation (3). 293 cells seem not to respond significantly to Wnt7a in the absence of Fz7. Transfection of Fz7 confers a 27% increase in β-catenin levels. On the other hand, cells transfected with Fz7 and treated with Wnt7a CM have an increase in β-catenin of 67% on average. Although this result does not achieve statistical significance in this cell type, these experiments highlight the potential for Fz7 and Wnt7a to functionally co-operate to stabilise β-catenin.

of the synergism between Wnt7a and Fz7 is confirmed in this summary plot, with Fz7 transfected cells exposed to Wnt7a CM displaying a 62% increase in intracellular ß-catenin. Yet, data variation prevents these data from achieving statistical significance.

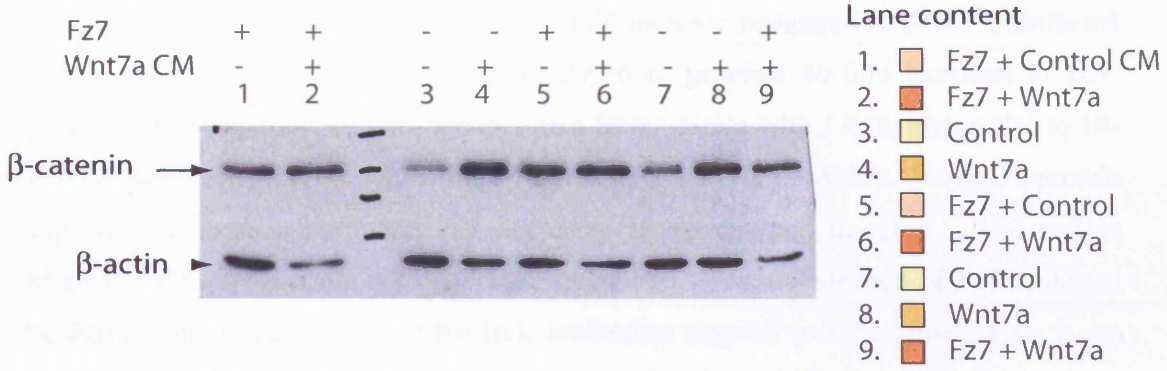
Similar experiments were therefore carried in another cells line, QT6 cells (Figure V. 6). QT6 cells were chosen for ß-catenin stabilisation experiment because they can be transfected with high levels of DNA, unlike 293 cells which may trigger apoptosis following Fz transfection (van Gijn *et al.*, 2001). An example of such a western blot is presented in panel A. of Fig.V. 6, and quantification of the bands of this blot is shown in panel 2. QT6 cells showed more significant increases in ß-catenin in response to treatment. When looking at the summary of two experiments in QT6 cells (panel 3), Wnt7a CM treatment of un-transfected cells led to a nearly 3-fold increase in intracellular ß-catenin. Fz7 transfected cells treated with control conditioned medium showed a 70% increase in ß-catenin compared to untransfected cells. *Fz7*-transfected cells treated with Wnt7a displayed on average a 5-fold increase in ß-catenin compared to untransfected cells treated with control CM. Although it cannot be excluded that the change in ß-catenin observed in *Fz7* transfected cells treated with Wnt7a CM could be caused by the additive but independent effect of Fz7 and Wnt7a, these data strongly suggest that Fz7 and Wnt7a can synergise to activate canonical Wnt signalling.

V. 2. 4. Co-transfection of LRP6 and Frizzled stimulates the canonical response

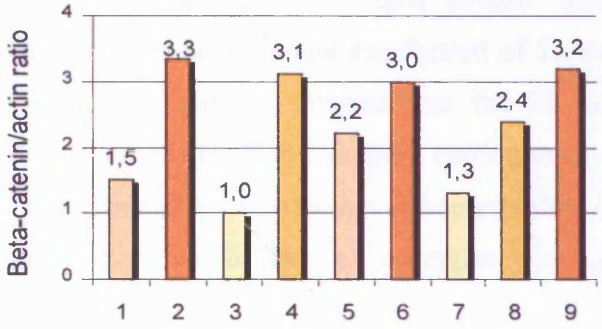
It is well established that the LRP5/6 co-receptor is required to significantly activate the canonical pathway (Pinson *et al.*, 2000; Tamai *et al.*, 2000). In fact, many publications that used 293 cells to carry-out Topflash assays often co-transfected *LRP5* or *LRP6* alongside *Frizzled* and/or *Wnt* in order to detect noticeable changes in TCF mediated transcription (Holmen *et al.*, 2002; Wang *et al.*, 2005). Therefore it was possible that some functional interactions between Wnt and Fz in the experiments described so far could have been overlooked, because of the absence of sufficient amounts of LRP5/6 in the cells used.

To determine whether the addition of LRP6 could affect the response of cells to Fz, a series of Topflash assays were carried out on cells co-transfected with *LRP6* and *Fz3*,

1. Effect of Wnt7a and fz7 on β-catenin levels in QT6 cells



2. Quantification of band intensity of (1)



3. Wnt7a and Fz7 synergistically stabilise β-catenin in QT6 cells

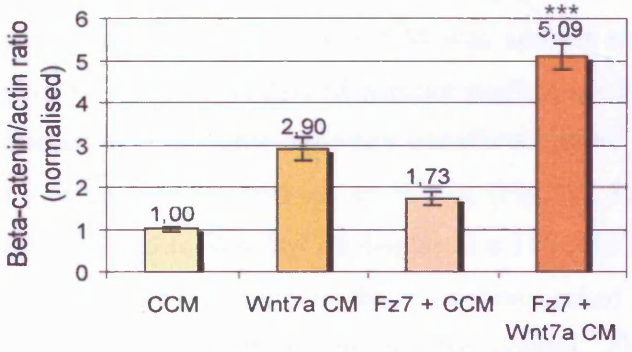


Figure V. 6. Wnt7a co-operates with Fz7 to stabilise β-catenin in QT6 cells

Identical analysis as in Figure V.5 was carried-out in QT6 cells.
The western blot (1) contains repeated loadings of four samples. Indeed, for a precise measurement of a given sample's β-catenin and actin content, samples must be loaded at least twice. The quantification of band intensity of this western blot is shown in (2). QT6 respond to Wnt7a CM (orange bars) and in cell expressing Fz7 exposed to Wnt7a displayed consistently high levels of Wnt7a. The data obtained from multiple loading of two experiments are presented in panl 3. Wnt7a induces a three fold increase in β-catenin and transfection of Fz7 further increases the response to obtain a five fold increase in β-catenin levels ($P<0.005$). Taken together, thes data confirm that Wnt7a co-operate with Fz7 to trigger the stabilisation of β-catenin in both 293 and QT6 cell lines.

Fz7 or *Fz8* (Figure V.7). Co-expression of *LRP6* and any of the three Fz tested showed a robust increase in TCF-mediated transcription of the luciferase reporter. *Fz3* appeared to co-operate most efficiently with *LRP6* (45-fold increase compared to EGFP transfected cells). *Fz7* also strongly synergised with *LRP6* to generate 40-fold increases in TCF mediated transcription. *Fz8* co-operated to a lesser extent with *LRP6*, giving rise to 18-fold increases activation of signalling but this effect was very variable. The LRP6 protein expressed in these experiments did not carry an epitope-tag, therefore LRP6 protein levels in the samples could not be directly quantified. Also, transfection of *Fz8* could not be directly measured because of the lack antibodies targeted to Fz8. Nonetheless, it can be considered that the luminescence readings for the *Renilla* luciferase reflect cell numbers and transfection efficiency of each sample. Similar readings for *Renilla* luciferase activity were observed for single transfection of Fz constructs and LRP6/Fz co-transfections, therefore it can be inferred that the *Fz* and *LRP6* constructs were transfected to similar extents in all the samples. Having made this assumption that *LRP6* transfection did not increase the transfection efficiency of the *Fz,* it can be concluded that Fz3, Fz7 and to a lesser extent Fz8, all synergise with LRP6 to activate canonical signalling in 293 cells.

The discovery that Fz3 was able to signal in 293 cell in the presence of LRP6 prompted the re-assessment of whether Fz3 could increase the cellular response to Wnt7a signals in the presence of *LRP6*. Wnt7a CM was applied to cells co-transfected with *LRP6* and *Fz3* or *Fz7* (Figure V. 8). *Fz8* was not studied, because of its inability to bind Wnt7a or influence Wnt7a signalling when transfected alone. Cells co-transfected with *Fz3* and *LRP6* were able to respond to Wnt7a (Fig. V. 8). On average, *Fz3/LRP6* transfected cells exposed to Wnt7a CM displayed a 110-fold increase in TCF mediated transcription, but in some experiments, this activation peaked to 250-fold and exceeded the activation obtained with BIO, our positive control. *Fz3/LRP6* transfected cells exposed to Wnt7a increase their TCF-mediated transcription by 165% compared to cells exposed to control CM. In contrast, cells expressing only *LRP6* have their TCF-mediated transcription increase by 47% when they are exposed to Wnt7a instead of control CM. These date therefore indicate that the Fz3/LRP6 receptor complex can function as receptor for Wnt7a.

