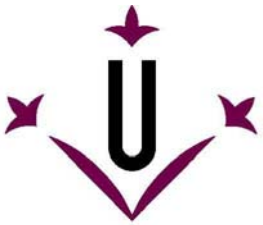

Universitat de Lleida

Departament de Ciències Mèdiques Bàsiques

Differentiation of spinal cord neural precursors and hippocampal neurons: a role for Wnt and β-catenin

Monica Delia David

Doctoral Thesis

Judit Herreros Danés

Supervisor

Judit Herreros Danés, doctor in *Biological Sciences* and *Ramón y Cajal* researcher of the *Departament de Ciències Mèdiques Bàsiques* of *Universitat de Lleida*, as supervisor of this thesis

Hereby states that,

Monica Delia David, who majored in *Medicine* at the *Medicine and Pharmacy University Cluj-Napoca, Romania*, has performed under mi direction and supervision, and within the *Cell Signalling and Apoptosis Group* of the *Departament de Ciències Mèdiques Bàsiques,* the experimental work entitled "**Differentiation of spinal cord neural precursors and hippocampal neurons: a role for Wnt and β-catenin".**

The work accomplishes the adequate conditions in order to be defended in front of the corresponding Thesis Committee and, if it is case, to obtain the **Doctor degree** from *Universitat de Lleida.*

Signed:

Dr. Judit Herreros Danés, *PhD*

Lleida, January 2009

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Abbreviations

ActD	Actinomycin D
Akt	Protein Kinase B
APC	Adenomatous Polysis Coli
BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CaMKII	Calcium/Calmodulin- Dependent Protein Kinase II
cGMP	Cyclic Guanosine Monophosphate
CK1	Casein Kinase 1
CNS	Central Nervous System
CRD	Cystein-Rich Domain
CREB	Cyclic AMP-response Element Binding
DIV	Days *in Vitro*
Dkk	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dymethilsulfoxide
DNA	Desoxirribonucleic Acid
DTT	1,4-Dithiothreitol
DVL	Dishevelled
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
GSK-3β	Glycogen Synthase Kinase 3 β
HBSS	Hank's Balanced Salt Sodium
HS	Horse Serum
JNK	Jun N Terminal Kinase
kDa	kiloDalton
Kremen	Kringle-Containing Transmembrane Protein
LDCVs	Large Dense Core Vesicles
LEF	Lymphoid-Enhancing Factor
LRP	Low Density Lipoprotein Receptor-Related Protein
MAP1B	Microtubule Associated Protein 1B
NF-kB	Nuclear Factor-kappa B
NGF	Nerve Growth Factor
NPC	Neural Progenitor Cell
NEC	Neuroepithelial Cell
NTs	Neurotrophins
PBS	Phosphate Buffer Saline
PCP	Planar Cell Polarity Pathway
PEI	Polyethylenimine
PFA	Paraformaldehyde

PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PLC-γ	Phospholipase C-γ
RT	room temperatura
RTK	Receptor Tyrosine Kinase
SCNP	Spinal Cord Neural Precursor
sFRP	Secreted Frizzled Related Protein
Shc	Src Homology Collagen-Like
Shh	Sonic Hedgehog
shRNA	Short Hairpin RNA
TCF	T-Cell Factor
TEMED	N,N,N',N'-tetramethylethylendiamine
TGF-β	Transforming Growth Factor-β
TLE	Transducin-Like Enhancer of Split
Trk	Tropomyosin Receptor Kinase
WIF	Wnt Inhibitory Factor
WT	Wild Type
RGC	Radial Glia Cell
IPC	Intermediate Progenitor Cell
SNP	Short Neural Precursor
VZ	Ventricular Zone
SVZ	Subventricular Zone

Abstract

Proper development of the nervous system requires a precise coordination of neurogenesis and neuronal morphogenesis. Neural progenitors, exposed to specific local environmental signals that regulate their proliferation and specification, give rise to neurons. Newborn neurons start extending neuronal processes that develop into a complex arbour. This step results in the formation of synapses, usually between axons and dendrites, and the assembly of functional brain networks. Among the extracellular factors that control these processes, Wnts, neurotrophins (NTs) and hepatocyte growth factor (HGF) are of special interes for this work.

Wnts have been shown to regulate proliferation of neural precursors, neurogenesis, terminal neuronal differentiation and synaptogenesis. Classical Wnt signalling involves stabilization of cytoplasmic β-catenin and regulation of transcription by LEF/TCF transcription factors. Here we demonstrate that Wnt-3a and Wnt-3 signalling through the canonical/β-catenin pathway regulate neuronal differentiation (neurogenesis and neuritogenesis) of spinal cord neural precursors.

NTs and HGF belong to different growth factor families signalling through receptor tyrosine kinase (RTK) that are important regulators of neuron survival and differentiation. In this work we show the involvement of β-catenin tyrosine phosphorylation downstream of NTs and HGF signalling during the regulation of axon outgrowth and branching in postmitotic neurons. NTs and HGF signalling phosphorylate β-catenin at different residues (Y654 and Y142, respectively). Interestingly, these differential tyrosine phosphorylations target β-catenin to distinct subcellular locations and activate different downstream mechanisms (TCF-independent and dependent) to regulate axon morphogenesis.

Collectively, this work highlights the importance of β-catenin, as a signalling component of Wnt-dependent and growth factor/RTK pathways, in the control of different aspects of the neuronal differentiation.

Abstract

El correcto desarrollo del sistema nervioso requiere de una precisa coordinación de la neurogénesis y morfogénesis neuronal. La señalización por moléculas específicas del entorno regula la proliferación y especificación de precursores neurales, que se diferencian en neuronas. Durante su diferenciación, las neuronas extienden procesos neuríticos que se ramifican dando lugar a complejos arbóreos. Este paso da como resultado la formación de sinapsis entre axones y dendritas y el ensamblamiento de la red funcional cerebral. Entre los factores extracelulares que controlan estos procesos se encuentran los factores Wnts, las neurotrofinas (NTs) y el factor de crecimiento hepático (HGF), que son de especial interés para este trabajo.

Está demostrado que los factores Wnt regulan la proliferación de precursores neuronales, la neurogénesis, la diferenciación terminal de la neurona y la sinaptogénesis. La vía de señalización clásica por Wnt implica la estabilización de β-catenina citoplásmica y la regulación de la transcripción por los factores de transcripción LEF/TCF. Aqui demostramos que la señalización por Wnt-3a y Wnt-3 a través de la via canónica/β-catenina regula la diferenciación neuronal (neurogénesis y neuritogénesis) de precursores neurales de la médula espinal.

Las NTs y el HGF pertenecen a diferentes familias de factores de crecimiento que señalizan a través de receptores tirosina quinasa (RTK), siendo importantes reguladores de la supervivencia y la diferenciación neuronal. En este trabajo se demuestra la implicación de la fosforilación en tirosina de β-catenina desencadenada por la señalización por NTs y HGF durante la regulación del crecimiento y ramificación del axón en neuronas postmitóticas. La señalización por NTs y HGF induce la fosforilación de β-catenina en diferentes residuos (Y654 y Y142, respectivamente). Es importante esta diferencia en las tirosinas fosforiladas ya que dirige β-catenina a diferentes localizaciones subcelulares y resulta en la activación de diferentes mecanismos (dependientes e independientes de TCF) durante la morfogénesis del axón.

En resumen, este trabajo destaca la importancia de β-catenina como un componente de señalización de las vías de Wnt y de los factores de crecimiento/RTK, en el control de distintos aspectos de la diferenciación neuronal.

Per al correcte desenvolupament del sistema nerviós cal una precisa coordinació entre la neurogènesi i la morfogènesi neuronal. L'exposició de les cèl·lules neurals progenitores a un seguit de senyals específiques de l´entorn, que regulen la seva proliferació i especificació, donarà lloc a les neurones. Les neurones recent formades comencen a extendre processos neuronals fins a formar una complexa estructura arborescent; durant aquesta etapa es formen les sinapsis entre els axons i les dendrites, i s'estableix una xarxa cerebral funcional. Entre els factors extracel·lulars que controlen aquests processos destaquem per la relació amb aquest treball els membres de la família Wnt, les Neurotrofines (NTs) i el Factor de Creixement Hepàtic (HGF).

S'ha demostrat que els factors Wnt regulen la proliferació dels precursors neurals, la neurogènesi, la diferenciació terminal de neurones i la sinaptogènesi. La via de senyalització clàssica per Wnt implica l'estabilització de β-catenina citoplasmàtica i la regulació de la transcripció controlada pels factors de transcripció LEF/TCF. En aquesta tesi demostrem que la senyalització de Wnt-3a i Wnt-3 a través de la via de senyalització canònica regula la diferenciació neuronal (neurogènesi i neuritogènesi) dels precursors neurals de la medul·la espinal.

NTs i HGF pertanyen a diferents famílies de factors de creixement, i senyalitzen a través de receptors tirosina quinasa (RTK), regulant supervivència i diferenciació neuronal. En aquest treball hem demostrat que la senyalització de NTs i HGF durant la regulació del creixement i la ramificació axonal en neurones postmitòtiques implica la fosforilació de β-catenina en tirosina. Les vies de senyalització de NTs i HGF fosforilen β-catenina en diferents residus (Y654 i Y142, respectivament). Es interessant la fosforilació de residus diferents, que dirigeixen β-catenina a diferents localitzacions subcel·lulars i activen diferents mecanismes (dependents o independents de TCF) durant la morfogènesi de l'axó.

En resum, aquest treball remarca la importància de β-catenina, com a un component de la via de senyalització dependent de Wnt i de la via activada per factors de creixement/RTK, en el control de diferents aspectes de la diferenciació neuronal.

Introduction

1. Neurogenesis

The adult vertebrate central nervous system (CNS) is composed by four major cell types: the neurons, the myelin-forming oligodendrocytes, the astrocytes, and the ependymal lining of the ventricle. All of these cell types are generated during development from a common source, the neuroepithelial cells of the neural tube.

Neurons are specialized cells with a complex morphology that represent the functional unit of the nervous system. The process of neuron generation from stem cells is known as neurogenesis.

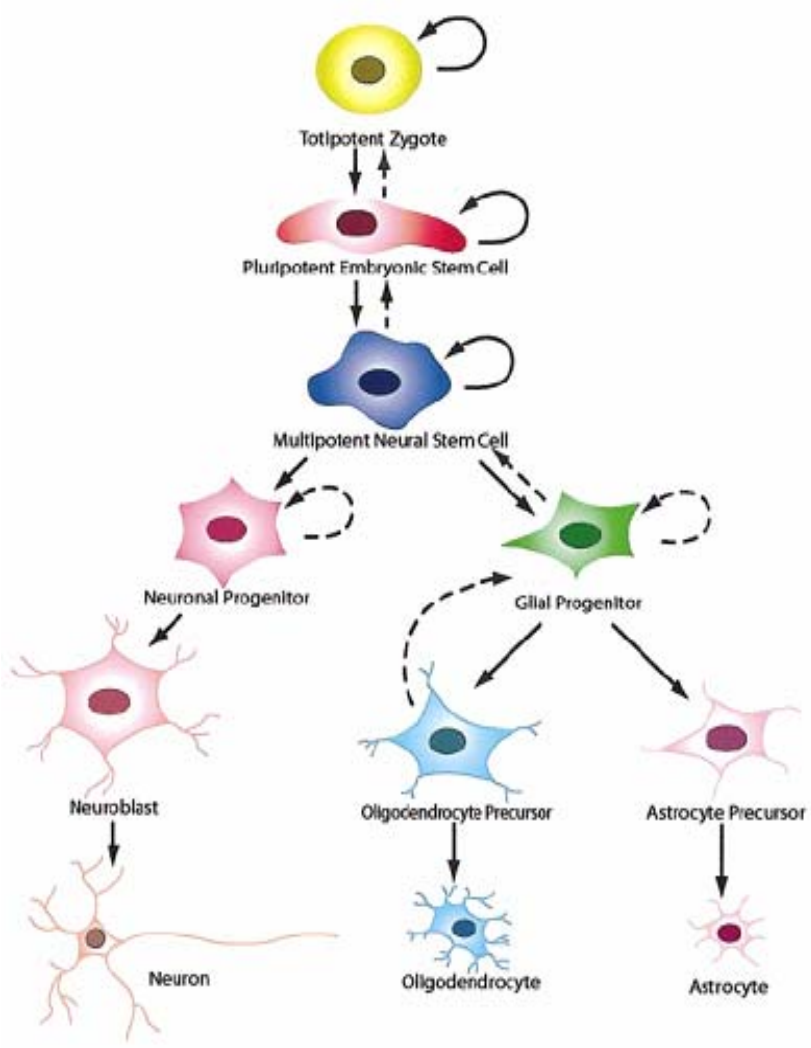

Figure 1. ***Relationships between stem cells, progenitors, precursors, and differentiated neural cell types*** *(Ahmad, 2001). Totipotent cells have total potential. They specialize into pluripotent cells that can give rise to most, but not all, of the tissues necessary for embryonic development. Pluripotent cells undergo further specialization into multipotent cells that are committed to give rise to cells that have a particular function.*

The whole process of neurogenesis involves multiple, sequential steps (Kempermann et al., 2004): neuroepithelial cells proliferate to give rise to rapidly amplifying neural progenitor cells (NPCs); these multipotent NPCs undergo cell-fate determination, and some become committed to the neuronal lineage that then differentiate into immature neurons and migrate to their final locations (Fig. 1).

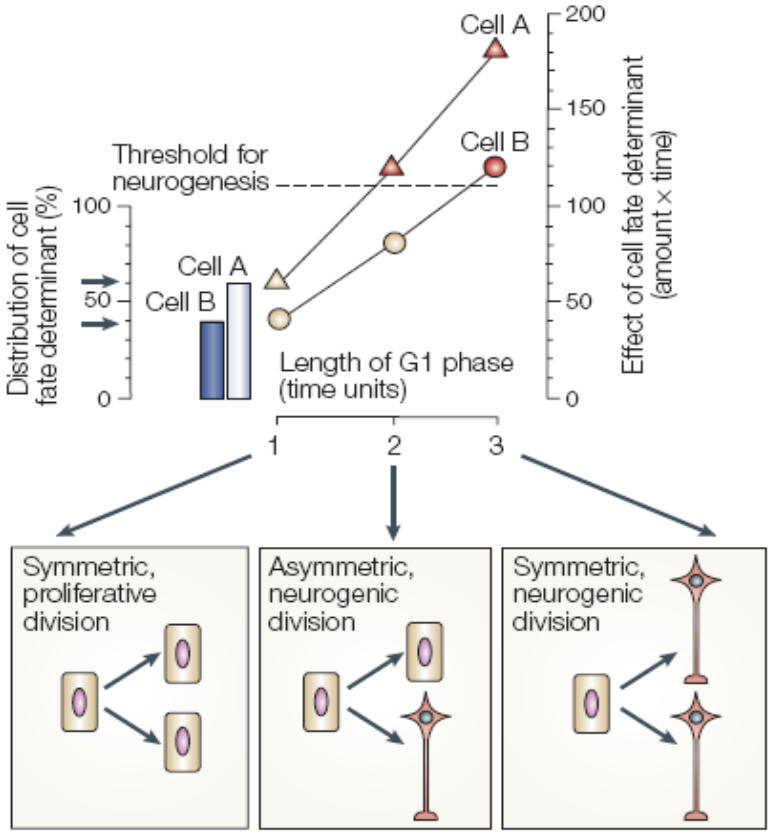

Figure 2. ***The cell-cycle length hipotesis*** *(Calegari and Huttner, 2003). A neurogenic cell fate determinant that, following cell division, is distributed unequally to daughter cells A and B (60% and 40%, respectively) can induce one or both of the daughter cells to become a neuron depending on whether G1 phase is sufficiently long for the cell fate determinant to achieve its neurogenic effect. So, neither cell A nor cell B will become a neuron after one unit of time. Cell A, but not cell B, will become a neuron after two units of time. Both cell A and cell B will become a neuron after three units of time.*

Neuroepithelial cells (NECs) in the neural tube are self-renewing and multipotent cells which can generate intermediate and mature cells of both neuronal and glial lineages (Gage, 2000). NECs change their competences and developmental potential over time. During neurogenesis, there is a progressive increase in neuron production and in the frequency of differentiative divisions. In an early expansion stage, NECs primarily undergo to symmetric divisions to enlarge their cell

population (Alvarez-Buylla et al., 2001). As development proceeds, NECs undergo to differentiative (symmetric and asymmetric) divisions giving rise to cells that progressively initiate the expression of proneural genes. It has been proposed that symmetric and asymmetric divisions are related to the length of G1 phase. A prolonged G1 phase coud be a characteristic feature of differentiative divisions, facilitating the integration of extrinsic signals that influence cell fate and/or allowing an unequally inherited cell-fate factors to act over a sufficient time period (Calegari and Huttner, 2003; Gotz and Huttner, 2005) (Fig. 2). With the initiation of proneural gene expression, cells in the neural tube become heterogeneous, including neuronal progenitors and NECs which express and do not express proneural genes, respectively (Gage, 2000). Subsequently, neuronal progenitors exit the cell cycle and differentiate into postmitotic neurons, whereas NECs remain in an undifferentiated state and give rise to later born cell types (Temple, 2001). Newborn neurons develop functional neuronal properties, differentiate axons and dendrites and gradually develop the capacity to fire action potentials and receive synaptic inputs. Ultimately, these cells mature into fully functional neurons that are integrated into a neural network (Song et al., 2002; van Praag et al., 2002).