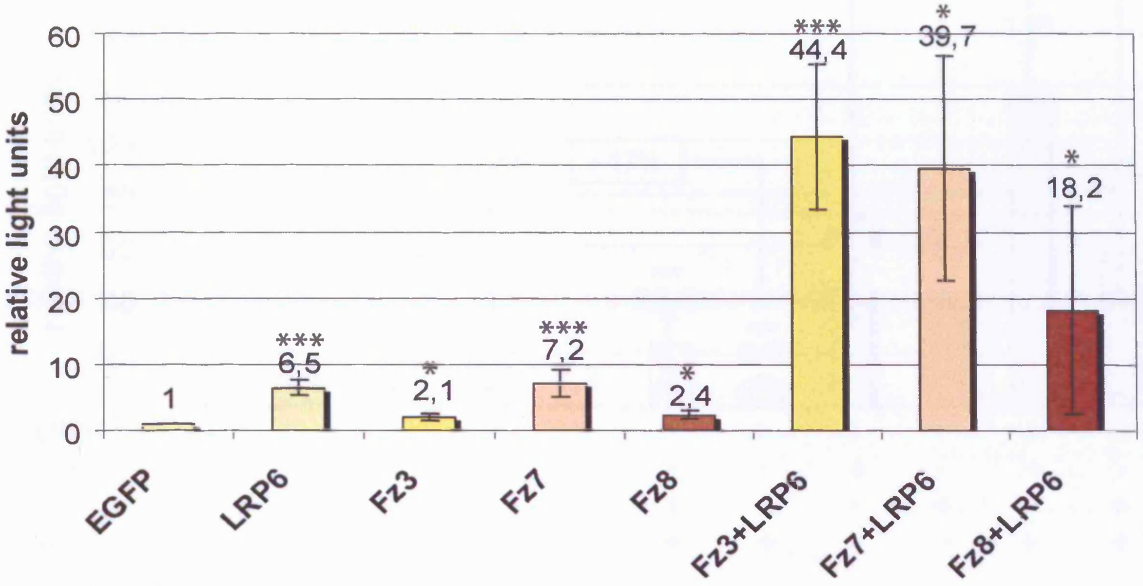


Figure V. 7. Synergism between Fz receptors and LRP6

293 cells expressing the Topflash reporter gene were transfected with *EGFP, LRP6* or co-transfected with *LRP6* and *Fz3, Fz7* and *Fz8* alongside the *Renilla* luciferase control plasmid. Activation of TCF-mediated transcription was quantified in the topflash assay. To achieve satistical significance, the data presented for samples transfected with only one construct was pooled with data of other experiments.

Both Fz3 and Fz7 display a strong synergism when co-transfected with LRP6, increasing TCF-mediated transcription around 40-fold. compared to control EGFP-transfected cells. Fz8 also synergised with LRP6 although to a lower extent than Fz3 and Fz7 , and with much variance. These experiments highlight that 293 cells require the expression both Fz and LRP6 in order to generate a strong response.

Error bars represent SEM of triplicate readings of a least three independent experiments. P values: ***<0,001, *<0,005

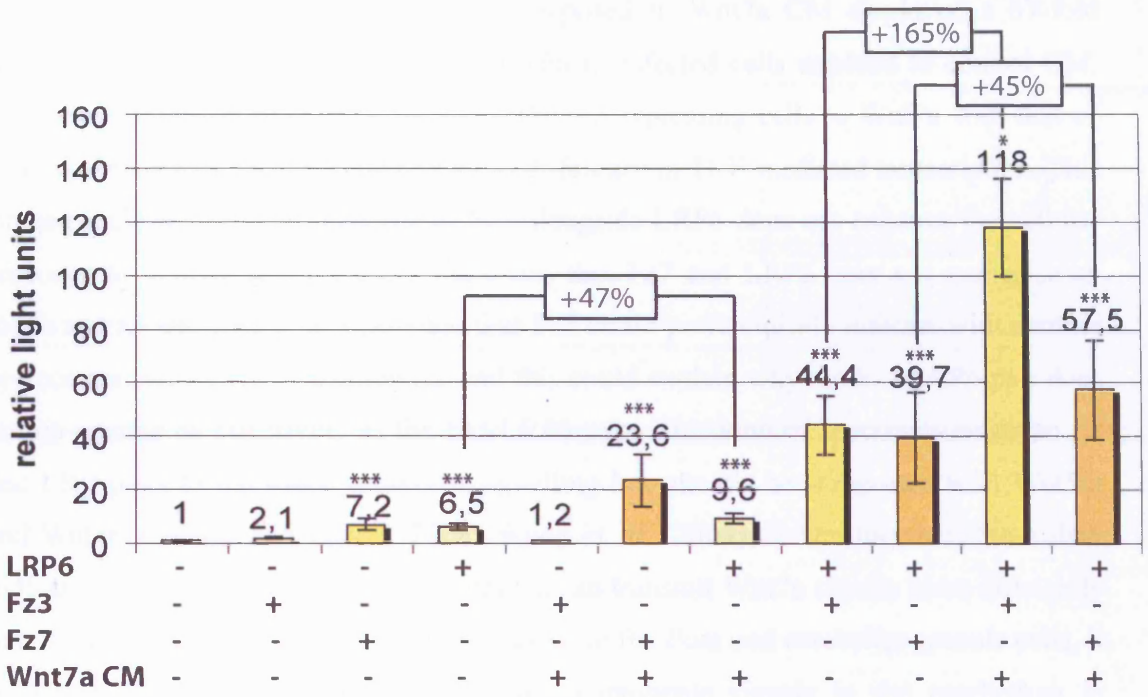


Figure V. 8. Fz3 and LRP6 synergise to transduce Wnt7a signals

293 cells expressing the Topflash reporter gene were transfected with *EGFP*, *LRP6* or co-transfected with *LRP6* and *Fz3* or *Fz7* alongside the *Renilla* luciferase control plasmid and subsequently exposed to Wnt7a or control conditionned medium.
Cells expressing LRP6 showed a 47% increase in transcription of the Topflash reporter when exposed to Wnt7a CM. This response to Wnt7a was not changed in cells co-transfected with *Fz7* and *LRP6*, suggesting that Fz7 and LRP6 did not co-operate to transduce Wnt7a signals. In contrast, cells co-expressing Fz3 and LRP6 displayed a very strong increase (+165%) in TCF-mediated transcription when they were exposed to Wnt7a. The Fz3/LRP6 receptor-ligand pair is therefore a strong candidate to transduce Wnt7a signals to the canonical pathway. Error bars are SEM from triplicate readings of three experiments. Some data points were obtained by pooling normalised values obtained in previous experiments. P values: ***<0,001; *<0,005.

The presence of LRP6 along-side Fz7 affected the cells' response to Wnt7a less dramatically. Fz7/LRP6 transfected cells exposed to Wnt7a CM displayed a 57-fold increase in signalling compared to Fz7/LRP6 transfected cells exposed to control CM. Yet, when comparing the response of LRP6/Fz7 expressing cells to Wnt7a with that of cells expressing LRP6 alone, there was no difference in TCF-mediated transcription. This finding indicates that the presence of Fz7 alongside LRP6 does not enhance the cellular response to Wnt7a, and therefore illustrates that Fz7 and LRP6 may not synergise as much as Fz3 and LRP6. It is possible that Fz7 could preferentially interact with another co-receptor, with LRP5 for example, and this could explain why the Fz7/LRP6 pair does not co-operate as effectively as the Fz3/LRP6 pair. This kind of specificity of given Fz and LRP pairs to transduce canonical signalling has already been reported with Wnt5 a and Wnt7b (Mikels and Nusse, 2006; Wang *et al.*, 2005). Taken together, these data indicate that the Fz3/LRP6 receptor complex can transmit Wnt7a signals more efficiently that the Fz7/LRP6 pair. Since Fz3 is expressed in the Pons and cerebellar granule cells, it is possible that Fz3 transmit the Wnt7a synaptogenic signals in the cerebellum in collaboration with LRP6. The results presented in this chapter are summarised in table V. 1.

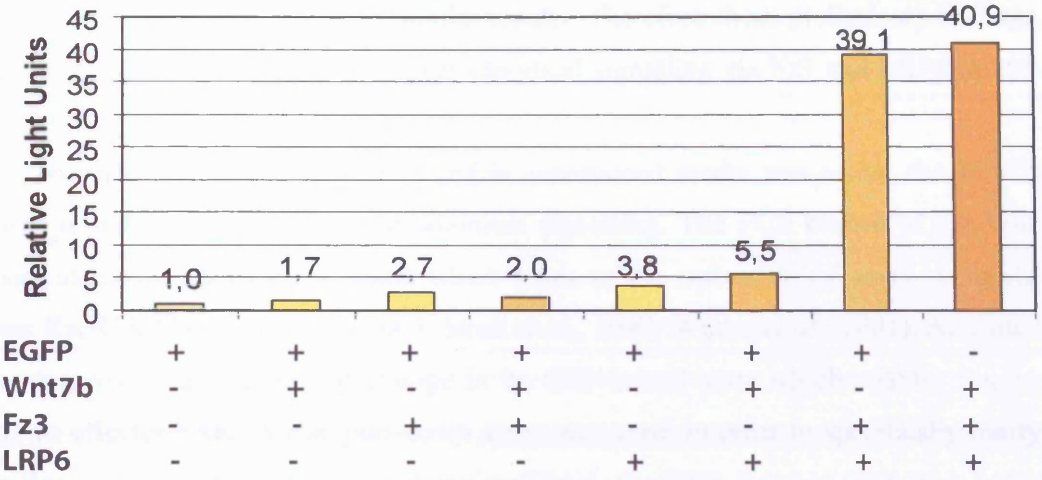
V. 2. 5. Wnt7b is unable to signal to the canonical pathway in HEK293 cells but affects Rac activation

Wnt7b is known to stimulate the dendritic arborisation of hippocampal pyramidal neurons, but the receptors mediating these processes are unknown. 293 cells are known not to respond to Wnt7b and the initial experiments were carried-out in the experimental system used here confirmed this finding (Fig. V.1) (Wang *et al.*, 2005). Yet, it remained possible that co-transfection of LRP6 and Fz could enable cells to respond to Wnt7b. Fz3 was of candidate receptor of particular interest because of its strong expression in the hippocampus (Fig. III. 8) and it s ability to bind Wnt7b (Fig. IV. 2). Indeed, having established that the Fz3/LRP6 pair was a potent co-receptor complex to trigger canonical Wnt signals (Fig. V. 7), signalling in response to Wnt7b CM was re-examined. Wnt7b failed to activate canonical signalling in cells transfected with LRP6 or co-transfected with Fz3/LRP6 (Figure V. 9). This data was generated from a single experiment and thus

Wnt7a CM		○				○	○			○	○
Fz3			▮			▮		▮		▮	
Fz7				▮			▮		▮		▮
LRP6					▮			▮	▮	▮	▮
TCF-mediated transcriptional activation	-	+	-	+	+	-	++	+++	+++	++++	+++

Table V. 1. Summary of canonical / β-catenin signalling activation in 293 cells by Wnt7a, Fz3, Fz7 and LRP6

Exposure of 293 cells to Wnt7a conditioned medium activated TCF-mediated transcription to similar levels than in cells expressing Fz7 or LRP6, whereas Fz3 expression alone does not affect canonical signalling. Wnt7a exposure of cells expressing Fz7 activated signalling further, whilst cells expressing Fz3 remain irresponsive to Wnt7a. Co-expression of Fz3 or Fz7 with LRP6 strongly activated TCF-mediated transcription. Co-expression of Fz3 and LRP6 enables cells to respond very strongly to Wnt7a. In contrast, exposure to Wnt7a of Fz7/LRP6 expressing cells does not result in higher signalling activity than cells exposed to control CM. Therefore, these results indicate that Wnt7a signalling can be transduced by Fz7 and the Fz3/LRP6 pair.



V. 9. Wnt7b is unable to trigger canonical signalling in cells expressing *Fz3* and/or *LRP6*

293 cells expressing the Topflash reporter gene were transfected with EGFP, Fz3, LRP6 or combinations of the above alongside and subsequently exposed to Wnt7b or control conditionned medium.
Wnt7b was unable to trigger a significant response in TCF-mediated transcription in any of the conditions tested here. These data correspond to the duplicates of a single experiment, nonetheless similar results were obtained in inadequately controlled experiments.

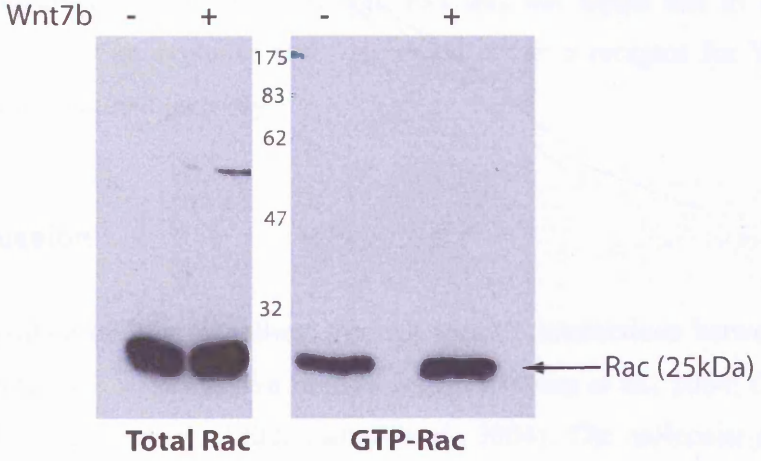


Figure V. 10. Wnt7b conditionned medium activates Rac in 293 cells (premiliminary result)

293 cells were treated with either control or Wnt7b conditionned medium for six hours. Cell lysates were collected and their protein content quantified. Equal amounts of protein were incubated with CRIB-GST in order to purify active (GTP-bound) Rac. The start material of the purification (Total Rac) and the result of the purification (GTP-Rac) were loaded on an SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-Rac antibody.
The left lanes illustrate that both control and Wnt7b treated samples contained equivalent levels of total Rac. Quantification of band intensity revealed Wnt7b treated sampled seem to have 7% more total rac. Samples exposed to Wnt7b display a marked increase in purified active Rac compared to cell exposed to control CM. Quantification revealed there is a 15% increase in Rac activation. This experiment demonstrates that the Wnt7b CM used in the experiments presented is functional and can activate the non-canonical pathway. (This represents a single experiment)

cannot achieve statistical significance. Other experiments which lacked the Renilla luciferase internal control produced similar results. Therefore these preliminary findings suggest that Wnt7b was unable to trigger canonical signalling via Fz3 and LRP6 in 293 cells.

To verify that the Wnt7b produced in conditioned media was active, the Wnt7b CM was tested in an assay for non-canonical signalling. The PCP branch of the Wnt pathway utilises a signalling cascade which leads to the activation of small molecule GTPase Rac/Cdc42 (Habas *et al.*, 2003; Strutt *et al.*, 1997; Winter *et al.*, 2001). Activated Rac undergoes a conformational change in its GTP-bound state which enables Rac to bind to its effector PAK. A Rac pull-down assay was used, in order to specifically purify active Rac, using the Rac-interacting domain of PAK, the PBD, fused to GST. This assay was carried-out and demonstrated that the Wnt7b CM used was capable of increasing active Rac by 15% in 293 cells (Figure V. 10). This experiment demonstrates that the Wnt7b in the conditioned media is active and can activate the non-canonical pathway in 293 cells. Taken together, our results suggest that Fz3 and LRP6 are unable to confer the ability of Wnt7b to signal to the canonical pathway in 293 cells. However, Wnt7b-induced non-canonical signalling through Fz3 was not tested due to time constraints. Therefore it cannot be excluded that Fz3 could act as a receptor for Wnt7b signalling through a non-canonical pathway.

V. 3. Discussion

Activation of Wnt signalling through specific interactions between Wnt with Fz and LRP proteins is a very active field of research (Cong *et al.*, 2004; Gonzalez-Sancho *et al.*, 2004; Holmen *et al.*, 2002; Tamai *et al.*, 2004). The molecular mechanisms that activate ß-catenin signalling are beginning to emerge but some controversy remains regarding whether the Fz/LRP only functions as a receptor complex or whether Fz and LRP5/6 can carry-out some functions on their own (Cong *et al.*, 2004; Tamai *et al.*, 2004). Here, a subset of components of the Wnt signalling machinery was studied to establish their ability to co-operate to stabilise ß-catenin and trigger TCF mediated transcription. The main assay used was the Topflash assay, the most common read-out

for canonical signalling used in the field (Molenaar *et al.*, 1996; Xu *et al.*, 2004). Fz7 was shown to be able to induce signalling in 293 cells (Fig. V. 2). Fz7 transfected cells displayed an increased response to Wnt7a conditioned medium compared with un-transfected cells (Fig. V. 4), indicating that Fz7 could transduce Wnt7a signalling to ß-catenin. Furthermore, Fz3 and Fz7 co-operated with LRP6 to induce TCF mediated transcription (Fig. V. 7). Finally, Fz3/LRP6 co-transfected cells show considerable synergism at transmitting the Wnt7a signal, indicating that this specific receptor complex can transduce Wnt7a signalling (Fig. V. 8). The fact that Fz3 is expressed in the Pontine nuclei and that Fz3, Fz7 and Wnt7a are all expressed by cerebellar granule cells during development points out a potential role of these receptor/ligand pairs in neuronal remodelling and synaptogenesis in the cerebellum. The data presented here indicates that both Fz7 and Fz3 may act as receptors for Wnt7a.