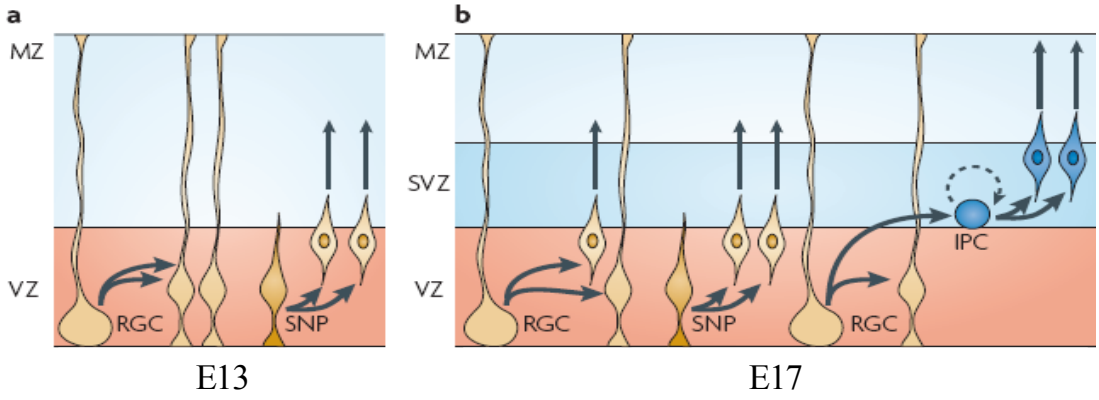

Figure 3. ***The proliferative behaviour and progeny of cortical progenitors*** *(Dehay and Kennedy, 2007). Schematic transects of the rodent cortex at embryonic day 13 (E13) (**a**) and at E17 (**b**). Cortical neurons are generated from precursors: radial glia cells (RGCs), short neural precursors (SNPs) and intermediate progenitor cells (IPCs). RGCs and SNPs divide at the apical surface of the ventricular zone (VZ) (**a,b**). RGCs undergo several types of symmetrical and asymmetrical divisions including self-renewing ones (**a**) or neurogenic divisions (**b**). SNPs are committed neural precursors (**a,b**). IPCs divide away from the ventricular surface in the VZ and in the subventricular zone (SVZ) (**b**). IPCs have been reported to undergo mostly neurogenic divisions with a small fraction undergoing symmetrical proliferative divisions. Through asymmetrical divisions, RGCs give rise to IPCs that migrate to the SVZ (**b**). The VZ generates lower layer neurons (red) and the SVZ generates upper layer neurons (blue).*

Studies in the developing cerebral cortex, classified the neural progenitors into two principal groups depending on whether they undergo mitosis at the apical or basal side of the ventricular zone (VZ). NECs and the related cells they transform into, the radial glial cells (Kriegstein and Gotz, 2003; Gotz and Huttner, 2005) and the recently described short neural precursors (Gal et al., 2006; Mizutani et al., 2007), undergo mitosis at the luminal surface, which corresponds to their apical side, therefore collectively named apical progenitors. Neuronal progenitors dividing in the basal region of VZ and in the subventriculat zone (SVZ), generically referred to as basal progenitors, have been called intermediate (Noctor et al., 2004; Kriegstein et al., 2006), basal (Haubensak et al., 2004; Gotz and Huttner, 2005) or SVZ (Miyata et al., 2004) progenitors (Fig. 3).

1.1. The role of morphogens in neurogenesis

Every step during the neurogenesis should be strictly controlled. Proper development of tissues requires appropriate growth and differentiation of each constituent cell according to its position within that tissue (Wolpert, 1989). Proliferation and maintenance of the progenitors pool is essential for normal neural development, as precocious neurogenesis would allow generation of early born cell types only and disorganize the shape and cytoarchitecture of the brain. Proper development of tissues requires appropriate growth and differentiation of each constituent cell according to its position within that tissue (Wolpert, 1989).

The acquisition of a specific neural fate depends on the initial spatial coordinates of a precursor cell within the neural plate. This initial position defines the exposure of a progenitor cell to specific local environmental signals (morphogens) that progressively restrict its development potential. Morphogens are secreted proteins that, diffusing away from their sources, induce distinct cellular responses in a concentration-dependent manner. Morphogens direct cell fate by activating the expression of transcriptional regulators, which in turn, control the genetic network necessary for the proper function of each neuronal cell (Jessell, 2000; Briscoe and Ericson, 2001; Helms and Johnson, 2003). Members of fibroblast growth factor (FGF), Wnt, hedgehog (Hh), and transforming growth factor β (TGF-β) families appear to work as morphogens in specific cellular contexts (Kiecker and

Niehrs, 2001; Freeman and Gurdon, 2002; Smith and Gurdon, 2004; Bovolenta, 2005; Mehlen et al., 2005). In the vertebrate CNS development, possibly one of the best examples of a morphogen type of function is provided by the antagonistic role of bone morphogenetic proteins (BMPs) and Sonic Hedgehog (Shh) to specify neuronal identities along dorso-ventral axis of the developing spinal cord (Marti et al., 1995a). Restricted progenitors will also differ according to their position along the rosto-caudal and dorso-ventral axis of the neural tube. Signalling along rosto-caudal axis of the neural tube (FGF, Wnt, retinoic acid (RA)) establishes the main subdivisions of the CNS: the forebrain, midbrain, hindbrain and spinal cord (Lumsden and Krumlauf, 1996). The dorso-ventral signalling system (Wnt, Shh, BMPs, RA) has a more prominent role in establishing cell type diversity within each of these rosto-caudal subdivisions (Pituello, 1997).

Changing signalling of paraxial mesoderm, from FGF secreted by presomitic mesoderm to RA produced by somites, controls the switch between proliferation and the onset of neural differentiation in the extending vertebrate body axis (Diez del Corral et al., 2003; Diez del Corral and Storey, 2004).

Shh is secreted by floor plate and notochord cells (Marti et al., 1995b) and is essential for the establishment of the ventral pattern along the entire neural axis. Shh can induce the differentiation of floor plate, motoneurons, and ventral interneurons (Echelard et al., 1993; Marti et al., 1995a; Roelink et al., 1995; Ericson et al., 1996). Progressive changes in Shh concentration generate five molecularly distinct classes of ventral progenitors cells at defined positions within the ventral ventricular zone, characterized by the expression of different homeodomain and basic helix-loop-helix transcription factors (Jessell, 2000; Briscoe and Ericson, 2001). In addition to this fundamental role played in pattern formation of the ventral CNS, Shh has been demonstrated to have a mitogenic role in the development of dorsal brain structures, including the cerebellum (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Pons et al., 2001), neocortex and tectum (Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004) and the maintenance of neural stem cells in late development and adult CNS (Lai et al., 2003; Machold et al., 2003).

Multiple BMPs, as well as non-BMP members of TGF-β family, are expressed in the roof plate and in the dorsal neural tube and are able to induce dorsal

phenotypes in vitro (Basler et al., 1993; Liem et al., 1997). A gradient of BMP-dependent positional information extends throughout the entire dorsal-ventral neural tube controlling neuronal determination (Barth et al., 1999; Nguyen et al., 2000). BMP signalling might also control proliferation of neuronal precursors.

Wnts are important regulators of neurogenesis. In the spinal cord Wnt-1 and Wnt-3a specify dorsal interneurons (Muroyama et al., 2002; Murashov et al., 2005). Wnt-5a induces the differentiation of the ventral midbrain dopaminergic neuron (Castelo-Branco et al., 2003; Castelo-Branco et al., 2006). Furthermore, Wnt-3 signalling promotes adult hippocampal neurogenesis (Lie et al., 2005).

There is little cell division in the adult nervous system of vertebrates, and in the most areas, the final number of neurons is determined early in development. In the adult mammalian central nervous system, generation of new neurons from stem cells is restricted to two regions (neurogenic niche): the subventricular zone of the lateral ventricle which generates neurons destined for the olfactory bulb, and the subgranular zone of the hippocampal dentrate gyrus. Neurogenesis has recently been observed within a perivascular niche in the SVZ (Palmer et al., 2000; Tavazoie et al., 2008). Within the adult neurogenic niche, a subset of glial fibrillary acidic protein (GFAP)-expressing astrocytes (type B cells) are stem cells (Doetsch et al., 1999; Laywell et al., 2000; Imura et al., 2003; Garcia et al., 2004; Sanai et al., 2004; Ahn and Joyner, 2005). Stem cell astrocytes divide to give rise to transit-amplifying type C cells, which, in turn, generate neuroblasts (type A cells) (Doetsch et al., 1999). Neuroblasts migrate as chains through a network of pathways extending along the length of the VZ and SVZ and once reaching their destination they differentiate into neurons (Lledo et al., 2008). Adult neurogenesis can be modulated by physiological and behavioral events such as aging, stress, seizures, learning, and exercise (van Praag et al., 1999b; van Praag et al., 1999a).

1.2. Establishment of the neuronal morphology

After they are born and differentiate, neurons break their previous symmetry, dramatically change their shape, and establish two structurally and functionally distinct compartments: axons and dendrites. Axons and dendrites of neuronal cells differ from each other in the composition of their proteins and organelles. Axons are

typically long and thin, with a uniform width, and they branch at right angles from the cell body. Dendrites are relatively short; as they emerge from the cell body they appear thick, but become thinner with increased distance from the cell body and then undergo Y-shaped branching (Craig and Banker, 1994; Fukata et al., 2002). Axons contain synaptic vesicles from which they release neurotransmitters at axon terminals in response to electrical signals. Dendrites, especially dendritic spines, contain receptors for these neurotransmitters. Axons and dendrites are fundamental for neuronal function, as they enable neurons to receive and transmit electrical signals.

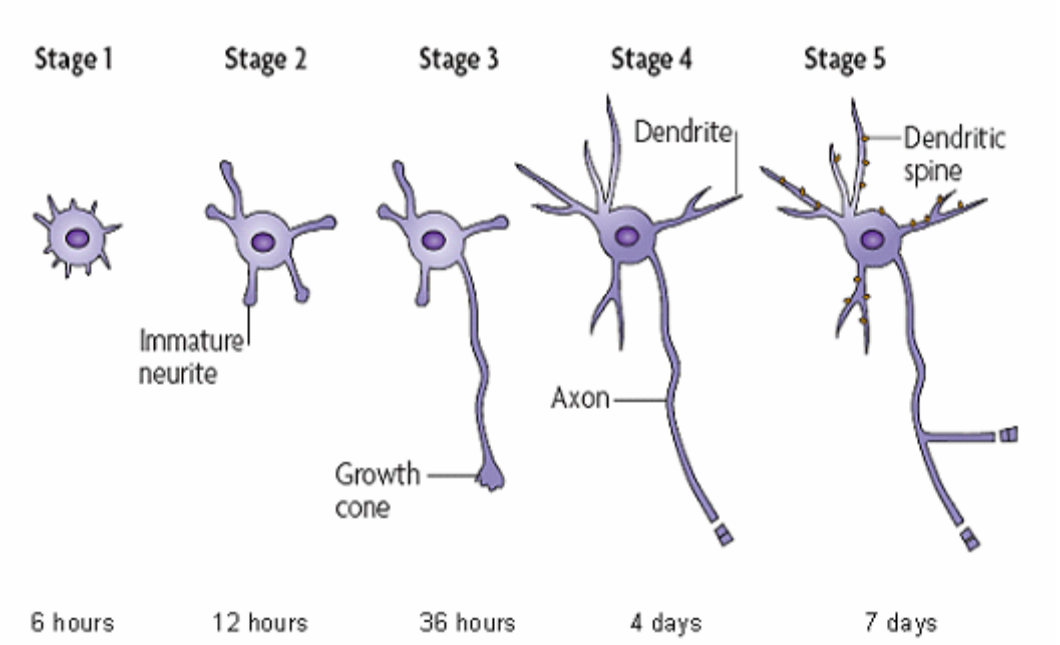

Figure 4. ***Changes in neuronal morphology during neuronal polarization*** *(Govek et al., 2005a; Arimura and Kaibuchi, 2007). Schematic representation of neuronal polarization in cultured rat embryonic hippocampal neurons. Shortly after plating, the neurons form small protrusion veils and a few spikes (stage 1). These truncated protrusions have growth cones at their tips, and develop into several immature neurites (stage 2). One neurite then starts to break the initial morphological symmetry, growing at a rapid rate, and immediately establishing the polarity (stage 3). A few days later, the remaining neurites elongate and acquire the characteristics of dendrites (stage 4). Approximately seven days after plating, neurons form synaptic contacts through dendritic spines and axon terminals, and establish a neuronal network (stage 5)*

Cultured rat hippocampal neurons have been extensively used to study the establishment of neuronal polarity. The morphological changes that occur during polarization are divided into five stages (Fig. 4). First, few hours after plating, hippocampal neurons form several thin filopodia (stage 1). After several hours, the neurons form a number of immature neurites, so-called ‘minor processes’ (stage 2).

These neurites are morphologically equal, and undergo repeated, random growth and retraction. Half a day after plating, one of these minor processes begins to extend rapidly, becoming much longer than the other neurites (Ruthel and Hollenbeck, 2000; Jacobson et al., 2006). This extended process becomes an axon; the other minor processes continue to undergo brief spurts of growth and retraction, maintaining their net length, for up to a week, when they then become mature dendrites (stage 4). During this process, dendrites become thicker and shorter than the axon and begin to establish dendritic components and to construct premature dendritic spines (stage 5). When the axon and dendrites are mature, neurons form synaptic contacts that enable the transmission of electrical activity (da Silva and Dotti, 2002; Govek et al., 2005a; Arimura and Kaibuchi, 2007).

Studies of neurogenesis *in vivo* highlight the fact that most neuroblasts break the original sphere to make a solitary neurite, usually the further axon, which will serves as a guide for migration. Once the migrating neuron has reached its final position in the brain, the neuronal spheres breaks again to make more neurites, which will become the dendrites (da Silva and Dotti, 2002).

Actin cytoskeleton changes are required for migratory behaviour of cells in response to growth factor stimulation or matrix interaction. Neurite extension and retraction are controlled by positive and negative signalling molecules respectively, secreted and adhesion molecules, which regulate the actin filaments and microtubules (Andersen and Bi, 2000). β-catenin, a member of the adhesion complex, is present at high levels in neuronal processes early in development, well before synaptic proteins are detected (Benson and Tanaka, 1998). It has been shown that overexpressing β-catenin and other members of the cadherin-catenin complex enhances neurite outgrowth (axonal growth and branching and dendritic arborisation), whereas sequestering endogenous β-catenin prevents the enhancement of axon and dendritic morphogenesis (Yu and Malenka, 2003, 2004).

Sprouting and elongation of axons and dendrites, at the right time and in the right direction, forms the basis of proper neuronal connectivity and, consequently, brain function. Mutations that affect this process cause diseases in humans, ranging from varying degrees of mental retardation to severe heterotopias that lead to early death (Ramakers, 2002; Chechlacz and Gleeson, 2003).

2. Wnt signalling

In 1982, Nusse and Varmus reported the identification of a proto-oncogene *Wnt-1* (originally called Int-1) as a signalling molecule involved in the development of mammary tumors. Since then, numerous Wnt proteins have been described including 19 human and murine Wnt proteins. Wnts are secreted glycoproteins with an unusual post-translational modification, palmitoylation at a conserved cysteine, that is essential for their function (Willert et al., 2003). Wnts are important in the regulation of almost every aspect of neural development including patterning of the neural tube, neural stem cell maintenance, proliferation, fate determination, axon guidance, dendrite development, and synapse formation. They can influence tissue organization and growth by functioning locally, in an autocrine manner or on immediately adjacent cells (paracrine), and can also act at a distance, by generating a gradient across a tissue.

Wnt effects are highly context dependent. Wnts are potent morphogens promoting the expansion and maintenance of stem cell populations, proliferation or differentiation of neuronal progenitor cells depending of the stage of neural development (Nusse, 2008). In post-mitotic neurons, Wnts control axon behaviour (axon outgrowth and guidance, axon remodelling, growth cone enlargement) and synapse formation (Ciani and Salinas, 2005).

A recent work using Axin-reporter mice uncovered a role for Wnt signaling as an important regulator of stem cell self-renewal in the developing brain, suggesting that Wnt signaling is a hallmark of self-renewing activity *in vivo* (Kalani et al., 2008). Studies using the glycogen synthase kinase-3β (GSK-3β) specific inhibitor Bio (Sato et al., 2004) or the Wnt antagonist soluble Frizzled related proteins (sFRPs) (Aubert et al., 2002) also demonstrate the importance of Wnt canonical pathway to maintain the pluripotency of embryonic stem cells. Wnt-3a, a "classical" Wnt member, but not Wnt-11 supports self-renewal of embryonic stem cells (Singla et al., 2006). Consistently, Wnt-11 mediate repression of canonical Wnt signalling pathway (Maye et al., 2004).

In neuronal committed cell, Wnts are important regulators of the proliferation, specification and differentiation of NPCs in different brain regions. Wnt-3 derived from hippocampal astrocytes signalling by Wnt/β-catenin pathway promotes adult

hippocampal neurogenesis (Lie et al., 2005). Wnt-1 and Wnt-3a play a role in dorsal interneuron specification in the spinal cord (Muroyama et al., 2002; Murashov et al., 2005). Ectopic expression of Wnt-1 in transgenic mice causes an overgrowth of the neural tube without altering the primary patterning of cell identities along the dorso-ventral axis (Dickinson et al., 1994). In the absence of Wnt-1, neural precursor populations in the developing mid/hindbrain fail to expand, leading to an almost complete loss of the mid/hindbrain region (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Wnt-3a is required to promote the proliferation of cortical progenitor cells at the caudomedial margin of the cerebral cortical neuroepithelium (Lee et al., 2000a). Wnt-5a plays a pivotal role in the ventral midbrain dopaminergic neuron differentiation (Castelo-Branco et al., 2003; Castelo-Branco et al., 2006; Andersson et al., 2008). A clear example of stage-specific Wnt effect is Wnt-7a that promotes proliferation and suppresses neuronal differentiation of cortical NPCs dissected from E10.5 embryos, while promotes neuronal differentiation in cortical NPCs derived from E13.5 embryos (Hirabayashi et al., 2004).

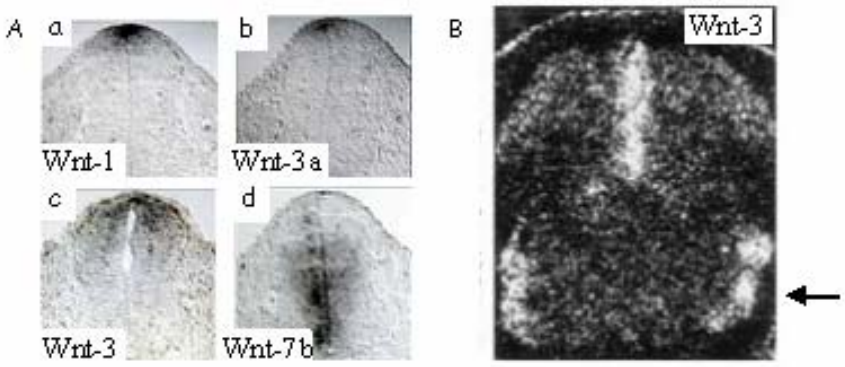

Figure 5. ***Wnt expression in the spinal cord.*** *A) Wnts expression in E 9.5 chick. Wnts expressed in the dorsal midline of spinal cord (Wnt-1, Wnt-3a) have an important proliferative effect, while the non-dorsal Wnts (Wnt-3, Wnt-7b) do not influence proliferation of neuronal precursors (a,b) Wnt-1 and Wnt-3a have similar expression patterns in the dorsal midline. (c) Wnt-3 is expressed in broad dorsal domains. (d) Wnt-7b is expressed in broad ventral domains (Megason and McMahon, 2002). B) Wnt-3 expression in E 13.5 mice. Wnt-3 expression was found in the VZ and in the lateral motor column(arrow) at cervical and lumbar levels (Krylova et al., 2002).*

In the spinal cord, Wnt proteins have been identified as components of roof-plate signalling (Fig. 5). *Wnt-1* and *Wnt-3a* are both expressed soon after the neural plate forms in the dorsal third of the neural tube. The expression of *Wnt-1* and *Wnt-3a* is restricted to the dorsal midline of the neural tube by E9.5, and remains only in

the dorsal midline throughout morphogenesis of the spinal cord. *Wnt-3* and *Wnt-4* are also initially expressed in the dorsal third of the spinal cord, but as development proceeds their expression broadens ventrally to include the entire dorsal half of the VZ. *Wnt-7a* and *Wnt-7b* are initially expressed at low levels throughout much of the VZ and their expression increases at intermediate-ventral levels with time, until they are expressed at high levels throughout the ventral three quarters of the VZ (Parr et al., 1993; Hollyday et al., 1995; Megason and McMahon, 2002) (Fig. 5).

Studies in the cerebellum led to the discovery that Wnts modulate axonal behaviour (Lucas and Salinas, 1997). Wnt-7a expressed by cerebellar granule cells functions as a retrograde signal to regulate the terminal arborization of axons and presynaptic differentiation of the granule cells (Hall et al., 2000; Ahmad-Annuar et al., 2006). In proprioceptive NT-3-responsive sensory neurons, Wnt-3 expressed by motoneurons at limb levels, induces axon branching and increases growth cone size (Krylova et al., 2002). Wnt-3a rapidly reduces the rate of axonal extension and subsequently increases membrane protrusion, also causing a significant increase in growth cone size (Purro et al., 2008). Wnts function as repulsive and attractive signals during axon guidance. The first evidence of a role of Wnts in axon guidance came from studies of the *D. melanogaster* Wnt-5 (Yoshikawa et al., 2003; Fradkin et al., 2004). In vertebrates Wnt-4 signalling stimulates axon outgrowth and induces turning of commissural axons (Lyuksyutova et al., 2003).

The finding that Wnts increased axonal branching led to the suggestion that Wnt signalling could also regulate dendritic branching. Wnt-7b, expressed in the mouse hippocampus, has been shown to increase dendritic outgrowth and arborization of hippocampal neurons during dendritogenesis by signalling through Dishevelled (DVL), Rac and Jun N terminal kinase (JNK) (Rosso et al., 2005).

Several studies demonstrate that Wnts regulate synapse formation in mammals and at the neuromuscular junction of *D. melanogaster* (Krylova et al., 2002; Packard et al., 2002). Wnt-7a was shown to increase synapsin I clustering when added to mossy fibre axons (Lucas and Salinas, 1997). More recent studies illustrate the role of Wnt signalling regulating synaptic physiology (Hall et al., 2000; Ahmad-Annuar et al., 2006).

The ability of Wnt proteins to control these biologically divergent and

sometimes opposing processes during neural development may be explained by their great potential to elicit several signalling cascades and coordinate multiple cellular signals, their different regulation of downstream molecular targets, and the specific cellular contexts that modulate different Wnt effects.

2.1. Wnts receptors

The main receptors of Wnts, the Frizzled proteins, consist of at least ten family members that share a highly conserved seven transmembrane domain separated by short extracellular and cytoplasmic loops, a cysteine-rich extracellular N-terminal domain and a less conserved cytoplasmic domain of variable size. An additional level of complexity is provided by the fact that a single Wnt can bind to different Frizzled proteins with different affinities, suggesting that the specific action of Wnt might be determined by the individual Frizzled receptor that is expressed in the target cell. The interaction of Frizzled proteins with their co-receptors, low-density-lipoprotein receptor-related protein 5 (LRP5) and LRP6, a single-pass transmembrane protein, is specifically required for signalling through the canonical Wnt pathway. A third identified coreceptor for Wnt is a transmembrane tyrosine kinase receptor from the Ryk family (Halford and Stacker, 2001). The kinase domain of Ryk is atypical because it contains mutations in the evolutionarily conserved tyrosine kinase residues (Hovens et al., 1992; Yee et al., 1993) and lacks protein tyrosine kinase activity. Ryk proteins contain an extracellular domain with homology to Wnt inhibitory factor (WIF1) and a conserved PDZ-binding motif, which links Ryk to downstream molecules of the canonical Wnt pathway, such as DVL. Ryk can also activate the non-canonical pathway through Frizzled-independent signalling cascades; but the signalling events downstream of these cascades are not known. Recently, it has been shown the importance of Ryk cleavage in neuronal differentiation, the cleaved intracellular domain of Ryk translocates into the nucleus to induce neurogenesis in response to Wnt-3 signalling (Lyu et al., 2008).

2.2. Extracellular modulators of Wnt signalling

A common theme in many signalling pathways is the presence of regulatory proteins capable of dampening down the effects of the ligands. On this level, the Wnt

activity is controlled by transmembrane as well as secreted proteins. Secreted antagonists include sFRPs, members of the Dickkopf (Dkk) family of proteins, Cerberus and WIF proteins, which inhibit Wnt signalling either by binding directly to Wnts or to the receptor components (Kawano and Kypta, 2003). The sFRP proteins contain the cystein-rich domain (CRD) of the Frizzled family that is sufficient and necessary for Wnt binding, but no transmembrane regions (Hoang et al., 1996). Therefore, they are able to bind and sequester Wnts preventing their interaction with Frizzled and LRP5/6 receptors and inhibiting both canonical and non-canonical signalling (Finch et al., 1997; Leyns et al., 1997; Hsieh et al., 1999). Dkk proteins such as Dkk1 interact with LRP5/6, which prevents the formation of the complex between Wnt, Fz and LRP and, therefore, they specifically inhibit the Wnt canonical pathway (Glinka et al., 1998). Another level of control is achieved through the interaction of Dkk1with the kringle-containing transmembrane protein 1 (Kremen1) and Kremen2 receptors, the Dkk1-LRP5/6-Kremen complex is internalized and degraded, thus depleting the cell surface from the LRP5/6 coreceptor, and inhibition of the canonical pathway. Dkk family is formed by three members. While Dkk1 acts as a specific Wnt antagonist, Dkk2 also binds to LRP5/6, but depending on the cellular context it can either activate or inhibit the canonical pathway (Li et al., 2002). Dkk3 fails to bind to LRP5/6 and inhibit Wnt signalling (Krupnik et al., 1999). Cerberus functions as a more general, multivalent growth-factor antagonist, as in addition to Wnt it binds to Nodal and BMP ligands via independent sites (Piccolo et al., 1999). WIF1 also appears to antagonize Wnt signalling by sequestering Wnts from their Frizzled receptors; however in contrast to sFRPs, it lacks a CRD-like domain (Hsieh et al., 1999). The regulation of the Wnt pathway at the extracellular level becomes even more complex considering that depending on the context, extracellular inhibitors can also enhance signalling by facilitating Wnt secretion, transport or stability (Logan and Nusse, 2004). In addition, canonical Wnts are sensitive to the action of other non-canonical Wnts, which are cabable of interfering with the canonical pathway (Logan and Nusse, 2004; He and Axelrod, 2006). For example, Wnt-5a can antagonize the Wnt-3a canonical pathway via the tyrosine kinase receptor Ror2 (Mikels and Nusse, 2006).

2.3. *Wnt pathways*

Wnts signal through three main pathways: the canonical Wnt/β-catenin, the planar cell polarity (PCP) and the calcium pathway (Fig. 6).

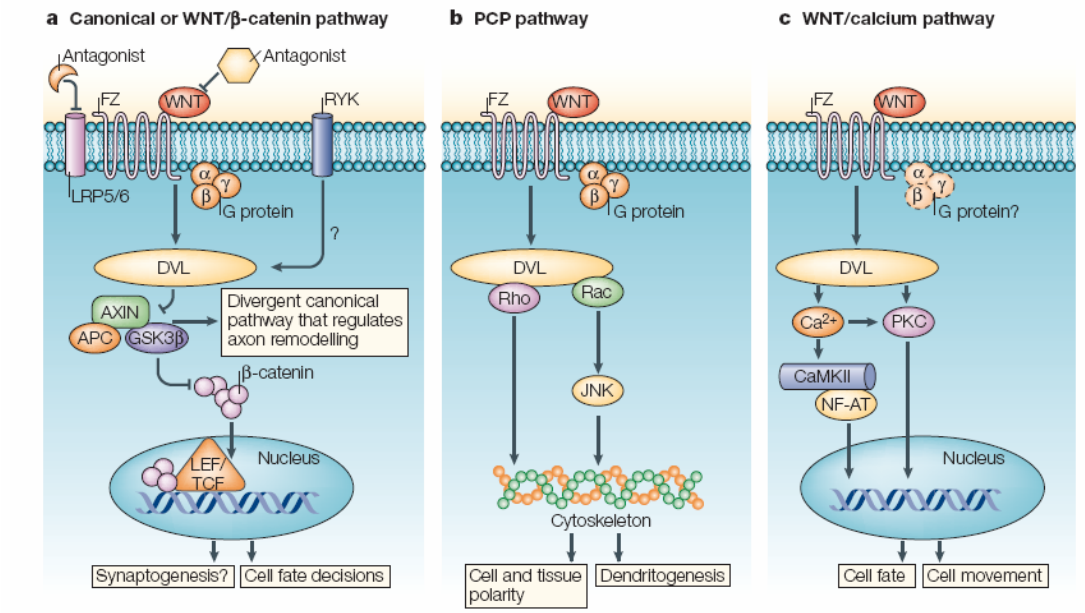

Figure 6. ***The three main branches of the Wnt signalling pathway*** *(Ciani and Salinas, 2005). a) In the canonical or Wnt/β-catenin pathway binding of Wnt to the Frizzled (FZ) and low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 receptor complex activates Dishevelled (DVL). Activation of DVL results in the inhibition of glycogen synthase kinase 3 β (GSK-3β) and accumulation of β-catenin in the cytoplasm by inducing the disassembly of the destruction complex that is formed by adenomatosis polysis coli (APC), AXIN and GSK-3β. Subsequently, β-catenin translocates to the nucleus where it activates transcription mediated by T-cell specific transcription factor (TCF)/ lymphoid-enhancing factor (LEF). A pathway that diverges downstream of GSK-3β has also been shown to control microtubule dynamics. b) In the planar cell polarity (PCP) pathway, FZ functions through G proteins to activate DVL, which, in turn, signals to Rho GTPases (Rho or Rac or both). Activation of Rho GTPases induces changes in the cytoskeleton. c) In the Wnt/calcium pathway, activation of DVL induces the release of intracellular calcium and activation of protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII).*

2.3.1. *Canonical Wnt/β-catenin pathway*

In the absence of a Wnt signal, free cytoplasmic β-catenin is recruited in a large "scaffolding" complex consisting of Axin, APC (adenomatous polyposis coli), casein kinase 1 (CK1) and the serine/threonine kinase GSK-3β. GSK-3β of the complex phosphorylates β-catenin triggering its subsequent destruction by the proteasome machinery. In the presence of Wnt, binding of the ligand to a Frizzled receptor and the LRP5/6 coreceptor triggers the recruitment of the two cytoplasmic

components, DVL and Axin, respectively. GSK-3β is then released from the scaffolding complex, resulting in accumulation of unphosphorylated β-catenin, which enters the nucleus and forms complexes with members of the TCF and LEF family transcription factors. In the absence of β-catenin, TCF/LEF proteins act as transcriptional repressors by forming complexes with Groucho/TLE (transducin-like enhancer of split) proteins. The interaction with β-catenin converts these repressors into activators, which via recruitment of diverse coregulatory molecules, induce the expression of downstream target genes. The ability to switch between a repressor and an activator status is a regulatory strategy that appears to be utilized also by other transcription factors mediating signalling (Barolo and Posakony, 2002) and establishes a tight control of target gene expression at the transcriptional level (van Es et al., 2003). Wnt signalling through the canonical pathway regulates expression of Gli3, a transcription factor that regulates the response to Shh, thus controlling the balance between Shh and Wnt signalling important to pattern the spinal cord along its dorso-ventral axis (Jacob and Briscoe, 2003; Alvarez-Medina et al., 2008). Wnt signalling has been shown to regulate the cytoskeleton through a branch of the canonical pathway that diverges downstream of GSK-3β. DVL binds to microtubules (Krylova et al., 2000) and, through inhibition of GSK-3β, changes the organization of microtubules and increases microtubule stability (Krylova et al., 2000). DVL signals locally to regulate the phosphorylation of GSK-3β targets, such as microtubule-associated protein 1B (MAP1B) - a protein that regulates microtubule dynamics. Signalling to microtubules is independent of TCF-mediated transcription (Ciani et al., 2004; Purro et al., 2008). This divergent branch has recentlly been demonstrated to signal driving *Drosophila* neuromuscular junction synapse formation (Miech et al., 2008).

Canonical Wnt pathway controls early cell fate decisions and it has been suggested a role in neurite outgrowth (Lu et al., 2004).

2.3.2. Non-canonical pathways

In non-canonical signalling, Wnts still bind to Frizzled receptors to activate Dvl, but the downstream pathways activated do not involve GSK-3β or β-catenin.

Planar cell polarity pathway

In the Wnt/PCP pathway, Wnt proteins bind to Frizzled receptors on the cell surface and activate Rho/Rac small GTPases (Habas et al., 2003) or the JNK kinase (Moriguchi et al., 1999), thereby regulating gene expression and subsequent cytoskeletal organization in crucial morphogenetic events during development (Veeman et al., 2003; Wang and Malbon, 2003; Kuhl, 2004). The precise orchestration of these signalling events is not yet fully characterized. PCP is involved in the cell and tissue polarity and dendritogenesis (Rosso et al., 2005).

Calcium pathway

In the Wnt/Ca2+ pathway, the binding of Wnts to Frizzled receptors stimulates heterotrimeric G proteins and phospholipase C, thereby increasing intracellular Ca2+ release and decreasing cyclic guanosine monophosphate (cGMP) levels. This results in activation of Ca2+-calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC), which in turn can stimulate nuclear factor AT and other transcriptional regulators (Veeman et al., 2003; Wang and Malbon, 2003; Kuhl, 2004). Wnt/Ca2+ pathway is involved in the cell movement (Kohn and Moon, 2005).

It is not yet clear whether the PCP and Wnt/Ca2+ pathways are overlapping or represent distinct, context-specific non-canonical branches.

Wnt signalling has been studied primarily in developing embryos, in which cells respond to Wnts in a context-dependent manner through changes in survival and proliferation, cell fate and movement. But Wnts also have important functions in adults, and aberrant signalling by Wnt pathways is linked to a range of diseases like Crohn´s disease (Gersemann et al., 2008), schizophrenia, genetic diseases, age-related diseases (Moon et al., 2004) and most notably cancer (Fuerer et al., 2008).

3. Growth factors

Growth factors in the central nervous system play a crucial role in many aspects of the neuron biology regulating neuron birth, survival, migration, and neuronal connectivity by modifying the structure and function of synapses. Between the different growth factor families, neurotrophins and hepatocyte growth factor are of special interest for this work.

3.1. Neurotrophins

Neurotrophins (NTs) belong to a highly homologous family of secreted growth factors, essential for their roles in proliferation, survival and differentiation of different cell populations in the mammalian nervous system. In the mammalian brain, four NTs have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4).