V. 3. 1. Topflash assays versus ß-catenin level measurements

The 293 cell line used for the Topflash assays was stably transfected with the topflash reported construct (Xu *et al.*, 2004). This cell line was a major asset, because it guaranteed that all cells tested in different conditions have comparable levels of the reporter plasmid. Therefore, *luciferase* expression from the TCF promoter was directly linked to ß-catenin signalling and independent from transfection efficiency. Other methods to detect activation of the canonical Wnt pathway were used in the present study. Intracellular levels of ß-catenin were measured by Western blot (Fig. V.5 and Fig. V. 6) and translocation of ß-catenin to the nucleus was visualised by immunofluorescence (Fig. V.1) Although increases in ß-catenin levels were detected, these methods did not provide a reliable and quantitative method to measure signalling activation, especially when signalling is activated at low levels. This is consistent with other reports which show that transcription of Wnt target genes can occur without a detectable change in ß-catenin levels (Holmen *et al.*, 2002). This may occur in untransfected cells treated with Wnt7a which show a small Topflash response (Fig. V. 1) without a significant increase in intracellular ß-catenin levels (Fig. V.5). Perhaps fractionation of cell lysates to separate cytosolic and membrane fractions would have led to more clear-cut increases in ß-catenin. Indeed, a proportion of ß-catenin is constitutively associated with cadherins at the

membrane (Kemler, 1993). Since this association is independent from Wnt signalling, it is possible that including membrane fractions made the detection of changes in total levels of ß-catenin more difficult to detect in the data presented here (Lee *et al.*, 1999; Willert *et al.*, 1997). Also, the duration of exposure to Wnt7a-CM (6 hours) was longer than other studies which often use 2-3 hour treatments for these assays (Mikels and Nusse, 2006). 6-hour treatment were used as this is the minimum length of time our laboratory exposes neurons to Wnt and thus all our Wnt treatments were 6-hour in length. It is possible that by reducing the duration of exposure to Wnt7a, more striking changes in ß-catenin could have been observed. Alternatively, phospho-specific anti-ß-catenin antibodies is another method that can be used to monitor the phosphorylation state of ß-catenin in cell lysates (Wu and He, 2006). Similarly, although detection of ß-catenin in the nucleus by immunofluorescence was clear in cells treated with BIO (Fig. V.1), analysis of Wnt7a treated cells did not give rise to a clear nuclear accumulation of ß-catenin that could be detected by immunofluorescence. Therefore, the Topflash assay which enables a quantification of the outcome of ß-catenin stabilisation, transcription of genes controlled by TCF, was used as a precise read-out for canonical signalling.

V. 3. 2. Receptor trafficking and cell-surface expression

Topflash assays carried-out in cells transfected with various receptor components relied on the successful expression of the constructs. Proper expression of the different receptors was imperative for the validity of the presented studies. Because the anti-frizzled antibodies available did not give specific signals in western blots, it was not possible to directly measure Fz proteins levels. Instead, transfection efficiency was assessed by immunostaining of the transfected cells. *Frizzled* expression plasmids were transfected at lower efficiency than *EGFP* vectors, and *Fz7* was more efficiently transfected than *Fz3* (30-50% vs 10-30%). Therefore it is possible that the failure of *Fz3*-transfected cells to activate of Wnt signalling is a consequence of *Fz3* poor expression rather than an intrinsic inability of *Fz3* to signal to the ß-catenin pathway. Indeed, improper surface trafficking of Fz receptors transfected in mammalian cells has been reported as another hurdle in studying Wnt signalling and the expression of other proteins

is sometimes beneficial to Fz or LRP5/6 surface expression (Hering and Sheng, 2002; Yao *et al.*, 2001).

Some mammalian Fz have been shown to associate with the scaffold protein PSD-95 and co-expression of PSD-95 with Frizzled receptors can modify the intracellular distribution of Frizzled (Hering and Sheng, 2002). In this study, mFz7 or rFz2 were detected in an intracellular reticular pattern when transfected alone in COS-7 cells, suggesting the majority of the over-expressed receptors remained in the endoplasmic reticulum (ER). Upon PSD-95 co-expression, mFz7 and rFz2 are re-located to large clusters as seen by immunofluorescence. Although Hering and Sheng failed to demonstrate that the change in Frizzled localisation is in fact an increased cell surface targeting of Frizzled, these data suggest that some additional factors may be required for the functional localisation of Frizzled receptors in heterologous systems. In addition, two Frizzled-binding proteins, GOPC (Golgi-associated PDZ Coiled-coil motif containing protein) and Kermit have been described as protein assisting Fz in either signal transduction and/or membrane expression (Tan *et al.*, 2001; Yao *et al.*, 2001). GOPC is a mammalian protein of the Golgi apparatus that associates with mFz5 and mFz8 during the secretory pathway and co-localises with Fz at the membrane. (Yao *et al.*, 2001). Kermit is a Xenopus protein that binds specifically to XFz3 and that is required for XFz3 function in neural crest induction. Although the function of Kermit has not been demonstrated, it is homologous to proteins involved in vesicular and membrane trafficking. Alternatively, Kermit could function as an adaptor protein to couple Fz to its downstream effectors (Tan *et al.*, 2001). PSD-95, GOPC and Kermit share the feature that they all contain PDZ domains and these PDZ domains are required for their interactions with Frizzled. Therefore it appears that some Frizzled may require assistance from specific proteins in order to be targeted to the membrane for signalling. Indeed, the sub-optimal surface of expression of Fz3 in the data presented here constitutes one of the major caveats of our study. Future work aiming to optimise Fz3 expression should be carried-out in order to study the role of this receptor further.

Frizzled are cystein-rich proteins, and thus may have a tendency to form un-wanted disulfide bonds between cystein residues during their maturation. Therefore Frizzled require proteins to chaperone their folding. In fact, a mutation in *Frizzled*-4

which causes the protein to remain trapped in the ER has been shown to be the cause of a degenerative disease of the retina, familial exudative retinopathy (FEVR) (Kaykas *et al.*, 2004; Robitaille *et al.*, 2002). Therefore, absence of one such factor contributing to Fz surface expression may be another factor accounting for the absence of response in cells expressing *Fz3* or *Fz8*.

The LRP5/6 co-receptors are necessary for the activation of canonical Wnt signalling (Logan and Nusse, 2004; Tamai *et al.*, 2000)). It was therefore necessary to evaluate their co-operation alongside *Fz* in 293 cells. *LRP6* co-transfection with *Fz3, Fz7* and *Fz8* (albeit to a lesser extent and with much variance), led to significant increases in transcription, as measured by the Topflash reporter (Fig.V. 7). Further more, Wnt7a CM potently activated TCF-mediated transcription in cells expressing *LRP6* and *Fz3* (Fig. V. 8). Although the most straight-forward conclusion of these results is that LRP6 synergises with Fz to activate canonical / ß-catenin signalling, it is also possible LRP6 expression contributed to an increase the cell surface expression of Fz. Yet, immunostaining of cell expressing *LRP6* and *Fz3* did not display different Fz3 expression than cells expressing *Fz3* alone. Therefore, although it remains possible that co-expression of Fz and LRP5/6 slightly increases their surface targeting, the functional synergism observed for Fz3 and LRP6 indicate these two proteins are likely to function as a receptor complex *in vivo*.

Similarly to Frizzled, the correct expression of LRP5/6 at the membrane also requires additional factors. The importance of the ER chaperones in Wnt signal transduction came to light when mutants for *Boca* in *Drosophila* and *Mesd* in the mouse revealed striking similarities with mutants for members of the canonical Wnt pathway transduction machinery (Culi and Mann, 2003; Hsieh *et al.*, 2003). Both Boca and Mesd are ER-resident proteins that are required for the correct surface expression of Arrow and it s homologue LRP5/6, respectively. It is therefore possible that the signalling activation by LRP6 reported here would have been enhanced if Mesd had been co-expressed but this remains a hypothesis to be verified. Never the less, since activation of signalling upon LRP6 transfection was detected to similar levels as reported in other publications (Cong *et al.*, 2004; Holmen *et al.*, 2002), the requirement for Mesd was not considered essential. Although the studies mentioned above state that Fz receptor surface expression is not affected by the reduced surface expression of LRP5/6/Arrow in the mutants (Culi

and Mann, 2003; Hsieh *et al.*, 2003), it remains possible that increased-expression of LRP6 favours Fz accumulation. We could speculate that a functional receptor complex (e.g. Fz3/LRP6) would be maintained at the surface (or recycled) whilst a receptor that is not being activated (e.g. Fz3) would be preferentially targeted for degradation.