NTs arise from precursors, proneurotrophins (30-35 KDa), which are proteolytically cleaved to produce mature protein (12-13 KDa) (Seidah et al, 1996). After synthesis in the endoplasmic reticulum, proneurotrophins need to be folded correctly, sorted into the constitutive or regulated pathway, and transported in the appropriate subcellular compartment. Regulated secretion is prevalent in neurons. NTs containing secretory granules are transported to dendrites and spines, and are secreted postsinaptically. NTs containing large dense core vesicles (LDCVs) undergo anterograde transport to axonal terminals.

Mature NTs exist in solution as noncovalently linked homodimers. Although some NT monomers are able to form heterodimers with other NT monomers *in vitro*, there is no evidence that these heterodimers exist at significant concentrations *in vivo*.

NTs bind to two different classes of transmembrane receptor proteins, the tropomyosin receptor kinase (Trk) and the neurotrophin receptor p75 (p75NTR) (Fig. 7). This dual system allows the transduction of very different signals following ligand binding, which can be as contrasted as signalling cell death through p75NTR or cell survival through the Trk receptors. These two classes of receptors also directly interact, allowing fine tuning and cross talk.

3.1.1. Trk receptors

In vertebrates, a family of tyrosin kinase proteins codified by *trk* proto-oncogenes serves as high affinity signal-transducing receptors for NTs. In mammals, three main groups of Trk receptors have been identified; denominated TrkA, TrkB and TrkC. Trk proteins are mainly expressed in nerve cells but can also be found in non-neuronal tissues (Shibayama and Koizumi, 1996; Tessarollo, 1998).

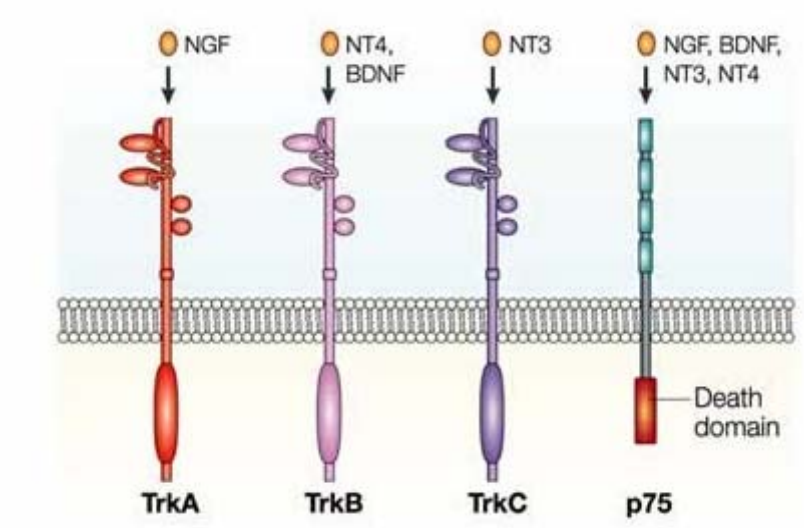

Figure 7. ***Neurotrophin receptors*** *(Chao, 2003). The actions of neurotrophins are mediated by two principal transmembrane-receptor signalling systems. Each neurotrophin receptor — TrkA, TrkB, TrkC and the p75 — is characterized by specific affinities for the neurotrophins.*

The extracellular domain of each of the Trk receptors consists of a cysteine-rich cluster followed by three leucine-rich repeats and another cysteine-rich cluster followed by two immunoglobulin-like domains. Immunoglobuline-like domains, especially the one that is closer to the membrane, seem to be involved in the interaction between NT and receptor (Perez et al., 1995; Urfer et al., 1995; Urfer et al., 1998; Ultsch et al., 1999). Each receptor spans the membrane once and is terminated with a cytoplasmic domain consisting of a tyrosine kinase domain surrounded by several tyrosines that serve as phosphorylation-dependent docking sites for cytoplasmatic adaptors and enzymes. In contrast to interaction with p75NTR, the NTs dimerize Trk receptors, resulting in activation through transphosphorylation of the kinases present in their cytoplasmatic domains. Different NTs show binding specificity for particular receptors: NGF binds preferentially to TrkA, BDNF and NT-4 to TrkB (Klein et al., 1990; Cordon-Cardo et al., 1991; Klein et al., 1991), and NT-3 to TrkC (Barbacid, 1995; Bothwell, 1995). With less affinity, NT-4 binds to TrkA and NT-3 binds to TrkA or TrkB (Segal and Greenberg, 1996).

3.1.2. p75NTR receptor

p75NTR was identified as a low-affinity receptor for NGF, but was subsequently shown to bind each of the NTs with a similar affinity (Rodriguez-Tebar

et al., 1990; Frade and Barde, 1998). Also p75NTR acts increasing ligand specificity to Trk receptors.

3.1.3. Neurotrophin receptor expression

NTs are not ubiquitously expressed. The exquisite neuronal specificity of NTs results from the selective expression pattern of the Trk receptors, and the localization of the NTs in the target areas innervated by responsive axons (Davies, 1994). In the CNS, few neurons express *TrkA* (mostly the basal forebrain cholinergic neurons), whereas *TrkB* is widely expressed, explaining the multitude of actions of BDNF in the CNS. *TrkC* is typically expressed early in development (Tessarollo et al., 1993). p75NTR is coexpressed with Trk receptors in many neuronal populations (Chao and Hempstead, 1995) but hippocampal and cortical neurons have a low level of p75NTR.

3.1.4. Neurotrophin signalling

Trk and p75 receptors show independent signalling properties (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001; Hempstead, 2002; Roux and Barker, 2002), and downstream signal transduction pathways significantly contribute to individual physiological responses. NTs bind as dimers to p75- and Trk-family members. Trk receptor dimerization leads to trans-autophosphorylation and to the activation of intracellular signalling cascades. The Src homologous and collagen-like (Shc) adaptor protein links the activated Trk receptor to two separate intracellular signalling pathways (Fig. 8). Neuronal survival requires Shc binding to the Trk receptor, which results in increases in phosphatidylinositol 3-kinase (PI3K) and Akt (protein kinase B) activities (Fig. 8). Phosphorylation of Shc by Trk also leads to increases in the activity of Ras and the extracellular signal-regulated kinase (ERK). These events in turn influence transcriptional events, such as the induction of the cyclic AMP-response element binding (CREB) transcription factor. CREB has effects on the cell cycle, neurite outgrowth and synaptic plasticity (Lonze and Ginty, 2002). The small G protein Rap1 accounts for the ability of NTs to signal through ERK for sustained periods (York et al., 1998). In addition, phospholipase C γ (PLC-γ) binds to activated Trk receptors and initiates an intracellular signalling cascade,

resulting in the release of inositol phosphates and activation of protein kinase C (PKC).

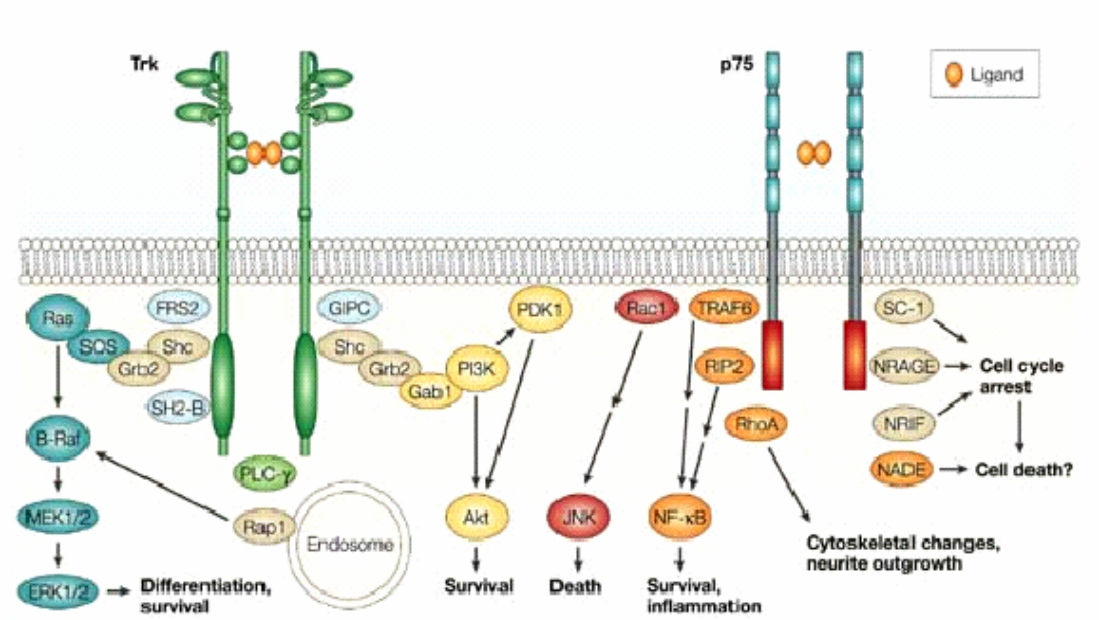

Figure 8. ***Neurotrophin signalling*** *(Chao, 2003) Trk receptors mediate differentiation and survival signalling through extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase C‘ (PLC-‘) pathways. The p75 receptor predominantly signals to activate NF-κB and Jun N-terminal kinase (JNK), and modulates RhoA activity. which can exert effects on apoptosis, survival, neurite elongation and growth arrest.*

Surprisingly, proneurotrophins are more selective ligands for the p75 receptor than mature forms, and are more effective at inducing p75-dependent apoptosis (Lee et al., 2001; Beattie et al., 2002). This indicates that the biological actions of NTs can be regulated by proteolytic cleavage, with pro-forms preferentially activating p75 to mediate apoptosis and mature forms selectively activating Trk receptors to promote survival.

3.1.5. Roles of neurotrophins in the nervous system

NTs are known to have a wide range of roles in development and function of the nervous system, controlling survival, cell fate, axon growth and guidance, dendrite structure and pruning, synapse formation and synaptic plasticity.

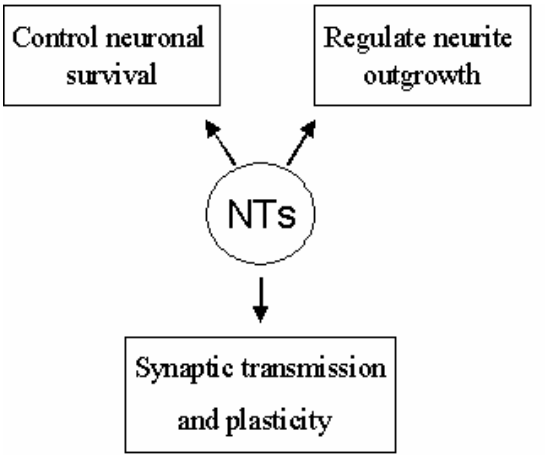

Figure 9. ***Neurotrophins functions in the nervous system***

NTs regulate programmed cell death

One of the most studied properties of the NTs is their ability to keep alive subpopulation of sensory neurons. The demonstration that NGF antibodies cause the loss of peripheral sympathetic ganglia was the first experimental evidence demonstrating an absolut requirement of certain cells for a specific survival factor (Cohen, 1960; Levi-Montalcini and Booker, 1960). BDNF prevent the death of sensory neurons not responding to NGF (Barde et al., 1982). NT-3 is required for the survival of about 50% of cranial sympathetic neurons.

On the contrary, the observation that NTs kill cells during development is comparatively recent and still incompletely understood. It was shown that p75NTR could signal independently and promote the death of several cell types, including neurons (Casaccia-Bonnefil et al., 1996; Frade et al., 1996). Overexpression of p75 causes neuronal death in developing CNS (Majdan et al., 1997). NGF and BDNF are able to activate p75 to induce cell death (Bamji et al., 1998).

NTs regulate the growth of neuronal processes

The role of NTs to induce neurite outgrowth is well established. NGF was purified following its ability to induce neurite outgrowth from peripheral ganglia embedded in a plasma clot (Levi-Montalcini et al., 1954; Cohen, 1960), thus suggesting from the beginning that NTs may promote axon elongation. NGF levels regulate dendritic growth in sympathetic ganglia (Ruit et al., 1990), accompanied by a large increase in the number of preganglionic axons inervating NGF-responsive ganglia (Schafer et al., 1983). BDNF injection into the optic tectum led to increased arborization of ganglion axon terminal, whereas blocking endogenous BDNF had the

opposite effect (Cohen-Cory and Fraser, 1995; Cohen-Cory, 1999). In slices of ferret visual cortex, BDNF, NT-3, and NT-4 were shown to increase the length and the complexity of dendrites of pyramidal neurons (McAllister et al., 1995).

NTs and synaptic transmission and plasticity

Many observations have indicated that NTs modulate the number of synapses, frequency and amplitude of synaptic currents. NGF levels regulate both the strength and the number of presynaptic inputs. In mice overexpressing BDNF in sympathetic neurons, increased numbers of synapses were observed. In mice carrying mutation in *trkB, trkC,* or in both genes, lower densities of synaptic contact were recorded in hippocampus. Presynaptic alterations were also noted, including decreased numbers of synaptic vesicles. BDNF and NT-3 produce increases in excitatory postsynaptic current in hippocampal neurons, as well as rapid increases in synaptic strength in nerve-muscle synapses (Lohof et al., 1993; Kang and Schuman, 1995; Levine et al., 1995) and hippocampus.

Due to the diversity and complexity of the processes that modulate, NTs and their receptors are essential molecules from the early embryonic development (survival of specific classes of neurons and contribute to the functional phenotype of CNS) and continue during life (survival, synaptic transmission and plasticity).

3.2. Hepatocyte growth factor (HGF)

HGF, also called scatter factor, was discovered as a mitogen for hepatocytes and as a factor that induces migration of epithelial cells (Nakamura et al., 1989). HGF is a large protein consisting of six domains: an amino-terminal domain (N), four Kringle domains (K1-K4) and a serine proteinase homology (SPH) domain, which lacks enzymatic activity as a result of mutations in essential residues (Lokker et al., 1992) (Fig. 10a). HGF is synthesized as a single-chain, largely inactive, precursor (pro-HGF) that is cleaved by extracellular proteases to form a biological active 69 KDa α chain and 34 KDa β chain heterodimer (Naka et al., 1992; Naldini et al., 1992).

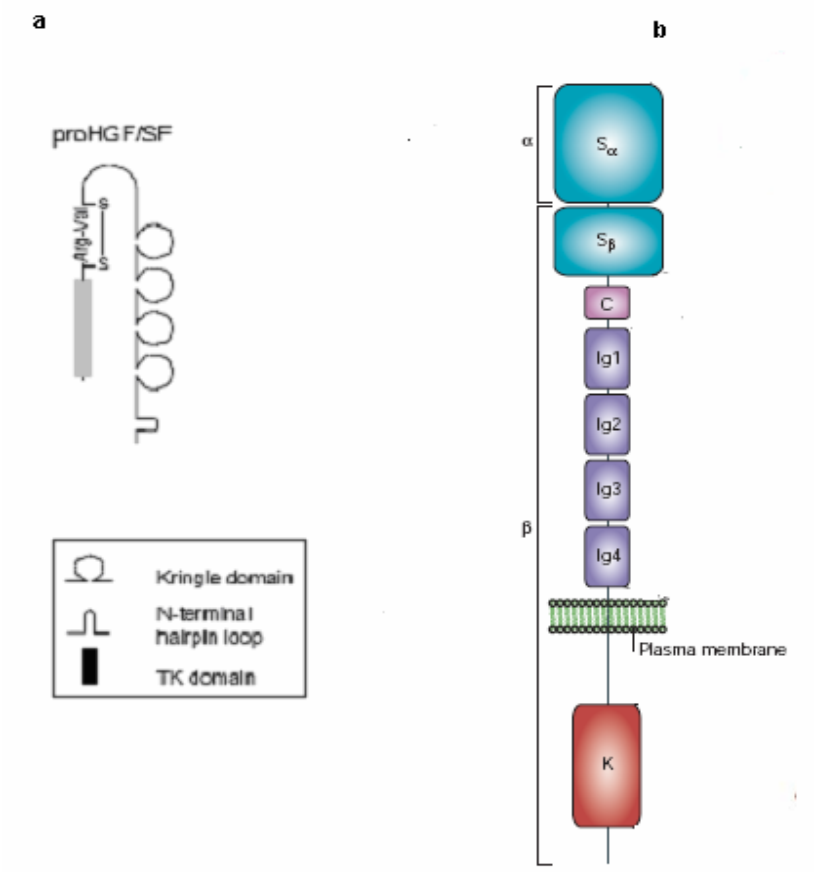

Figure 10. *a) The domain structure of hepatocyte growth factor/scatter factor. b) The domain structure of Met. S, sema domain; C, cysteine-rich domain; Ig, immunoglobulin domain; K, kinase domain; α and β refer to the subunits of the receptor that are present after proteolytic cleavage*

3.2.1. HGF receptor

HGF signals through its receptor tyrosine kinase (RTK) Met. Met was first identified in the 1980s as an oncogene (Cooper et al., 1984). Like its ligand, the Met receptor is a disulphide-linked heterodimer: an extracellular α chain and a longer β chain. The β chain encompasses the remainder of the Met ectodomain, the transmembrane helix and the cytoplasmic portion. The latter contains the juxtamembrane and kinase domains as well as a carboxy-terminal tail that is essential for downstream signalling (Fig. 10b). New insights into the domain structure of Met have shown that the α chain and the first 212 residues of the β chain are sufficient for HGF binding (Gherardi et al., 2003). The remainder of the Met ectodomain contains a small cysteine-rich sequence, which is followed by four repeats of an unusual type of immunoglobulin domain. The latter might form a 'stalk' structure that holds the propeller domain in the correct orientation for ligand binding. HGF also binds

heparin-sulphate proteoglycans with high affinity, which limits the diffusion of the factor *in vivo,* but the interaction is not essential for receptor activation (Schwall et al., 1996; DiGabriele et al., 1998; Hartmann et al., 1998). A fragment containing the amino-terminal and K1 domains (known as NK1) contains the main receptor-binding site. Mutagenesis studies indicate that residues in the SPH domain of HGF might make additional contacts with Met.