V. 3. 3. Fz/Wnt receptor-ligand pairs in canonical signal transduction

The cellular response to Wnt factors has been studied in a variety of cell lines and different animal models. The results presented here contribute to the current understanding of Wnt signal transduction. An early study of the capacity of Wnt to transform mammalian cells established Wnt1, Wnt3a and Wnt7a as "highly transforming" Wnt, whilst other Wnts were classified as "intermediate" (e.g. Wnt7b) or "non-transforming" (e.g. Wnt5a) (Moon *et al.*, 1993; Wong *et al.*, 1994). The transformation observed in response to a subset of Wnts were changes in cell morphology and excessive cell proliferation. In another study, the transforming capacity of Wnt1, Wnt3a and Wnt7a was linked to their ability to increase cytosolic levels of ß-catenin and this provided the distinction between "canonical" or transforming Wnts and "non-canonical" or non-transforming Wnts (Shimizu *et al.*, 1997). Interestingly, Shimizu and colleagues described Wnt7b as a non-canonical Wnt, whereas Wong and colleagues had observed some Wnt-7b-induced transformation in the same cell line. In hippocampal neurons, Wnt7b has been showed to activate a non-canonical pathway leading to the activation of Rac and JNK but not the canonical pathway (Rosso *et al.*, 2005). The results presented here (Figures V. 1, 9 and 10) are consistent with the findings reported by Shimizu and colleagues. Wnt7b was unable to trigger a canonical response, despite being able to activate a non-canonical signalling cascade (Fig. V. 10). The data illustrating that both Wnt7a and Wnt3a may activate the canonical Wnt signalling cascade are also consistent with other reports (Shimizu *et al.*, 1997; Wong *et al.*, 1994). It is likely that Wnt7b cells are unable to signal in 293 cells because they lack the adequate receptor proteins to trigger a canonical response upon Wnt7b reception, whereas these cells express receptors to transduce Wnt7a signals. In fact, *Fz7* is the most highly expressed Frizzled receptor by 293 cells (Wang et al., 2005). Although we did not demonstrate that Wnt7a is using endogeneous Fz7 to signal in 293 cells, this is a possibility. Indeed,

Fz7CRD binds Wnt7a (Fig. IV. 6) and transfection of *Fz7* further increases the response to Wnt7a in 293 cells (Fig. V. 4). Therefore our results indicate that Fz7 is able to transduce Wnt7a signals through the canonical pathway.

Frizzled receptors have also been characterised according to their capacity to activate a particular branch of the signalling pathway, but in many cases, over-expression of Frizzled may activate both canonical and non-canonical cascades (Holmen *et al.*, 2002; Medina *et al.*, 2000) . For example, this is the case in Drosophila for dFz1 and dFz2. Over-expression in cell lines and epistastic studies in *Drosophila* indicate some level of redundancy between dFz1 and dFz2 (Bhanot *et al.*, 1999). But further studies demonstrated that Fz1 preferentially activate the Planar Cell Polarity pathway whilst dFz2 is mainly responsible for the transduction of Wg signals to the canonical pathway (Boutros *et al.*, 2000; Strapps and Tomlinson, 2001). In this chapter, Fz7 expression triggered the activation of Topflash reporter, illustrating the ability of Fz7 to activate the canonical Wnt Pathway. In contrast, Fz3 expression did not affect this pathway when LRP6 was not co-expressed. The effect of *Fz3* expression on Wnt7b signalling through the non-canonical pathway would have been interesting to study using a Rac Assay. Yet, the lack of Fz3 antibodies and the low levels of Fz3 expression in cell lines prevented these experiments from being undertaken.

Both the inability of Fz3 to activate the canonical pathway and the capacity, albeit of intermediate strength, of Fz7 to signal to this pathway go along observations made by other researchers (Holmen *et al.*, 2002; Medina *et al.*, 2000). Nonetheless, in Xenopus, XFz3 was shown to induce Wnt-target genes expression when overexpressed in Xenopus whilst XFz7 was not, therefore contradicting the above (Carron *et al.*, 2003). These types of discrepancies are common in the field and are often explained by differences in expression levels of Fz, the types of assay used, and the time after transfection/injection when the experiments are carried out. Also, it is possible *Fz* orthologs have evolved to have slightly different functions. Our results indicate that expression of *Fz7* mildly activates transcription of Wnt target genes in 293 cells, whilst *Fz3* or *Fz8* does not. Yet, once *LRP6* is co-expressed with *Fz3*, *Fz8*, or *Fz7*, TCF-mediated transcription is activated.

V. 3. 4. Synergism between Fz and LRP affects the response to Wnt signals

Synergism between all the Fz receptors studied (Fz3, Fz7, Fz8) and LRP6 was detected (Fig. V. 7). Strikingly, Fz3 and Fz8 which were unable to signal when transfected alone, considerably enhanced signalling as compared to cells expressing Fz or LRP6 alone. As the mechanism for Fz/LRP co-receptor activation is emerging, strong activation of signalling upon Fz and LRP6 co-expression was expected. Indeed, it appears that Wnt may activate signalling by contributing to the formation of LRP and Frizzled oligomers (Cong *et al.*, 2004). This study from the Varmus laboratory indicates the Wnt ligand function to bring Fz and LRP together, through its interactions with their extracellular domains. In turn, Fz and LRP recruit Dishevelled and Axin, respectively, through their cytoplasmic domains, leading to signal activation (Cong *et al.*, 2004). These findings confirm a hypothesis put forward by Xe He when he demonstrated the role of LRP6 as Wnt co-receptors (Tamai *et al.*, 2000). Therefore, it is not surprising that over-expressing the two receptor components leads to a Wnt-independent activation of signalling. When expressed individually, the receptors (Fz or LRP5/6) do not encounter enough or any co-receptors (LRP5/6 or Fz, respectively) to trigger signalling, and thus activation of signalling is low. In contrast, at high concentrations of both Fz and LRP, when they are co-expressed, complexes are more likely to form and thus lead to intracellular signalling activation. This may explain the synergy between Fz3 and LRP6, which require to be over-expressed to come together in the absence of the appropriate ligand. Fz/LRP synergy has already been reported in the case of hFz5 and LRP6 (Tamai *et al.*, 2000). In this study in Xenopus, *hFz5* over-expression is unable to trigger *siamois* expression, a hallmark of canonical signalling. Yet, co-injection of *LRP6* alongside *hFz5* lead to robust *Siamois* transcription (Tamai *et al.*, 2000). Therefore, exogenous expression of LRP6 can release the signalling potential of certain Fz, and our results suggest Fz3 and LRP6 can successfully co-operate.

The activation of canonical / ß-catenin signalling in cells expressing *Fz7* or *Fz3/LRP6* led us to investigate how Wnt7a could further activate signalling in these cells. The most striking synergism was observed when Wnt7a conditioned medium was applied to cells expressing Fz3/LRP6 (Fig. V. 8). In contrast, application of Wnt7a CM to cells expressing LRP6 and Fz7 triggered a similar response in cell expressing only LRP6. This

finding is perhaps explained by the fact 293 cells endogenously express high levels of Fz7 (Wang *et al.*, 2005). If activation of the canonical Wnt pathway requires Wnt to bind and activate both a Fz receptor and a LRP5/6 co-receptor, the Fz3/LRP6 complex appears as a likely receptor complex for Wnt7a signals (Cong *et al.*, 2004; Tamai *et al.*, 2004). Indeed, Wnt7a binds Fz3 and application of Wnt7a to Fz3/LRP6 expressing cells very highly activated signalling. The Fz7/LRP6 complex may also be able to transduce Wnt7a signals. This could be the case when Wnt7a CM is applied to cells where endogenous Fz7 synergises with exogenous LRP6. Obtaining gene expression patterns for *LRP5/6* genes *in vivo* will shed some light regarding the receptor complexes likely to be formed *in vivo.*