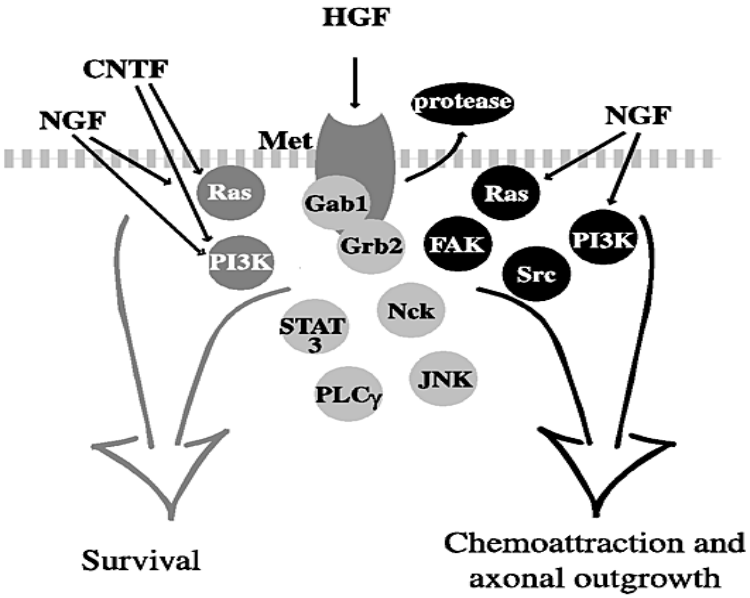

Figure 11. ***Schematic representation of signaling pathways activated by HGF*** *(Maina and Klein, 1999)**. HGF binds and activates a series of signaling proteins that may be differentially involved in distinct biological responses. The survival effect mediated by Met may involve the activation of Ras and PI3 kinase, which are also activated by NGF and ciliary neurotrophic factor (CNTF). Thus the cooperation between HGF and these neurotrophins may result from an enhancement of the kinetics of activation of these two pathways. Developing axons control motility of their growth cones by modulating both protease levels and cytoskeletal organization. Axonal outgrowth could resemble cell motility, which involves several pathways known to be activated by HGF. These include degradation of extracellular matrix by increased protease secretion, as well as activation of Ras, PI3 kinase, src and p125FAK. Other pathways activated by HGF/Met signaling include Nck, STAT3, JNK and PLC*γ*.*

Met is expressed in various cell types, including epithelial cells (Stoker et al., 1987; Di Renzo et al., 1991), endothelial cells (Bussolino et al., 1992; Grant et al., 1993), myoblasts (Anastasi et al., 1997), hematopoietic cells (Galimi et al., 1994; Nishino et al., 1995), spinal motoneurons (Ebens et al., 1996) and hippocampus (Korhonen et al., 2000). Binding of the proteolytically-activated form of HGF by Met induces autophosphorylation of its intracellular tyrosine kinase domain and

initiates signalling by multiple pathways (Fig. 11) including ERK-mitogen-activated protein kinase (MAPK) and PI3-K pathaways (Weidner et al., 1996; Fixman et al., 1997).

3.2.2. HGF roles

HGF has morphogenic, motogenic, and mitogenic effects on a wide variety of cells (Fig. 12) and can regulate gene expression, cytoskeletal structure, intercellular adhesion, and interactions among junctional proteins. In the CNS HGF acts as a neurotrophic factor that enhances survival of neurons (Ebens et al., 1996; Korhonen et al., 2000), promotes neurite outgrowth (Thompson et al., 2004), guides axons to targets (Ebens et al., 1996; Yang et al., 1998), promotes migration of interneurons during development (Powell et al., 2001), enhances elaboration of dendritic arbors (Gutierrez et al., 2004), and participates in the development of the cerebellum (Ieraci et al., 2002).

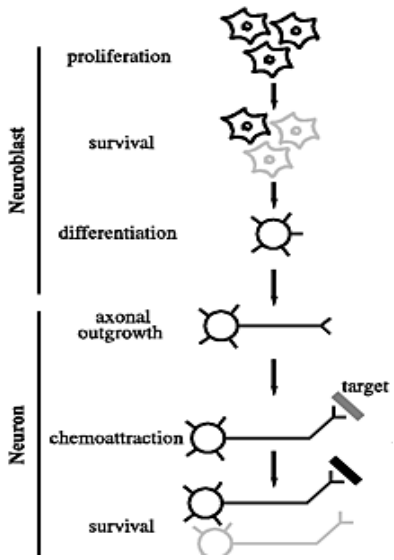

Figure 12. ***Biological functions of HGF at different time points during neurogenesis*** (Maina and Klein, 1999)**.**

3.3. Epidermal growth factor

Another growth factor signalling through a RTK is epidermal growth factor (EGF). The membrane receptor for EGF, the EGFR, is composed of an extracellular EGF-binding domain and a cytoplasmic kinase domain connected by a stretch of 23

amino acids encompassing the plasma membrane. EGF binding to the extracellular domain activates the cytoplasmic tyrosine kinase and subsequent autophosphorylation of tyrosine residues in the intracellular C-terminal domain provides docking sites for SH2 and PTB domain-containing adaptor proteins that couple extracellular signals and receptor activation to downstream signalling pathways (Yarden and Sliwkowski, 2001; Singh and Harris, 2005). EGF signalling through catenin/cytoskeleton pathways (Yasmeen et al., 2006) (Fig. 13) regulates cell adhesion and migration. EGF has been shown to induce tyrosine phosphorylation of β-catenin and plakoglobin after association with the cadherin–catenin complex. It has been argued that this induces disassembly of the cadherin–catenin complex from the actin filament network (Hoschuetzky et al., 1994), decreasing adhesion and increasing migration (Lilien and Balsamo, 2005).

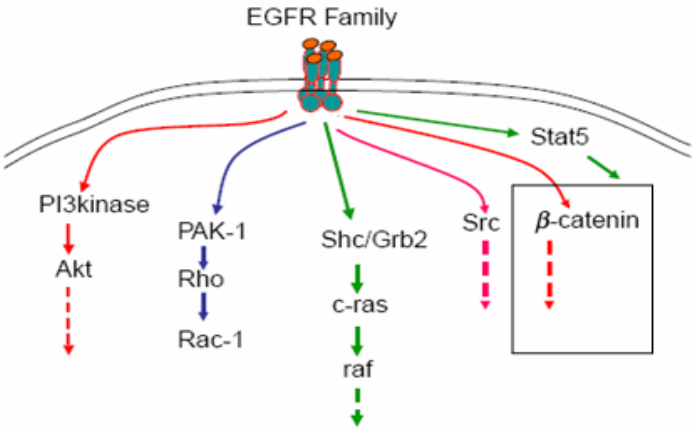

Figure 13. ***Intracellular signalling pathways activated by EGFR family members*** *(Burgess, 2008). Of special interest for this work is the cadherin/cytoskeleton pathway.*

The role of EGF has been extensively investigated in normal and pathological wound healing. EGF and its tyrosine kinase receptor regulate the production, survival, movement, and shape of many embryonic and adult cell types (Yarden and Sliwkowski, 2001). EGF has been shown to act as a potent mitogen for neural stem cells (Weiss et al., 1996). EGF acts not only on mitotic cells but also on postmitotic neurons, and many studies have indicated that EGF have a neurotransmitter-like or neuromodulatory role enhancing the differentiation, maturation and survival of a variety of neurons (Yamada et al., 1997). Transgenic mice lacking the EGFR developed neurodegenerative disease and die within the first month of birth (Gassmann et al., 1995; Lee et al., 1995).

4. β- catenin

β-catenin is a protein involved in cadherin-mediated cell–cell adhesion and in Wnt canonical signalling. β-catenin has 92 KDa, a primary structure composed by an amino-terminal domain, a carboxy-terminal domain and the central structure containing 12 repetition of 42 aminoacids known as armadillo domains (Fig. 14).

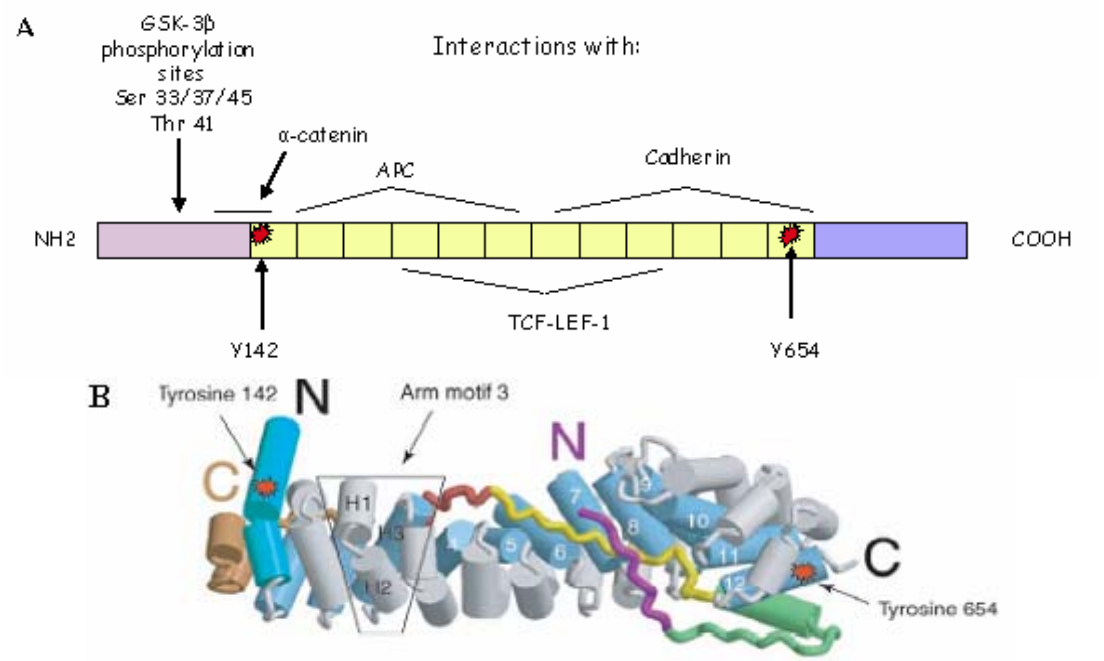

Figure 14. *A) The interaction domains of β-catenin. GSK-3β phosphorylates β-catenin in serine 33/37/45 and threonine 41 in the N-terminal domain .α-catenin binds to the N-terminus and the first two armadillo domains of β-catenin. The central core of β-catenin interacts with APC, TCF/LEF-1 and Cadherin. B) Three dimensional representation of β-catenin structure (Huber and Weis, 2001) showing the location of Y654 and Y142.*

β-catenin can be present in three cellular compartments: in the membrane (as a component of cadherin-catenin adhesion complex), in the cytoplasm (free or associated to other proteins like APC, Axin and GSK-3β) and in the nucleus (where it associates with LEF/TCF transcription factors).

Evidence accumulating during recent years underlines the importance of β-catenin in the development. Lack of β-catenin induces embryonic lethality (Haegel et al., 1995). Most of the genetic gain-of-function or loss-of-function studies of β-catenin have resulted in gross changes in brain morphology and neuronal precursor population (Thomas and Capecchi, 1990; Galceran et al., 2000; Brault et al., 2001; Chenn and Walsh, 2002).

Conditional knocking-out of β-catenin in hippocampal neurons alters synapse structure and function. At presynaptic sites , the number of reserve pool vesicles is reduced, with concomitant reduction in exocytosis in response to repetitive stimulation (Bamji et al., 2003). Postsynaptically, loss of β-catenin after synaptogenesis results in profound alterations in spine morphology (Okuda et al., 2007), leading to spines that are thin and elongated with no changes in spine density. In postnatal born dentate gyrus granule neurons, conditional knocking-out of β-catenin results in dendritic malformation (Gao et al., 2007).

4.1. The cadherin-catenin complex

The cadherin-catenin complex is a major signalling complex that regulates cell-cell adhesion and through which extracellular signals can influnence the cytoskeleton (Fig. 15).

Members of the cadherin superfamily are transmembrane proteins that operate mainly by homophilic interactions between neighboring cells, with a crucial importance in maintenance of solid tissues. The intracellular domain of cadherins binds cytoplasmic proteins that are thought to recruit and organize the actin cytoskeleton (Gumbiner, 2000; Jamora and Fuchs, 2002). The cytoplasmic tail of cadherin associates with high-affinity with the central region of armadillo domains of β-catenin and plakoglobin (γ-catenin). These interactions are necessary for proper cell functionality and tissue integrity. β-catenin region that binds to cadherin contains the tyrosine residue 654 (Y654). p120ctn and α-catenin are also Armadillo proteins. They were found to interact with another conserved cytoplasmic cadherin domain, which is more membrane-proximal. p120ctn seems to be involved in lateral clustering of cadherin molecules (Yap et al., 1998; Thoreson et al., 2000), in the regulation of cadherin stability (Ireton et al., 2002; Davis et al., 2003; Xiao et al., 2003), in the regulation of actin cytoskeleton (Noren et al., 2000), and in dendritogenesis (Elia et al., 2006). β-catenin region from amoniacid 118 to aminoacid 149 binds with low-affinity to α-catenin (Aberle et al., 1996). N-terminal portion and the first armadillo repeat of β-catenin, which contains tyrosine 142 (Y142), have been shown to be essential for α-catenin binding (Pokutta and Weis, 2000).

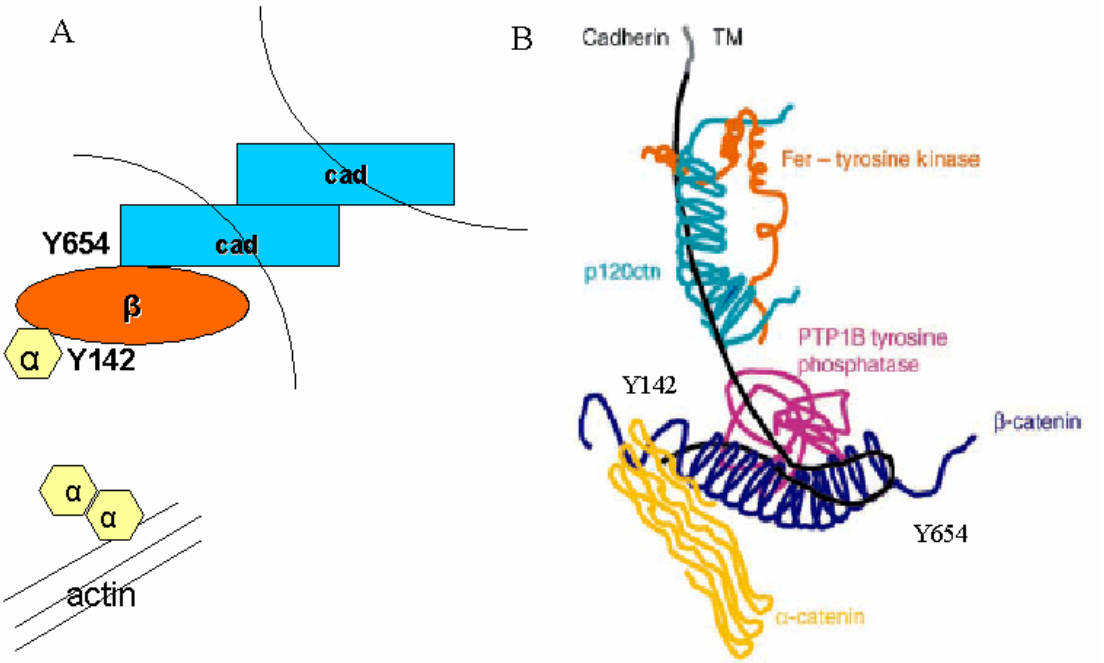

Figure 15. *A) β-catenin binds to the intracellular domain of cadherin and to α-catenin, which links the complex to the actin cytoskeleton. Phosphorylation of tyrosine 654 and 142 leads to the disassembly of the cadherin–β-catenin–α-catenin complex. Phosphoylation of tyrosine 654 reduces the affinity of β-catenin for cadherin, potentially disrupting their association. Phosphorylation of tyrosine 142 reduces the affinity of β-catenin for α-catenin. B) A schematic rendering of the relationships among the components of the cadherin/catenin complex (Lilien and Balsamo, 2005). Tyrosine kinase Fer is brought into the cadherin complex by p120ctn. The non-receptor tyrosine phosphatase PTP1B binds directly to the cytoplasmic domain of N-cadherin and dephosphorylates β-catenin. Note the location of Y142 and Y654 in the regions involved in α-catenin and cadherin bindings respectively.*

Since α-catenin can also bind actin filaments *in vitro* (Rimm et al., 1995; Pokutta et al., 2002), it was widely accepted that α-catenin bound to the cadherin-catenin complex bridges these components to actin. An additional link between cadherin-catenin complex and the actin cytoskeleton is established by the interaction of α-actinin or vinculin with α-catenin (Kobielak and Fuchs, 2004). Furthermore, the Rho family of small GTPases (Fukata and Kaibuchi, 2001; Braga, 2002), the actin nucleators formin (Kobielak et al., 2004) and Arp2/3 complex (Kovacs et al., 2002) are involved in cell-cell adhesion and may regulate actin dynamics around the cadherin-catenin complex. However, recent experiments with purified proteins demonstrated that the ternary complex of cadherin- β-catenin-α-catenin does not bind directly to actin filaments or indirectly through vinculin or α-actinin. Moreover, live-cell imaging experiments showed that the cadherin-catenin complex has dynamics that are very different to those of actin, consistent with the lack of a stable linkage

between the cadherin-catenin complex and actin in cells (Yamada et al., 2005). The inability of α-catenin to bind simultaneously to the cadhenin- β-catenin complex and actin indicates that α-catenin may function like a switch. α-catenin monomer preferentilly binds β-catenin, while α-catenin homodimer binds actin (Drees et al., 2005). As α-catenin binds numerous actin-binding proteins, these observations do not challenge the function of α-catenin as a link between the cadherin complex and the actin cytoskeleton.

The ability of cadherins to bind to catenins serves to link the cell-adhesion complex to a multitude of intracellular signalling pathways, affecting the cytoskeleton, cytoplasmic signalling pathways and gene expression. The cadherin-catenin complex is key regulator of cell adhesion, involved in the regulation of cell migration during development and in pathological conditions.

4.2. Regulation of β-catenin signalling by the Wnt pathway

The signalling function of β-catenin is regulated mainly through alteration of its stability. Wnt signalling induces the stabilization of the 'free' cytoplasmic pool of β-catenin (Bienz and Clevers, 2000; Polakis, 2000; Huelsken and Birchmeier, 2001). In the absence of Wnt signalling, the free pool of β-catenin is tightly regulated through phosphorylation at specific N-terminal residues by a destruction complex consisting of APC, axin (conductin homolog), GSK-3β and CK1 (Behrens et al., 1998; Kishida et al., 1998; Liu et al., 2002; Schwarz-Romond et al., 2002). Phosphorylated β-catenin is marked for rapid ubiquitylation and degradation by the proteasome. Binding of an extracellular Wnt ligand to the Frizzled receptor leads to the inhibition of GSK-3β and degradation complex. This, in turn, results in the nuclear accumulation of β-catenin and to the increased transcription of Wnt-β-catenin target genes by the LEF/TCF DNA binding proteins (Behrens et al., 1998; Hsu et al., 1998).

4.3. Regulation of β-catenin function by tyrosine phosphorylation

In addition to GSK-3β phosphorylation of β-catenin that regulates the protein stability, β-catenin function can be regulated by tyrosine phosphorylation. Tyrosine phosphorylation of β-catenin has been shown to lead not only to loss of adhesion but

also to increased transcription (Orsulic et al., 1999; Gottardi et al., 2001; Brembeck et al., 2004). Recent research revealed that phosphorylation of Y142 in β-catenin is crucial for this switch. Importantly, phosphorylation of Y142 disrupts binding to α-catenin but promotes binding of β-catenin to the nuclear co-factor BCL9-2, the homolog of BCL9–Legless, increasing β-catenin dependent transcription (Brembeck et al., 2004).

Interestingly, tyrosine phosphorylation of β-catenin regulates cell migration in non-neuronal system (Behrens et al., 1993; Roura et al., 1999; Lilien and Balsamo, 2005). Of particular importance are two tyrosine residues in β-catenin: Y142 in the first armadillo domain and Y654 in the helix 3 of the last armadillo repeat. It was shown that phosphorylation of these two key tyrosine residues leads to the disassembly of the cadherin–β-catenin–α-catenin complex at the plasma membrane and to increased β-catenin-dependent transcription, respectively (Aberle et al., 1996; Roura et al., 1999; Pokutta and Weis, 2000; Piedra et al., 2001; Piedra et al., 2003). The sequences surrounding tyrosines 142 and 654 of β-catenin are highly conserved in evolution, suggesting that regulation of the adhesive function of β-catenin by tyrosine phosphorylation might be conserved in different species. As mentioned above, Y654 in the last armadillo repeat of β-catenin is essential for binding to cadherin (Roura et al., 1999; Piedra et al., 2001). Phosphorylation of Y654, by EGF signalling or by c-Src, resulting in decreased affinity for cadherin and interferes with cell adhesion (Hoschuetzky et al., 1994; Takahashi et al., 1997; Roura et al., 1999; Piedra et al., 2001; Castano et al., 2002). Although cadherin and LEF/TCF proteins share overlapping binding sites in the central armadillo domain of β-catenin (Orsulic et al., 1999; Graham et al., 2000; von Kries et al., 2000; Huber and Weis, 2001), LEF/TCF binding does not require the last armadillo repeat, where Y654 is located. Consequently, binding of LEF/TCF to β-catenin is not affected by phosphorylation of this residue (Orsulic et al., 1999; Piedra et al., 2001). On the other hand, Y142 of β-catenin is important for binding to α-catenin (Aberle et al., 1996; Pokutta and Weis, 2000; Piedra et al., 2003; Brembeck et al., 2004). The aromatic ring of Y142 packs into a hydrophobic core and forms van der Waals contacts with several amino acid residues in α-catenin. The hydroxyl group of Y142, however, does not directly contact α-catenin but interacts with other residues in β-catenin to stabilize the first

armadillo repeats. Mutation of Y142 to alanine, therefore, abolishes interaction of β-catenin with α-catenin, whereas a change to phenylalanine does not affect binding (Aberle et al., 1996). Moreover, phosphorylation of Y142 or exchange to glutamic acid, which mimics tyrosine phosphorylation, disrupts binding to α-catenin (Piedra et al., 2003; Brembeck et al., 2004). In epithelial cells, phosphorylation of Y142 by the non-RTKs Fer and Fyn, or RTK Met inhibits the interaction of α-catenin with β-catenin (Piedra et al., 2003; Brembeck et al., 2004; Nelson and Nusse, 2004; Xu et al., 2004; Lilien and Balsamo, 2005). Activation of β-catenin signalling by Met and phosphorylation of β-catenin Y142 leads to its nuclear translocation and increased TCF-mediated gene transcription (Danilkovitch-Miagkova et al., 2001; Brembeck et al., 2004). In addition, Fer interacts with cadherin indirectly and phosphorylates PTP1B (PTPN1), regulating the binding of PTP1B to cadherin and the continuous dephosphorylation of β-catenin Y654 (Balsamo et al., 1996; Balsamo et al., 1998; Piedra et al., 2003; Xu et al., 2004).

In summary, the available data indicates that the tyrosine phosphorylation state of β-catenin, resulting from the balance of phosphatase and kinase activities, regulates cell migration by decreasing adhesion and activating β-catenin signalling in epithelial tissue.

In the nervous system, cadherin and β-catenin regulate axon extension (Riehl et al., 1996), dendritogenesis (Yu and Malenka, 2003), synaptic assembly and plasticity (Yu and Malenka, 2003; Bamji, 2005; Bamji et al., 2006; Tai et al., 2007). Interestingly, phosphorylation of β-catenin on Y654 regulates synapse formation and function (Murase et al., 2002; Bamji et al., 2006) and phosphorylation of β-catenin on Y489 regulates the growth cone response to the guidance cue Slit (Rhee et al., 2002; Rhee et al., 2007).

β-catenin switches from its adhesive or transcriptional functions (Fig. 16). Both functions of β-catenin are deregulated in human malignancies, thereby leading both to the loss of cell-cell adhesion and to the increased transcription of Wnt target genes.

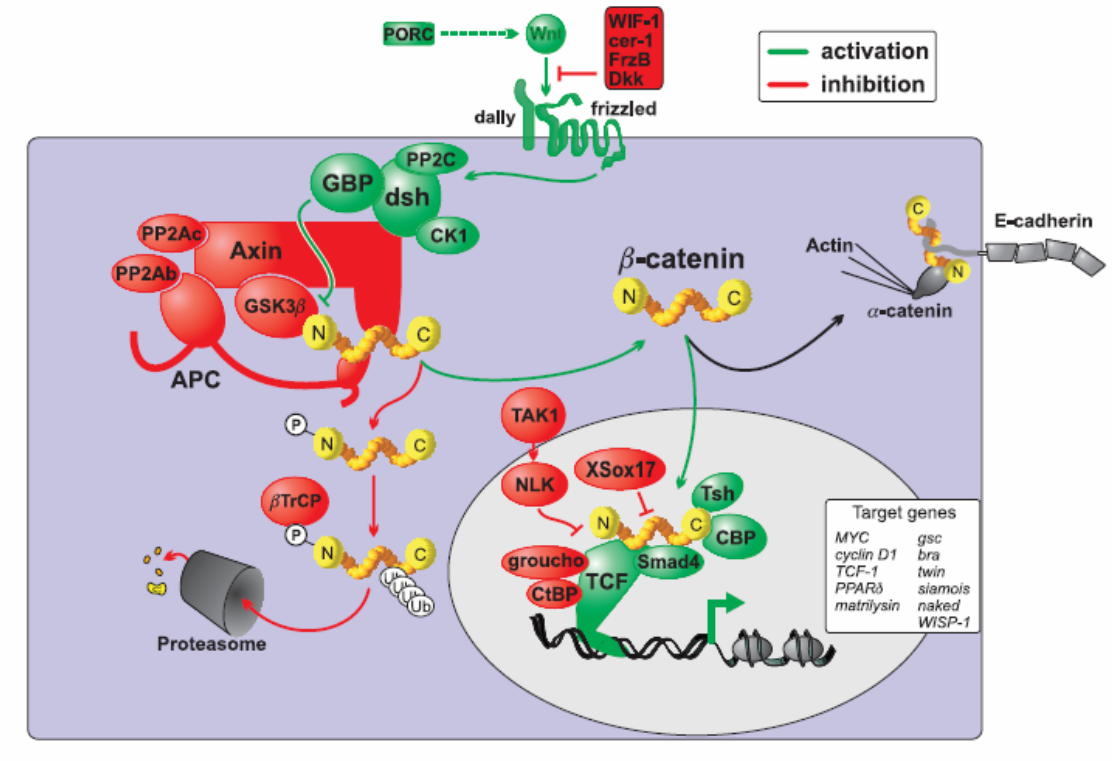

Figure 16. ***Regulation of β-catenin level in a normal colon cell*** *(Hulsken and Behrens, 2000). In the normal condition β-catenin level is regulated by the complex consisting of APC, axin, GSK-3β and CK1. Binding of Wnt to Frizzled receptor inhibits GSK-3β, allowing β-catenin accumulation in the cytoplasm and subsequent translocation to the nucleus. Disruption of cadherin-catenin complex leads to transitory accumulation of β-catenin in the cytoplasm.*

Objectives

β-catenin is a component of the canonical Wnt pathway and of the cell-cell adhesion complex. Here we studied the role of β-catenin in neurogenesis and neuritogenesis, two stages of neuronal differentiation: 1) its involvement in Wnt signalling (a classical β-catenin function) during neural progenitor differentiation and, 2) a novel role downstream of growth factor signalling in axon outgrowth.

1. To investigate the function of Wnt-3a and Wnt-3 and the pathway activated by these Wnt factors during the development and neurogenesis of spinal cord neural precursors (SCNPs).
   1.1) To study the role of Wnt-3a and Wnt-3 in SCNP proliferation.
   1.2) To study the role of Wnt-3a and Wnt-3 in the neurogenesis and neuritogenesis from SCNPs.
   1.3) To analyze the involvement of β-catenin and the components of the canonical Wnt/β-catenin pathway in the Wnt-mediated response.

2. Tyrosine phosphorylation of β-catenin downregulates cell adhesion and increases migration. We aimed to investigate the relationship between β-catenin and growth factor/RTK signalling in axon morphogenesis in primary hippocampal neurons.
   2.1) To study the interaction of β-catenin with the NT and HGF RTKs, Trk and Met.
   2.2) To determine if β-catenin is required downstream of NT and HGF signalling during axon morphogenesis.
   2.3) To investigate whether Trk and Met phosphorylate β-catenin and identify which β-catenin residues are involved in axon growth and branching downstream of these RTK.
   2.4) To study the molecular mechanisms involved in the regulation of axon morphogenesis by β-catenin tyrosine phosphorylation.

Materials and Methods

1. Materials

BDNF and NT-3 were obtained from Alomone Labs, NGF from Sigma, HGF from Calbiochem and Wnt-3a from R&D. K252a was from Calbiochem, Actinomycin D from Sigma. GSK-3 β inhibitors, Kenpaullone and Bio were from Sigma. TrkA and Met kinases were from Cell Signalling. Antibodies were purchased from the following companies: Met from Santa Cruz, P-Y654 and P-Y142 from Abcam, βIII-tubulin from Covance, P-Tyrosine (P-Tyr; 4G10), TrkB and TrkC from Upstate Biotechnology, β-actin from Sigma, β-catenin from BD Transduction Laboratories, N-cadherin from Zymed, α-catenin from the Hybridoma Bank, EYFP from Acris Antibodies, HA from Roche.

2. Cell culture

2.1. Cell lines and culture conditions

The PC12 rat pheocromocytoma cell line clone 6/15 overexpressing human TrkA (PC12 6/15) (Hempstead et al., 1992; Llovera et al., 2004) was grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO) supplemented with 6% fetal bovine serum (FBS) (GIBCO) and 6% heat-inactivated horse serum (HS) (GIBCO), 10mM HEPES, 20units/ml penicillin and 20 μg/ml streptomycin.

After 2 days in culture, when the plates were confluent, PC12 6/15 were deprived overnight from serum (DMEM only) and were stimulated in the next day with NGF 100ng/ml in deprive medium. If K252a treatment was required, the cells were preincubated in deprive medium with K252a 100nM for 15 min before adding NGF.

RAT 1B fibroblast lines stably overexpresing hemagglutinin-tagged Wnt-3 or mock (provided by Dr. A. Munsterberg, Norwich, UK) were maintained in DMEM with 10% FBS and Geneticin 500 μg/ml (G-418; GIBCO). To obtain control or Wnt-3 conditioned media (CM), cells were subplated in DMEM:F12 medium (GIBCO) plus B27 (Invitrogen) and conditioned overnight. CM was centrifuged to discard dead floating cells and the supernatant added to spinal cord neuronal precursors culture diluted 1:1 with fresh medium and supplemented with 1% FBS. CM was changed daily. We confirmed the expression of Wnt-3-HA protein in cell-lysates and

its seccretion in the CM by western blotting with an anti-HA antibody (Fig. 17).

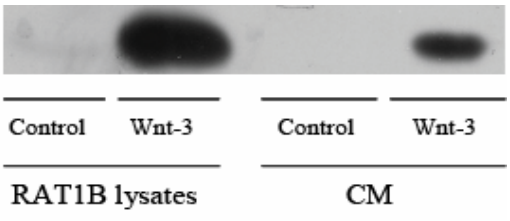

Figure 17. *Western blot with anti-HA antibody showing Wnt-3-HA protein expression in RAT-1B cell lysates and CM.*

The human embryonic kidney cell line HEK293T is a good model to overexpress and obtain proteins. HEK293T cell line (provided by Dr. Eduardo Soriano) was used to obtain lentiviral particles. The culture medium for HEK293T cell line was DMEM supplemented with 10% FBS and 10% non-essential aminoacids (NEAA) (GIBCO), 1mM Sodium Pyruvate (GIBCO), 20 units/ml penicillin and 20 µg/ml streptomycin.

Cell lines were grown at 37ºC in a humidified atmosphere of 5% CO_2.

2.2. Primary culture

Primary cell cultures were obtained using the minimum numbers of animals according with the scientific objectives of the research. Ethical procedures according to national and European guidelines were followed to sacrifice the animals.

2.2.1. Isolation and culture of mouse SCNPs.

Mouse embryonic day 12.5 - 13 spinal cord were dissected, the meninges cleaned off and the spinal cords mechanically dissociated in phosphate buffer saline (PBS) plus 0.6% glucose using fire-polished Pasteur pipettes as similarly described (Wu et al., 2003). Cells were plated on Petri dish plates coated with poly 2-hydroxyethyl methacrylate (Sigma). Cells were grown in serum-free DMEM: F12 medium supplemented with B27 and basic Fibroblast Growth Factor (bFGF; GIBCO) 20ng/ml. After minimum 7 days of growth in suspension, the resulting neurospheres were trypsinized (Trypsin 0.25%; GIBCO) for 10 min at 37˚C. Dissociated cells were plated on poly-D-lysine (10 µg/ml; Sigma) and laminin (4 µg/ml; Sigma) pre-coated glass coverslips or plastic wells in DMEM:F12

supplemented with B27 and 1% FBS in the absence of bFGF. For neurite growth cells were plated at 75 cells/mm^2 and for western blot at 400 cells/mm^2.

SCNPs treatment

SCNPs were stimulated with recombinant mouse Wnt-3a (R&D Systems; 50 ng/ml) or Wnt-3 conditioned medium for 48, 72 or 96 hours before fixation. Kenpaullone (15 μM) and Bio (6-bromoindirubin; 1μM) were added at plating or from *day in vitro* 2 (2DIV) to 3 DIV.

Actinomycin D (5nM) was added to the cells from plating and cells fixed at 2 DIV. In case of Wnt-3/ Control CM, Actinomycin D was added together with the fresh CM every day.

2.2.2. Isolation and culture of rat hippocampal neurons

At 18-19 days gestation (E18-19), pregnant females were killed and cultures of hippocampal neurons were established from each embryo. The dissected hippocampus were collected in ice-cold HBSS (GIBCO), trypsinized (15 minutes at 37ºC) and dissociated with a fire-polished Pasteur pipette. The resulting cells were suspended in DMEM supplemented with 10% HS, 1 mM Na Pyruvate, 20 units/ml penicillin and 20 μg/ml streptomycin. Neurons were plated on poly-D-lysine coated glass coverslips at the following cell densities: about 500-1000 cells/mm2 for transfection or biochemical experiments and 70 cells/mm2 for immunostaining after lentivirus infection. Three hours after plating the medium was replaced for serum-free DMEM mediu supplemented with B27 and N2 (GIBCO).