Fz7 is involved in the transmission of Wnt7a signals (Figures V. 4, 5 and 6). 293 cells are known to express low levels of *Fz1* and high levels of *Fz7* and it therefore possible that Wnt7a may use endogenously expressed Fz7 to activate signalling (Wang *et al.*, 2005). Furthermore, upon Fz7 transfection the response to Wnt7a is increased five-fold, suggesting that Fz7 is able to transduce Wnt7a signals (Fig. V. 4). Interestingly, LRP6 may not be the co-receptor involved in this situation, because cells expressing Fz7/LRP6 and cells expressing only LRP6 respond in similar ways to Wnt7a (Fig. V. 8). We can speculate the LRP5 would synergise strongly with Fz7 and Wnt7a. Indeed, LRP6 is expressed in 293 cells, whilst LRP5 is not (Wang *et al.*, 2005). Therefore it is possible that endogenous Fz7 and LRP6 contribute to the response to Wnt7a in cells expressing Fz7 or LRP6 individually, and this would explain why co-transfection of *LRP6* with *Fz7* does not dramatically enhance the cellular response. Perhaps co-expression of LRP5 with Fz7 would enable cells to respond better to Wnt7a, and therefore that Wnt7a/Fz7/LRP5 complex is more synergistic than Wnt7a/Fz7/LRP6. The fact *LRP5* is expressed by cerebellar granule cells supports this hypothesis (www.brain-maps.org). Such specificity for a Wnt ligand for a specific combination of Fz and LRP receptors was reported in two recent studies (Mikels and Nusse, 2006; Wang *et al.*, 2005). First, Wang and colleagues demonstrated that 293 cells required the co-expression of different LRP, LRP5 and Fz4 or Fz10 in order to respond to Wnt7b. In contrast, cells expressing LRP6 or Fz receptors alone failed to respond to Wnt7b thus demonstrating that each Wnt may require the presence of two specific receptor/co-receptor elements in order to signal (Wang *et al.*, 2005). Secondly, Mikels and Nusse demonstrate that Wnt5a is able to activate canonical

ß-catenin signalling in cells expressing Fz4 whilst it inhibits Wnt3a signalling in the absence of Fz4 (Mikels and Nusse, 2006). Therefore, within the limits of our experimental systems, it appears that Fz7 may also be a part of a Wnt7a receptor complex, but the LRP5/6 co-receptor of "preference" remains to be established.

V. 3. 5. Other receptors for Wnt7a

Other Frizzled receptors have been shown to be able to transduce Wnt7a signals. In the developing limb bud, Wnt7a is expressed by the dorsal ectoderm and is involved in dorso-ventral patterning of the limb mesenchyme (Parr and McMahon, 1995). In the chick, *cFz10* is expressed in posterior-distal region (Kawakami *et al.*, 2000). Wnt7a is able to bind *cFz10*. Upon co-injection in Xenopus animal caps, *Wnt7a* and *cFz10* synergise to drive the transcription of the Wnt target genes *Siamois* and *Xnr3* (Kawakami *et al.*, 2000). In a study combing gene expression analysis by RT-PCR and over-expression in PC12 cells, Wnt7a was shown to signal via the Fz5/LRP6 receptor complex (Caricasole *et al.*, 2003). This study failed to show any interactions between Wnt7a and Fz7, but a very low activation of Wnt signalling was detected in the positive controls (e.g. 10mM LiCl only induces a 2.7-fold induction of the Topflash report whilst other reports indicate a 10-fold activation) points out the poor effectiveness of the experimental setup used by the authors. Thus, the expression of *Fz10* in the cerebellum should be analysed and an interaction between Wnt7a and Fz10 tested. On the other hand, *Fz5* was not detected in the cerebellum and Fz5CRD was found not to bind Wnt7a. Therefore, Fz5 does not appear a likely candidate receptor for Wnt7a. *Fz3* and *Fz7* remain two very strong candidate receptors for Wnt7a signalling in the brain.

V. 4. Outlook

It is important to assess the expression patterns of *LRP6*, especially in the Pons and in the cerebellum, and compare them to *LRP5* expression. Indeed, although *LRP5* is known to be expressed in many regions where *Fz3* and *Fz7* are expressed, the expression patterns for *LRP6* are not known. This information is crucial in order to pin-down which receptor complex mediates Wnt7a synaptogenic effects *in vivo*. It is possible that *Fz3* and

Fz7 share a redundant function, but it is more likely that one of these two co-receptors carries a more important role in the synaptogenic signalling in the cerebellum. Although *Fz3* -/- mice have an apparently normal brainstem at E18, analysis of post-natal development of these knock-out is not possible because the mutant pups die at birth following respiratory failure (Wang *et al.*, 2002). *Fz7* -/- mutants have so far not been generated. Post-natal analysis of *Fz7* mutants and *Fz3* +/- (in order for the pups to live) and *Fz7* -/- mutants could be critical in evaluating the *in vivo* function of the receptors identified here in synaptic remodelling in the cerebellum. More simple *in vitro* experiments with primary neurons could be designed to test the signalling activities reported here in 293 cells in cerebellar or pontine neurons. Over-expression of the receptors or complexes identified here (e.g. Fz7 or Fz3/LRP6) in cerebellar granule cells or mossy fibres would perhaps mimic Wnt7a synaptogenic effects on these cultures (Hall *et al.*, 2000; Lucas and Salinas, 1997). Alternatively, neurons expressing *Fz3* or *Fz7* may show an increased response to Wnt7a conditioned medium. Identification of the precise molecular components mediating Wnt signalling in neurons during the formation of neuronal connections remains a challenge. The work presented here will provide the foundations for a line of research in neuronal cultures aiming to establish the role played by Fz3 and Fz7, as well as LRP5/6 co-receptors, in synaptogenesis and dendritogenesis.

Chapter VI

General Discussion and concluding remarks

VI. General discussion and concluding remarks

The work presented in this thesis focused on identifying the Fz receptors mediating Wnt signal transduction in neurons. Wnt factors regulate a number of processes including axonal guidance, dendritogenesis and synaptogenesis (Hall *et al.*, 2000; Lyuksyutova *et al.*, 2003; Rosso *et al.*, 2005; Schmitt *et al.*, 2006). The cell-surface receptors mediating these processes are only beginning to emerge. Wnts require specific receptor complexes to initiate particular cellular responses, such as TCF-mediated transcription or cytoskeletal remodelling (Logan and Nusse, 2004; Lucas and Salinas, 1997; Schmitt *et al.*, 2006). This thesis focused on post-natal Wnt signalling in the brain, in particular in the cerebellum where *Wnt7a* plays a key role in synaptic development and the hippocampus where Wnt7b stimulates dendritogenesis (Hall *et al.*, 2000; Lucas and Salinas, 1997; Rosso *et al.*, 2005). *Fz* gene expression was analysed by *in situ* hybridisation in post-natal brain slices. During post-natal development, *Fz3* and *Fz7* are expressed in the cerebellar system. In particular, *Fz3* is expressed in the Pons, the source of afferent mossy fibres. *Fz3* is also strongly expressed in many brain regions, including the cortex and the hippocampus. Interestingly, in the adult, *Fz3* expression in the neocortex is maintained and *Fz7* expression in the cerebellum is also maintained. The prevalence of *Fz3* expression in the postnatal forebrain were confirmed in a paper published during the course of this study (Shimogori *et al.*, 2004). Subsequently, we showed that Wnt7a bound to the surface of cells expressing Fz7CRD and Fz3CRD, whilst Wnt7b bound Fz3CRD and Fz5CRD. Finally, the candidate receptor/ligand pairs identified in the binding assay were tested for their capacity to activate the canonical Wnt pathway, using a Topflash reporter system under the transcriptional control of the TCF promoter. Both Fz3 and Fz7 were able to significantly increase the cellular response to Wnt7a, although Fz3 required LRP6 to signal effectively in the topflash assay. These results highlight a likely role for *Fz3* and *Fz7* as Wnt7a receptors in cerebellar neurons and mossy fibres. Future experiments using primary neuronal cultures and brain slices should be carried-out to confirm this hypothesis.

VI. 1. Wnt/Fz interactions

In this thesis I have demonstrated that Wnt7a could signal via Fz3 and Fz7. The approach I took, to combine gene expression analysis with biochemical studies of Wnt/Fz interactions, could be used to investigate the receptors mediating Wnt signalling in many other contexts. For example, the receptors mediating the effects of Wnt3 on the terminal arborisation of NT-3 sensitive DRG neurons are unknown and Fz gene expression pattern in the embryonic spinal cord could be the first steps to uncover the receptors for Wnt3 (Krylova *et al.*, 2002). Also, the *Wnt* expressed by cerebellar Purkinge cell (*Wnt3*) may signal retrogradely to granule cells which express *Fz3* and *Fz7,* therefore these two Wnts should be studied in binding assays *(Salinas et al., 1994)*. The lack of specific antibodies targeted to Wnt and Fz, along with the difficulty to produce high quantities of epitope-tagged Wnt3 remain the technical hurdles to overcome in order to carry-out these studies.

Although it has been demonstrated here that it is possible to assign binding of a Wnt to a FzCRD, the influence of the extra-cellular loops of the transmembrane segments of Fz were not taken into account. Furthermore, it is possible the LRP5/6 also contribute to Wnt binding to the receptor complex. In fact, a study in *Drosophila* demonstrated that the extracellular loops of Fz, alongside other components of the receptor complex, could be involved in Wg binding and signal transduction (Chen *et al.*, 2004). Yet, CRD domains are found in most Wnt-interacting proteins such as Fz, sFRP and Ror receptors, and their CRD are necessary to carry they function as Wnt receptors or inhibitors (Mikels and Nusse, 2006; Rattner *et al.*, 1997). Recent evidence has accumulated for a role of the CRD of Fz to bind Wnt to the cell surface and thus enable the recruitment of more Fz receptors, as well as LRP5/6 to the complex which becomes activated upon this assembly (Cong *et al.*, 2004; Liu *et al.*, 2005; Povelones and Nusse, 2005). Therefore, the binding data presented here must be taken with precaution, as full-length Fz receptors expressed in combination with other receptors, may have slightly different binding affinities or specificities *in vivo*. Yet, with the critical importance of specific Wnt/Fz interactions via the CRD domains, establishing which Wnts can bind FzCRD are significant steps forward in establishing the function of individual components of Wnt signalling.

VI. 2. Wnt receptors in post-natal development

Receptor complexes rather than individual receptors tend to transduce signals such as Wnt and Hh (Nusse, 2003). In this thesis, much attention was given to the Fz receptors, which remain to this date the receptors implicated in most, if not all, Wnt signalling events. Nevertheless, LRP5/6 is a necessary co-receptor for canonical Wnt signalling (Pinson *et al.*, 2000; Tamai *et al.*, 2000) and both Fz and Ryk receptors signal during guidance (Liu *et al.*, 2005; Lu *et al.*, 2004; Lyuksyutova *et al.*, 2003; Schmitt *et al.*, 2006). Here, I did not investigate the role of Ryk whereas the importance of LRP6 was highlighted in chapter V. Indeed, LRP6 over-expression was shown to greatly amplify the Wnt signalling response. The response of cells co-expressing *Fz3* and *LRP6* to Wnt7a was particularly striking. Yet, some Wnt may be specifically able to interact with either LRP5 or LRP6, and LRP5 was not tested in this study. For example, Wnt7b activates canonical signalling in cells over-expressing LRP5 but not LRP6 (Wang *et al.*, 2005). Therefore, it remains possible that Wnt7b is able to trigger a canonical response in the presence of both Fz3 and LRP5. Addressing the functional synergism of LRP5 with Fz3 and Fz7 is important to complement my work.

A functional interaction between Fz3 and Wnt7b to activate non-canonical signalling was not studied. Nonetheless, from their high levels of expression in overlapping brain regions, in particular in the hippocampus, *Fz3* and *Wnt7b* constitute a possible receptor-ligand that could mediate the dendritogenic effect of Wnt7b in the hippocampus. Further gene expression patterns need to be analysed in order to establish which receptor complexes may be signalling *in vivo*. It is of critical importance to determine the gene expression patterns for *LRP5, LRP6, Ryk* and *Ror* in the post-natal nervous system to complement our study. This data will become critical to begin the study of receptor complexes over-expression in primary cultures.

The finding that *Fz3* is expressed in the Pontine nuclei, the source of mossy fibres, at the time when mossy fibres reach the cerebellum suggest that Fz3 could mediate Wnt7a signalling at the mossy fibre-granule cell synapse. Indeed, Fz3-expressing mossy fibres form synapses with granule cells which express *Fz7, Fz3* and, most importantly, *Wnt7a* (Lucas and Salinas, 1997). The fact that Fz3 and Fz7 were able to signal by Wnt7a strengthens the hypothesis Fz3 could mediate the synaptogenic effects of Wnt7a in mossy

fibres. In addition, the fact cells co-expressing *Fz3* and *LRP6* strongly activated the canonical / ß-catenin pathway when exposed to Wnt7a suggest that Fz3/LRP6 may be a receptor complex for Wnt7a. In contrast, although Fz7 expression increased the cellular response to Wnt7a, this response was not greatly increased when *Fz7* and *LRP6* were co-expressed. It is therefore possible that Fz7 may utilise another co-receptor, LRP5 for instance, to transduce Wnt7a signalling.

The fact that *Fz3* and *Fz7* are expressed in overlapping patterns by cerebellar granule cells, suggest these receptors may either play overlapping or complementary roles. Fz3 and Fz7 have 47% protein sequence identity therefore it is a strong possibility they may carry overlapping function (protein alignment done using www.expasy.org). Interestingly, expression of *Fz3* in granule cells decreases in the adult whilst *Fz7* expression is maintained. What is the function of *Fz7* and *Fz3* in granule cells? It is possible that the role for these receptors change during cerebellar formation and function. *Fz3* being highly expressed in proliferating cells of the EGL before P10 suggest it could regulate cell division or early differentiation steps. In contrast, *Fz7* is strongly expressed both at early post-natal stages and in mature granule cells. Therefore, Fz7 could be transducing additional signalling events in the granular layer of the adult. For example, Fz7 could act within an autocrine loop within granule cells to either regulate neuronal behaviour or the expression of Wnt pathway components. Indeed, a microarray analysis of Wnt-stimulated cells highlighted that a large number of components of the Wnt signal transduction machinery were regulated by Wnt themselves (Willert *et al.*, 2002). Expression of *Fz7* in neuronal cultures will certainly clarify the role of this receptor in cerebellar neurons.

Granule cells synapse to Purkinge cells and this connection is central to the cerebellar system which co-ordinates movements, posture and balance by processing both sensory and cortical stimuli. *Wnt7a* expression by granule cells has been assigned a function of retrograde synaptogenic signal (Hall *et al.*, 2000), which may be mediated by Fz3 expressed by mossy fibres. Yet, because Wnt7a also affects granule cells in cultures, both *Fz3* and *Fz7* which are expressed in granule cells could be transducing Wnt7a. In addition *Wnt3* is expressed post-natally in cerebellar Purkinge cells, and its expression relies on an interaction with granule cells, since its expression in lost in mutants lack granule cells (Salinas *et al.*, 1994). Wnt signalling may thus not be limited to the mossy

fibre-granule cell relay. Are *Fz3* and *Fz7* expressed in granule cells to respond to *Wnt7a* within an autocrine feedback loop? Perhaps autocrine signalling regulates Wnt7a expression or controls their own maturation. Otherwise it is possible that Fz3 and Fz7 expressed in granule cells mediate a retrograde signal such as Purkinge cell-derived Wnt3 signalling. Furthermore, other *Wnt* and *Fz,* or even *Ryk,* may be expressed in the cerebellar system. Therefore, until the gene expression patterns for all *Wnt, Fz, Ryk* and *LRP5/6* are known, the receptor-ligand pairs signalling *in vivo* cannot be established. Similarly, the cellular localisation of Fz3 and Fz7 remains unkown. It is possible that certain Fz are preferentially targeted to dendrites or axons, and that Fz carry functions which are specific to cellular compartments. Clearly, both further gene expression studies and *in vitro* work with primary neurons may identify novel aspects of Wnt signalling in cerebellar neurons.

Figure VI. 1 is a summary diagram of the findings described in this thesis as well as hypotheses remaining to be verified. Here, we propose that mossy fibres which express Fz3 transduce Wnt7a signalling through the canonical pathway via the Fz3/LRP6 receptor complex. Indeed, this complex is highly efficient at Wnt7a signal transduction but it is not known whether LRP6 is expressed by mossy fibres *in vivo*. We also propose that granule cells could use both Fz7 and Fz3 to mediate Wnt7a signalling. It is not known if *LRP6* is expressed by granule cells, whereas *LRP5* is expressed in the granule cell layer (www.brain-map.org). Since LRP6 and Fz7 was not highly efficient in transducing Wnt7a signals to the canonical pathway, it is possible Fz7 preferentially synergises with LRP5 to transduce Wnt7a signals, but this synergism remains to be tested. It is also highlighted that Fz7 and Fz3 could be expressed in the granule cells axons, the parallel fibres of the molecular layer. These receptors could be transducing Purkinge cell-derived Wnt3 or other Wnts expressed in the cerebellum. Two critical studies should be carried out in order to validate this model. First, establishing the expression patterns of LRP5 and LPR6 in the cerebellar circuit is fundamental. Secondly, a comparative study of the synergism between LRP5/Fz and LRP6/Fz receptor complexes for Wnt7a signalling would shed some light regarding the receptor complexes contributing to Wnt7a signalling in the cerebellum.