Hippocampal neurons treatment

Hippocampal cells were transfected at DIV1, treated with growth factors (BDNF, NT-3, HGF) and fixed at 2 DIV. The pharmacological inhibitor of Trk activity, K252a, was preincubated for 10 min and added together with BDNF. For Western Blot assay, neurons were deprived of B27 overnight and stimulated with growth factors (50 ng/ml, 10 min, unless otherwise indicated) at 2 DIV. Cells were treated with pervanadate to inhibit tyrosine phosphatases (1mM final; 10 min together with the stimulus) where indicated.

3. Cell transfection

3.1. Polyethilenimine cell transfection

Polyethilenimine (PEI) cell transfection was performed in medium without serum and antibiotics. DNA was prepared in sterile NaCl 150 mM, as well as PEI solution, which is diluted to a final concentration of 10µM. PEI was directly added to DNA solution. After shaking (1 min), PEI-DNA complexes were obtained incubating the mix at room temperature (RT) for 10 min. The resulting cell transfection solution was added drop by drop to the HEK293T cell culture dish. Cell culture medium was changed after 3 hours to avoid toxicity.

3.2. Lipofectamine™2000 cell transfection

We used a ratio Lipofectamine™ 2000 and DNA 2µl:1µg. Cells were seeded in a 24 wells plate. 1.5µg of DNA and 3µl of Lipofectamine™ 2000 reagent, in different tubes, were diluted in the cell culture medium without serum and antibiotics. The Lipofectamine™ 2000 mixtures were incubated for 5 min at RT. The Lipofectamine™ 2000 and DNA solutions were finally mixed and incubated at RT (20 min) to allow the formation of DNA-Lipofectamine™ 2000 reagent complexes. The resulting cell transfection solution was added drop by drop to the cell culture dish (250 µl medium/24 well). Cell culture medium was changed after 3 hours.

Hippocampal neurons were transfected at 1 DIV, treated with the corresponding stimulus overnight and fixed at 2 DIV.

3.3. Nucleofection of SCNP cells.

Expression of EGFP alone or dominant-negative ΔN-TCF-4-HA plus EGFP (3:1) was achieved using the Amaxa Nucleofector according to manufacturer's instructions upon neurospheres disgregration. SCNPs were subplated at 300 cells/mm^{2}and treated 4 hours after plating with BSA/Wnt-3a (50 ng/ml) or control/Wnt-3 CM. Cells were fixed at 2 DIV and were co-immunostained with anti-GFP and anti-HA antibodies. Only cells co-immunostained for GFP and HA were measured for neurite length using the GFP immunostaining.

4. Lentiviral-driven shRNA expression

shRNA primers specific for mouse and rat β-catenin were designed using algorithms available on Promega and Invitrogen websites. The primers selected correspond to GTTTGTGCAGTTGCTTTAT (shRNA#1 -for both the mouse and rat proteins), to GGGTTCCGATGATATAAAT (shRNA#2 -rat β-catenin) and GGGTTCTGATGATATAAAT (shRNA#3 -mouse β-catenin).

4.1. Lentiviral constructs

pEIGW, pLVTHM, pSPAX2 and pM2G vectors were kindly given by Dr. Trono (Switzerland). β-catenin genes were subcloned into the pLVTHM plasmid together with the H1 promoter for the RNA pol III. shRNA vectors were transfected into HEK293T cells together with the plasmids psPAX2 and pMD2G using the PEI transfection method. Transfection efficiency was analyzed by GFP expression (driven by the pLVTHM plasmid).

4.2. Lentiviral production

In 2000 Dr. Trono and colleagues developed 3 generations of vectors used to product Lentivirus (Trono D., 2000). To perform this study Lentiviruses of second generation were used with a sufficient biosecurity level to what concerns genetic transference *in vitro*. Lentivirus production protocol was described by Naldini L. and collaborators in 1996 (Naldini L. et al, 1996). The plasmids used for Lentivirus production that belong to the second generation are the following:

1) Vectors pEIGW/pLVTHM were used to overexpress or inhibit gene expression, respectively. The vector itself is the only genetic material transferred to the target cells, as they lost the transcriptional capacity of the viral long terminal repeat (LTR). The vector sequence includes the trangene cassette flanked by cis-acting elements required for its encapsidation, reverse transcription and integration in the genome. In order to produce lentiviral siRNA construct, pLVTH was designed in such way that H1 Pol III promoter can be easily replaced by H1-siRNA cassette from pSUPER.retro.puro using EcoRI – Cla I.

2) psPAX2 vector codifies for viral packaging proteins. psPAX2 contains a very efficient promoter (CAG), which allows the expression of viral packaging compounds, among which TAT protein, DNA polimerase and Reverse Transcriptase are the most relevant. The CAG promoter includes CMV enhancer, chicken β-actin promoter and intron.

3) pM2G vector codifies for virus envelope. The viral protein comes from Vesicular stomatitis Virus (VSV) and triggers to a wide range of tissues and cell lines infection.

For Lentiviral production constructs, HEK293T cells were seeded at a density of $2{,}5x10^6$ cells in 0.1% gelatin-coated 100-mm cell culture dishes. The following day, cells were transfected with:

Vector pWPI / pLVTHM	20μg
pSPAX2	13μg
pM2G	7μg

The transfection was routinely performed by the PEI transfection method. Cells were allowed to produce Lentiviruses for 48 hours. Then the medium was centrifuged at 4000 g (5 min) to collect and eliminate died cells present in the supernatant, and subsequently clarified using a filter of 45μm. Lentiviruses were concentrated by centrifugation at 50.000 g (3 hours), thereby preventing that components of cell culture medium could affect the experiments. To obtain high infection efficiency, lentiviruses were finally resuspended in a solution containing a "carrier" protein which preserves their conservation at -80ºC without affecting the infectivity capacity, i.e. 1% BSA in PBS solution. The biological titer of the viral preparation was expressed as a number of transducing units per millilitre (TU/ml) and was determined by transducing HEK293T cells in limiting dilutions. After 48 hours incubation, the percentage of GFP-positive cells was counted and viruses at $5 \times 10^8 - 1 \times 10^9$ TU/ml were used in the experiments.

4.3. Cell transduction

For lentiviral transduction, hippocampal neurons or SCNPs were seeded in a 24-multiwell plate and 1μl of the concentrated lentivirus were added to the medium 2 hours after plating. Hippocampal neurons were treated with the corresponding

stimulus at 2 DIV and fixed at 3 DIV for GFP immunostaining and measurement of axon length. SCNPs were stimulated at 3 DIV and fixed at 5 DIV for GFP immunostaining and measurement of neurites length.

The efficiency of the infection was continuously monitored by direct counting of GFP-positive cells. According to GFP expression driven by the lentiviral vector ~ 90-95% of neurons/SCNPs were transduced.

5. Cellular lysis and Western Blot assay

5.1. Cell lysis

After the indicated treatments, cells were rinsed twice with cold PBS and resuspended in lysis buffer containing 2% SDS and 62.5mM Tris-HCl (pH 6.8) before loading into a SDS-polyacrylamide gels (SDS-PAGE).

5.2. Western blot

Cellular extracts were resolved in SDS-PAGE. According to the manufacturer instructions, proteins were elecrophoresed and electrotransferred from the gel to a Polyvinylidene difluoride (PVDF) Immobilon-P transfer membrane (Millipore, Bedford, MA) using a semidry Trans-Blot apparatus (Hoefer, Amersham Pharmacia Biotech). The membrane was blocked with Tris-buffered saline with Tween 20 (20mM Tris-HCl pH 7.4, 150mM NaCl and 0.05% Tween 20) containing 5% non fat dry milk or 5% BSA (1 hour, RT) and probed with the appropriate primary antibody according to the specific requirements indicated by each provider. After 1 hour incubation with the specific peroxidase-coniugated secondary antibody (Sigma, Jackson Immunoresearch or Cell Signalling), the membrane was finally developed with EZ-ECL (Biological Industries, Kibbutz Beit Haemek, Israel) or SuperSignal chemiluminescent detection kit (Amersham Pharmacia Biotech, Pierce, USA).

6. Immunoprecipitations

Immunoprecipitation is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. This

process can be used to isolate and concentrate a particular protein from a sample containing many different proteins.

Immunoprecipitation of intact protein complexes is known as co-immunoprecipitation (Co-IP). Co-IP works by selecting an antibody that targets a known protein that is believed to be member of a larger complex of proteins. By targeting this known member with an antibody it becomes possible to pull the entire protein complex out of solution and thereby study protein-protein interaction in the complex.

6.1. NP40 and RIPA solubilization

PC12 cells and hippocampal neurons were washed twice with cold PBS and were lysed in NP-40 (20 mM Tris-HCl, pH 7,4, 140 mM NaCl, 10% glycerol and 1% NP-40) or RIPA buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 5mM EDTA,1% NP-40, and 0.5% sodium deoxicholate) containing complete protease inhibitor, 40 mM β-glycerophosphate, 1mM sodium ortovanadate and 25 mM sodium fluoride. RIPA was used for all the experiments showing phosphospecific β-catenin phosphorylation in neurons. Protein concentration was quantified by Lowry Assay.

6.2. Immunoprecipitation

Typically we used 40 μg of protein obtained from hippocampal neuron lysates and 1 mg protein obtained from PC12 6/15 cell lysates. The lysates were rock for 20 min at 4ºC then cleared by centrifugation for 5 min at 12000 rpm. Cell lysates were incubated with 1 μg of antibody at 4°C for 1 hour and 35 μl Protein-G-Sepharose beads were added overnight at 4°C. The immunoprecipitates were analyzed by SDS-PAGE.

7. GST fusion protein

7.1. Growth and harvesting of bacteria

GST-β-catenin recombinant proteins were produced in BL21 bacteria (Fig. 18) following the below protocol:

- Set up an overnight culture in 4 ml LB buffer containing 100 mg/ml Ampicillin.

- In the morning, add overnight broth to 400 ml prewarmed LB supplement with 100 mg/ml Ampicillin. Grow for 3 hours at 37°C, shaking at 220rpm.
- When bacterial culture has grown enough (the absorbance measured at 600 nm is 0.6-0.7), induce the protein synthesis with 0.1mM isopropyl-beta-D-thiogalactopyranoside. Grow for 2 hours at 37°C. Samples are collected prior (S1) and after induction (S2).

- Pellet cells (4000 rpm 10 min at 4°C). The pellet is resuspended in 20 ml PBS supplemented with 1mM PMSF and sonicated for 20 min (10´´sonicate, 50´´off).

- Add 1% Triton X-100 (TX-100) and mix for 30 min at 4°C. Take a sample (S3).

- Pellet cellular debris at 10000 rpm for 10 min, 4°C. Take a sample from supernatant (S4).

7.2. Binding to beads and thrombine cleavage

- Incubate soluble lysate with 1200 µl of 50% GSH beads (Glutation-Sepharose 4B) at 4°C rocking for 120-150 min.

- Centrifuge at 3000 rpm, 3min. and remove the supernatant (before take a sample-S5). Wash beads three times in PBS.

- Wash with thrombin cleavage buffer (20 mM Tris, 100 mM NaCl, 2.5 mM $CaCl_2$; pH 8)

- To cleave 1 mg protein are necessary 8 units of thrombin (Sigma T4648). We assume a yield of 1 mg protein/ 400 ml cumture. Beads are incubated with the corresponding amout of thrombin (80 min, 30°C, 800 rpm).

- Centrifuge at 3000 rpm, 3min. Recover the supernatant. The digestion is blocked by adding complete protease inhibitors (Roche).

- Wash beads in 600 µl 0.5M NaCl-Tris pH 7 to release the remaining band protein. Add complete protease inhibitors. Take samples from both batches of protein (S6, S7) and beads (S8).

- Pool fractions containing a significant amount of protein and dialyse with 1000 x volume of dialysis buffer (50 mM Tris pH 7.8, 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 45% glycerol). Dialyse at 4°C for 16 hours.

- Proteins containing 50% glycerol are stored at -70°C. Load 2 µg of protein in a SDS-PAGE (S9).

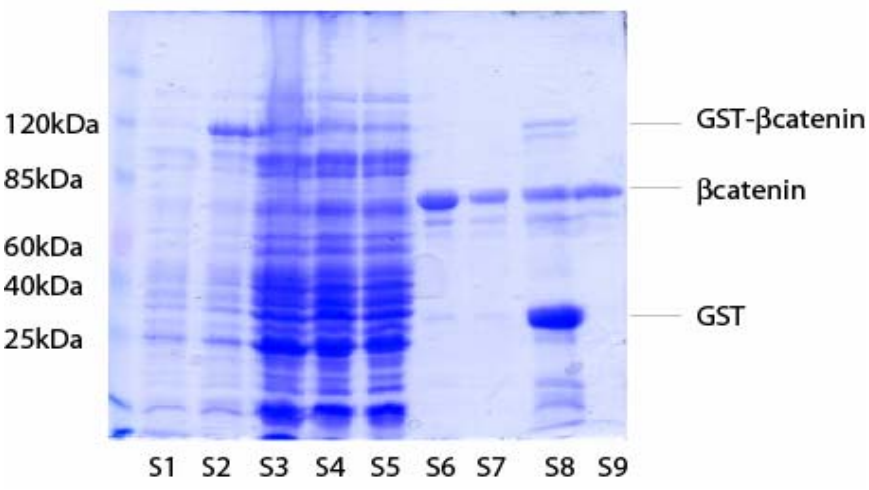

Figure 18. ***Coomassie staining illustrating the steps of protein obtaining and purification.*** *S1) Non-induced bacterial culture. S2)IPTG-induced bacterial culture. S3)Bacterial lysate after sonication and TX-100 permeabilization. S4) Supernatant containing GST-β-catenin protein. S5) Flow through after GSH binding. S6,7)Protein cleaved with thrombin on beads. S8)GSH beads. S9)Protein cleaved after dialyisis.*

8. In vitro kinase assays

A kinase is protein enzyme that adds a phosphate to a protein. The addition of a phosphate covalently modulates the substrate protein and typically alters the substrate conformation sufficiently to either activate or inactivate it.

We performed in vitro kinase assays to check β-catenin phosphorylation by different kinases. Recombinant TrkA kinase or TrkA immunoprecipitated from PC12 6/15 cells were incubated with GST-β-catenin or β-catenin wild-type (WT) or mutant in the presence or absence of the Trk kinase inhibitor, K252a. Recombinant Met kinase was incubated with GST-β-catenin WT or mutant. GST-β-catenin (0.8 μg; 6.7 pmols) phosphorylation by recombinant TrkA and Met kinases was performed following manufacturer's instructions (15 min, 30°C). β-catenin (1.5 μg; 17 pmols) phosphorylation by TrkA was assayed (20 min, 30°C) in kinase buffer (50 mM Hepes, pH 7,4, 10 mM MgCl2, 5 mM MnCl2, 0.5 mM EDTA, 1mM DTT, 0.1% NP-40 plus 1mM sodium ortovanadate in the absence or presence of 100 nM K252a (preincubated with the kinase 5 min, RT) and 0.1 mM ATP. Samples were analyzed by Western-blot.

9. Immunofluorescence

Cells were initially seeded on a coverslip pre-coated as specified. After the corresponding time in culture with the corresponding stimulus, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at RT. For cytoskeletal staining, detergent fixation (3% formaldehyde, 0.2% glutaraldehyde, 0.2% Triton X-100, 10 mM EGTA pH 7.2) was used (10 min, 37˚C). After detergent fixation, to block glutaraldehyde autofluorescence, cells were incubated with 50 mM NH4Cl and 0.3M Glycine. Cells were washed with PBS and blocked and permeabilized in PBS containing 5% FBS, 5% HS, 0.2% glycine and 0.1% Triton X-100. Binding of TRITC or FITC-Phalloidin (Sigma) (0.1 μg/ml in PBS for 45 min at RT) to localize actin filaments was performed in cells permeabilized with 0.2% Triton X100 in PBS without blocking. After the blocking step, cells were incubated overnight at 4°C with the primary antibodies. Secondary antibodies were Alexa Fluor 488 and 594 (Molecular Probes) and Rat IgG-FITC (GAR IgG-FITC; Alexis Biochemicals). Secondary antibodies together with Hoechst 33258 were incubated for 1 hour at RT in the dark. Finally, cells were washed three times with PBS and mounted in Moviol (Moviol 4-88, glycerol 50%, 0.2M Tris-HCl buffer pH 8.5).

9.1. Bromodeoxyuridine incorporation.

Cell cycle activity was investigated by incorporation of the thymidine analogue, bromodeoxyuridine (BrdU). BrdU 3.75 μg/ml was applied for 8 hours on 2 DIV or 4 DIV before fixation. Cells were fixed with 4% PFA in PBS for 20 min at RT. For BrdU immunotection, cells were first permeabilized with 0.05% TX-100 (1 min, RT), then incubated with 2N HCl (30 min, 37˚C) and neutralized with 0.1M sodium tetraborate, pH=8,5 (2 min, RT) before blocking.

10. Flow cytometry analysis (FACS)

For flow cytometry analysis SCNPs were stimulated with recombinant mouse Wnt-3a/BSA or Wnt-3/Control CM for 48 h. Cells were then harvested by trypsinization and fixed by resuspending in 0.5ml cold PBS and 1ml ethanol 70% for minimum 2 hours at 4°C. Cells were incubated with 1 ml staining solution containing

0.1% Triton X-100, 50 μg/ml of propidium iodide and 50 μg/ml of RNAse A for 30 min at 37°C before analysis for their DNA content on an EPICS XL flow cytometer (Coulter) according to the manufacturer instruction. The programme WinMDI 2.9 was used to determine the percentage of S phase of cell cycle.