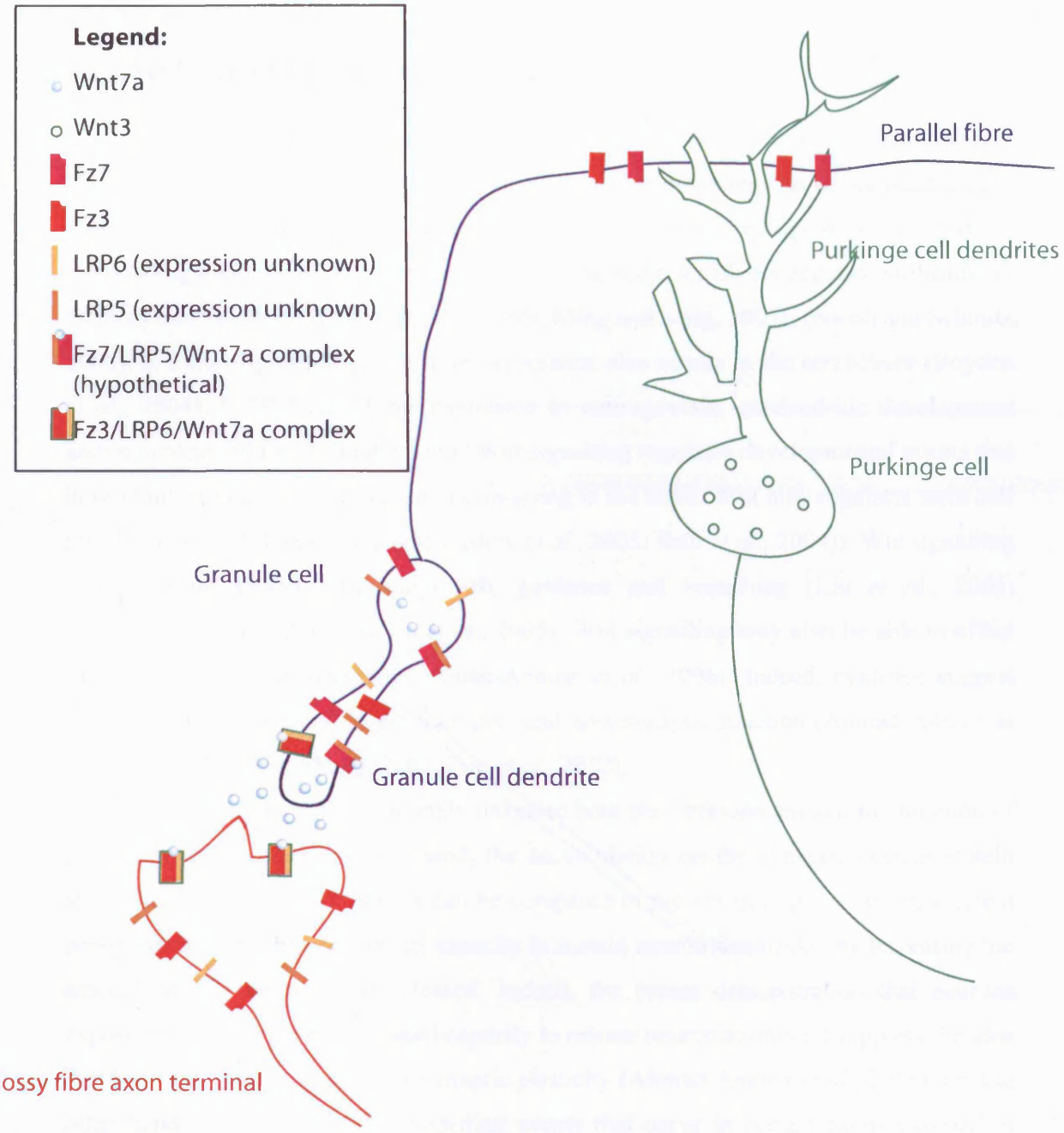


Figure VI. 1. Summary diagram of proposed receptor/ligand interactions in the cerebellar circuit

Incoming mossy fibre axons express *Fz3* and respond the Wnt7a produced by granule cells. Wnt7a binding to Fz3 receptors may act to nucleate a receptor complex composed of Fz3 and LRP6. Our findings suggest that the Fz3/LRP6 can transduce Wnt7a signalling to the canonical / β-catenin pathway in mossy fibres. Yet, expression of LRP5 and LRP6 remains to be investigated. Granule cells express both Fz3 and Fz7 and may use both of these receptors to mediate Wnt7a autocrine signalling. It remains to be clarified whether Fz7 and LRP5 can efficiently co-operate to transduce Wnt7a signalling, our results with Fz7 and LRP6 suggest this co-receptor complex may not be the most efficient at transducing Wnt7a signals. *Fz3* and *Fz7* receptors expressed by granule cells may also contribute to signalling in parallel fibres. Indeed, it is known Purkinge cell fibres express *Wnt3* and that this expression requires the presence of granule cells. Yet, it is not known whether the Wnt3 protein is released by PC dendrites in the molecular layer. It remains possible other Wnt and Fz are expressed by these cells and also contribute to Wnt signalling in the cerebellum.

VI. 3. Wnt signalling in the adult brain

One of the important finding of this thesis is the fact Fz receptors expression is maintained in the adult. *Fz3* has is highly expressed in many regions of the adult mouse brain, in particular in the hippocampus and the neocortex. Neurogenesis and synaptic remodelling continue to occur in these areas in the adult, which are regions implicated in memory and cognition (Eriksson *et al.*, 1998; Ming and Song, 2005); (Nicoll and Schmitz, 2005). A form of plasticity, long-term depression, also occurs in the cerebellum (Boyden *et al.*, 2004). Is Wnt signalling implicated in neurogenesis, axodendritic development and/or synaptic plasticity in the adult? Wnt signalling regulates developmental events that have many similarities with processes on-going in the adult. Wnt may regulates stem cell proliferation or differentiation ((Murashov *et al.*, 2005; Sato *et al.*, 2004)). Wnt signalling can stimulate axodendritic outgrowth, guidance and branching (Liu *et al.*, 2005; Lyuksyutova *et al.*, 2003; Rosso *et al.*, 2005). Wnt signalling may also be able to affect neurotransmission at synapses (Ahmad-Annuar *et al.*, 2006). Indeed, evidence suggest Wnt have the capacity to affect both pre- and post-synaptic function (Ahmad-Annuar *et al.*, 2006; Hering and Sheng, 2002; Luo *et al.*, 2002).

Wnt signalling is increasingly linked to both the development and the function of pre-synaptic terminals. On one hand, the accumulation on the synaptic vesicle protein synapsin-1 in response to Wnt7a can be compared to pre-synaptic potentiation, where a pre-synaptic terminal increases its capacity to sustain neurotransmission by increasing the amount of SV ready to be released. Indeed, the recent demonstration that neurons exposed to Wnts have an increased capacity to release neurotransmitters supports the idea that Wnt signalling may regulate synaptic plasticity (Ahmad-Annuar *et al.*, 2006). On the other hand, the cytoskeletal remodelling events that occur in certain axons exposed to Wnt3 or Wnt7a are reminiscent of the cysoskeletal re-arrangements that occurs during the growth of dendritic spines (Bonhoeffer and Yuste, 2002; Hall *et al.*, 2000; Krylova *et al.*, 2002; Lucas and Salinas, 1997). Therefore, Wnts have the potential to affect many aspects of neuronal behaviour and synaptic function. In fact, the findings that Dvl and Axin may signal locally to stabilise microtubules and that synaptic vesicle are affected within 15min of Wnt exposure emphasise that Wnt signalling can induce rapid changes in specific neuronal compartments (Ahmad-Annuar *et al.*, 2006; Ciani *et al.*, 2004).

Wnt signalling is known to affect both axons and dendrites, it is possible that new anterograde functions of Wnt signalling in regulating post-synaptic structure and activity are possible. Indeed, two findings already highlight the potential for Wnt signalling components to regulate post-synaptic function. Fz may bind PSD-95, a key scaffold component of the post-synaptic density (Hering and Sheng, 2002) and Dvl plays a central role in Agrin-mediated AChR clustering in developing myotubes (Luo *et al.*, 2002). Clearly, there are many potential roles for Wnt signalling in the developing brain and in the adult. Although many other receptors may be involved in Wnt signalling in the brain, this thesis highlights the potential role for Wnt/Fz signalling to affect neuronal connectivity in the adult.

I identified Fz3 and Fz7 as two receptors able to mediate Wnt7a signalling. These proteins may be central to neuronal development and function. Establishing the sub-cellular localisation of Fz receptors will be important to address whether they transduce Wnt signalling locally to pre- or post-synaptic compartments, or whether their activation affects the whole cell. The role of LRP5/6 co-receptors is another key aspect to study within the context of canonical / ß-catenin signalling, and the roles of Ryk and Ror receptors must also be clarified. Wnt signalling is a very complex and rapidly evolving field of study. I hope the findings presented here contribute to clarify the molecular mechanisms that make the nervous system such a fascinating and efficient system.

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Chapter VII

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VII. References

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