11. Morphometric measurements and statistical analysis

Micrographs were obtained using an inverted Olympus IX70 microscope (10x, 0.3 NA and 20x, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti). Images were acquired using DPM Manager Software. Alternatively, images were obtained using an Olympus confocal IX70 microscope and the FluoView 500 Software and processed using MacBiophotonics ImageJ software (www.macbiophotonics.ca).

Neurite length was measured using Adobe Photoshop software. The axon was identified as the longest neurite at this stage (2 DIV) of the hippocampal cell development. Branching was measured by counting Total Axonal Branch Tip Number (TABTN) (Yu and Malenka, 2004). Neurite length and axon branching plots represent values normalized to the corresponding untreated control, shown as average ± s.e.m. Significance was calculated by the Student T test. Asterisk (*) indicates statistical significance compared to the corresponding untreated control and hash (#) compared to stimulated controls.

Results

Objective 1

1. Wnt-3a and Wnt-3 regulate spinal cord neural precursor development by canonical Wnt/β-catenin signalling

1.1. Characterisation of SCNPs culture

SCNPs are multipotent progenitors whose proliferative and differentiation potentials are progressively restricted during development.

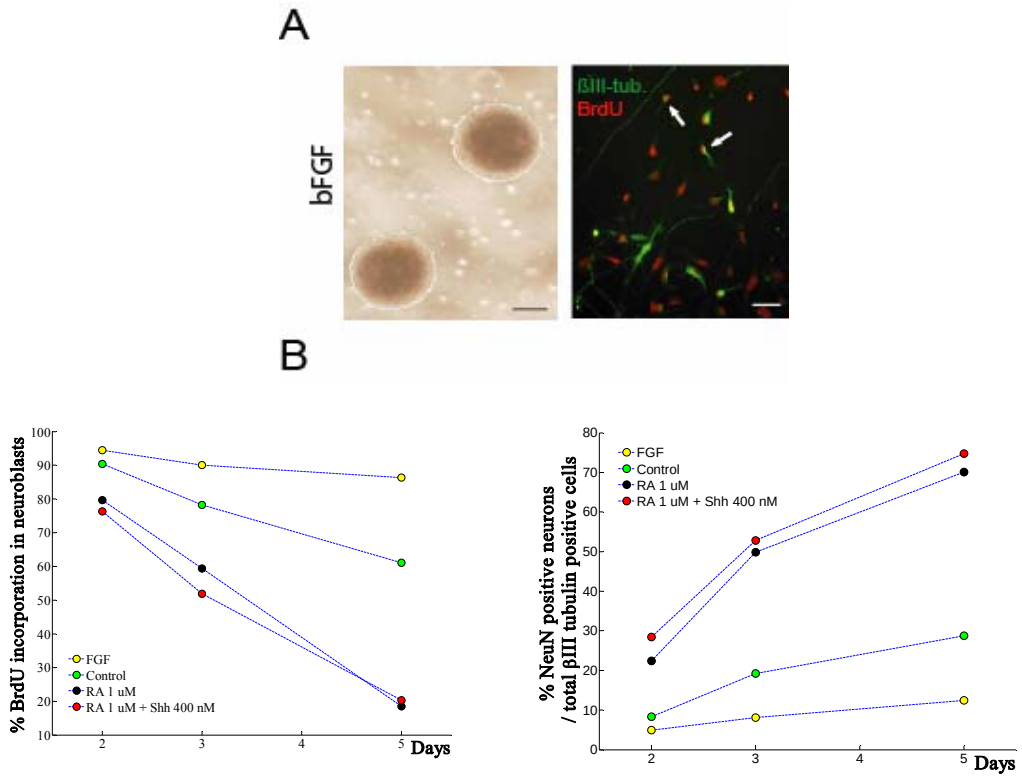

Figure19.***Characterisation of SCNPs culture.*** *A) SCNPs form floating neurospheres and show high BrdU incorporation and weak immunoreactivity for early neuronal marker βIII-tubulin (arrows) on adherent conditions in the presence of bFGF Bar= 40 µm. B) Plots representing changes in BrdU incorporation and NeuN expression in basal medium, proliferative medium (bFGF), and differentiation medium (RA and RA + Shh) and 1% FBS as mentioned in methods . Cells were pulsed overnight with BrdU.*

We have examined changes in developmental potential of SCNPs culture in basal conditions (without stimulus), in proliferative medium (with bFGF 20 ng/ml), or in differentiation medium (retinoic acid (RA) 1µM or RA 1µM and Shh 400nM). SCNPs proliferation was measured by BrdU incorporation. Neuronal differentiation of SCNPs was visualized by the expression of the early neuronal marker βIII-tubulin and the post-mitotic marker NeuN. Proliferation and differentian assays show that bFGF treatment keeps cells in a proliferative state (95 % at 2 DIV, 86 % at 5 DIV) with a low tendency to differentiate (5 % at 2 DIV, 12 % at 5 DIV) of SCNPs up to 5

days in culture. In contrast, treatment with RA or RA and Shh dramatically reduced BrdU incorporation (from 80% at 2 DIV to 19 % at 5 DIV and from 76 % at 2 DIV to 20 % at 5 DIV respectively) and increased neuronal differentiation (from 22 % at 2 DIV to 70 % at 5 DIV and from 29 %. at 2 DIV to 75 % at 5 DIV respectively) (Fig. 19B). Control culture of mouse SCNPs showed decreased proliferation (from 90 %. at 2 DIV to 61 % at 5 DIV) and increased neuronal differentiation (from 8 %. at 2 DIV to 29 % at 5 DIV) (Fig. 19B) over time, indicating a tendency of SCNP to differentiate on adherent conditions. From these studies we decided to concentrate our future analyses from 2 DIV to 4 DIV, a period in which SCNPs display proliferation versus differentiation capacity.

1.2. Wnt-3a and Wnt-3 increase the proliferation of SCNPs

The expression patterns of *Wnt-3a* and *Wnt-3* in the developing spinal cord suggested a role in SCNPs development (Parr et al., 1993; Krylova et al., 2002; Megason and McMahon, 2002). To study the Wnt capacity to regulate proliferation of SCNPs we performed two types of experiments. To first evaluate a possible effect of Wnt-3a and Wnt-3 in SCNPs proliferation, DNA content was analyzed in control or Wnt-treated SCNPs after propidium iodide staining. We found that Wnt-3a and Wnt-3 increased the percentage of cells in S phase compared to controls by 29% and 41%, respectively (Fig. 20A) at 2 DIV. To further confirm a proliferative effect of Wnt-3a and Wnt-3 signalling in SCNPs, we also performed BrdU incorporation assays. Adherent cultures treated for 2 or 4 days with recombinant Wnt-3a showed an increase of 69 % and 88 % in BrdU incorporation compared to control cultures. (Fig. 20C). Similarly, cultures treated with Wnt-3 CM for 2 days also showed an increase of 72 % in BrdU incorporation compared to control SCNPs. Contrary to the sustained stimulation of the proliferation of SCNP by Wnt-3a, Wnt-3 did not affect BrdU incorporation in SCNPs at 4DIV (Fig. 20C). We have also calculated BrdU incorporation in small neuroblasts (typically with a cell diameter of about 15 μm, nestin-immunoreactive and already displaying short neurites). Interestingly, Wnt-3a increased the proliferation of small neuroblasts by 19% at 2 DIV and 64% at 4 DIV. Furthermore, Wnt-3 increased BrdU incorporation in small neuroblasts by 20% at 2 DIV, but this effect was not maintained at 4 DIV (Fig. 20C).

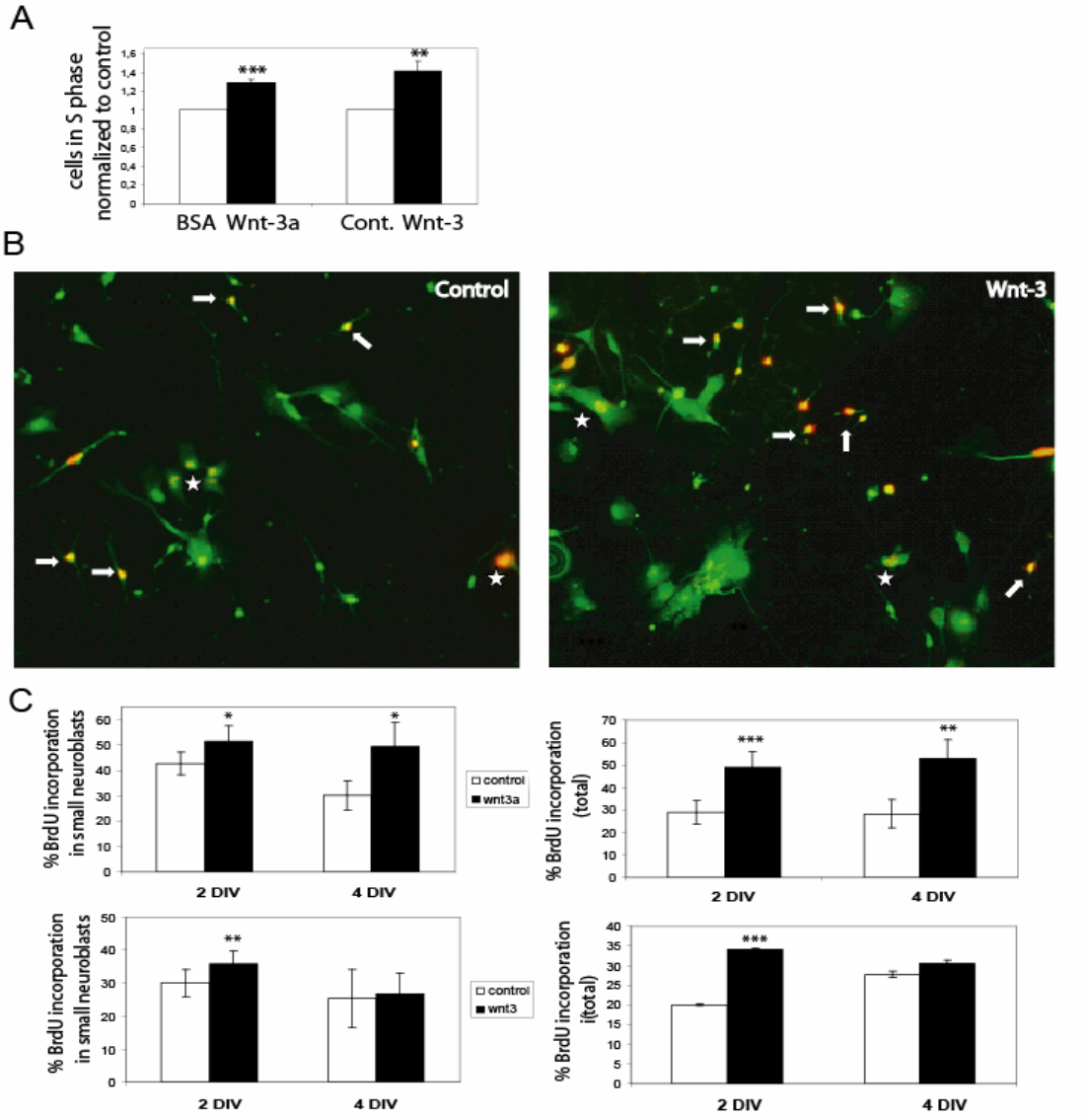

Figure 20. ***Wnt-3a produces a sustained increase of SCNP proliferation, whereas Wnt-3 transiently increases SCNP proliferation.*** *A) Plot represents the increase in the percentage of SCNPs in S phase in Wnt-treated SCNPs normalized to controls. B) Note that nestin immunocytochemistry reveals the presence of different nestin-positive cell populations in SCNP cultures (small neuroblasts of about 15 µm diameter –arrows and, cells with a diameter of 30-40 µm, that could correspond to GFAP-immunoreactive precursors-asteriks). SCNPs treated with Wnt-3 CM compared to control CM show increased percentage of SCNPs that have incorporated BrdU at 2 DIV. C) Plots represent the % of BrdU incorporation in small neuroblasts (left panels) and in total nestin-positive neural precursors (right panels) of control, Wnt-3a or Wnt-3-treated SCNPs (2 and 4 DIV). Wnt-3a and Wnt-3 treatments significantly increase BrdU incorporation over control cells at 2 DIV, but at 4 DIV only Wnt-3a treatment promotes SCNP proliferation. Bar= 65 µm*

Thus our results suggest that Wnts signal in different populations of nestin-positive neural precursors. Together, these results indicate that Wnt-3a maintains a proliferative SCNPs pool, whereas Wnt-3 transiently stimulates proliferation.

1.3. Wnt-3 increases neurogenesis of SCNP-derived neurons by canonical Wnt/β-catenin pathway

The transient increase in BrdU incorporation by Wnt-3 signalling suggested that SCNPs exit the cell cycle shortly after Wnt-3 treatment. Therefore we next asked whether the observed Wnt-induced increase in proliferation correlated with increased neurogenesis in SCNP cultures. We found that Wnt-3 increased about 1.6x the proportion of neurons (identified by the βIII-tubulin immunostaining) at 4 DIV, which also express the post-mitotic marker NeuU (Fig. 21). However, the percentage of βIII-tubulin positive neurons was not affected by Wnt-3a (Fig. 21A), consistent with its sustained proliferative effect at 4 DIV. We also studied the effect of the GSK-3β inhibitors Bio and Kenpaullone on neuronal differentiation. The selective GSK-3β inhibitor Bio (Sato et al., 2004) significantly increased the proportion of βIII-tubulin positive neurons, whereas the effect of Kenpaullone (that inhibits GSK-3β, Cdks and other kinases) was not significant (Fig. 21A). These results indicate that GSK-3β inhibition mimics Wnt-3 signalling, suggesting that Wnt-3 promotes neurogenesis by canonical Wnt/β-catenin in SCNPs.

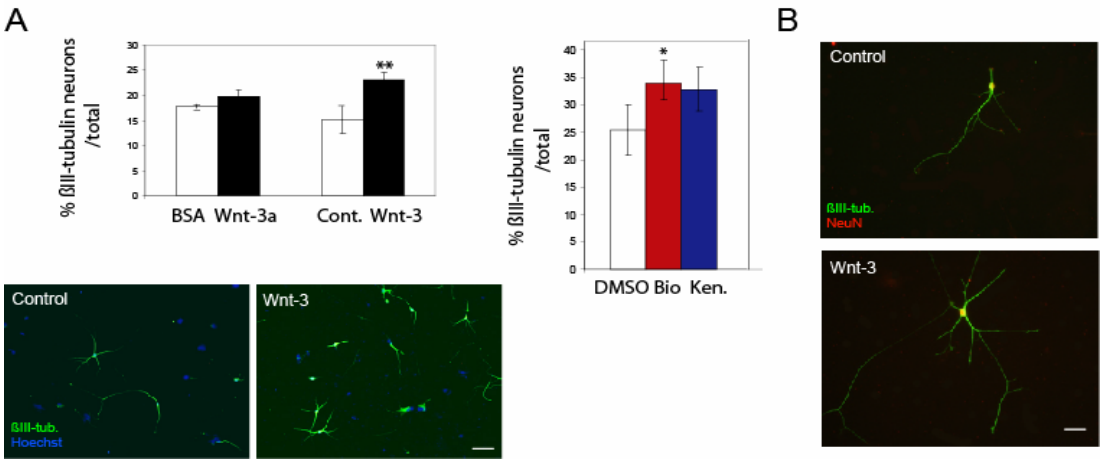

Figure 21. ***Wnt-3 increases neurogenesis of SCNP-derived neurons by canonical Wnt/β-catenin signalling.*** *A) Plot shows that Wnt-3, but not Wnt-3a, increases the percentage of βIII-tubulin-positive neurons in SCNP cultures at 4 DIV. Representative pictures of control and Wnt-3-treated SCNP cultures immunostained for βIII-tubulin. Note the increased number of βIII-tubulin-immunoreactive cells compared to total cells (stained for Hoechst) in Wnt-3-treated SCNPs. Treatment with the GSK-3β inhibitor Bio also increases the percentage of βIII-tubulin-positive neurons compared to control (DMSO), whereas the increase in the percentage of βIII-tubulin-positive neurons obtained with Kenpaullone was not significant (LEFt panel). Bar= 65 µm. B) Wnt-3 increases neurite extension in SCNP-derived postmitotic neurons, co-immunostained for βIII-tubulin and the transcription factor NeuN at 4 DIV. Bar= 40 µm.*

1.4. Wnt-3a and Wnt-3 increase neurite outgrowth of SCNP-derived neurons

In addition to the Wnt-3a and Wnt-3 effects on the proliferation of SCNPs, we noticed an effect of Wnt signalling on neurite extension of differentiating SCNPs. To further investigate a role of Wnt-3a and Wnt-3 signalling on neurite outgrowth, a hallmark of neuronal differentiation, Wnt-3a and Wnt-3 treated SCNP cultures were immunostained for βIII-tubulin and neurite length was measured. Wnt-3a increased neurite length by 23 % and 35 % at 2 and 4 DIV, respectively, compared to control treatment (Fig. 22A and 22C). Similarly, Wnt-3 CM increased neurite length by about 25 % and 59 % at 2 and 4 DIV compared to control CM (Fig. 22B and 22D).These results demonstrate that Wnt-3a and Wnt-3 signalling stimulate neurite outgrowth in SCNP-derived neurons. These findings also indicate that Wnt-3a and Wnt-3 act on different SCNP subpopulations to regulate different aspects of SCNP development (proliferation and neurite extension).

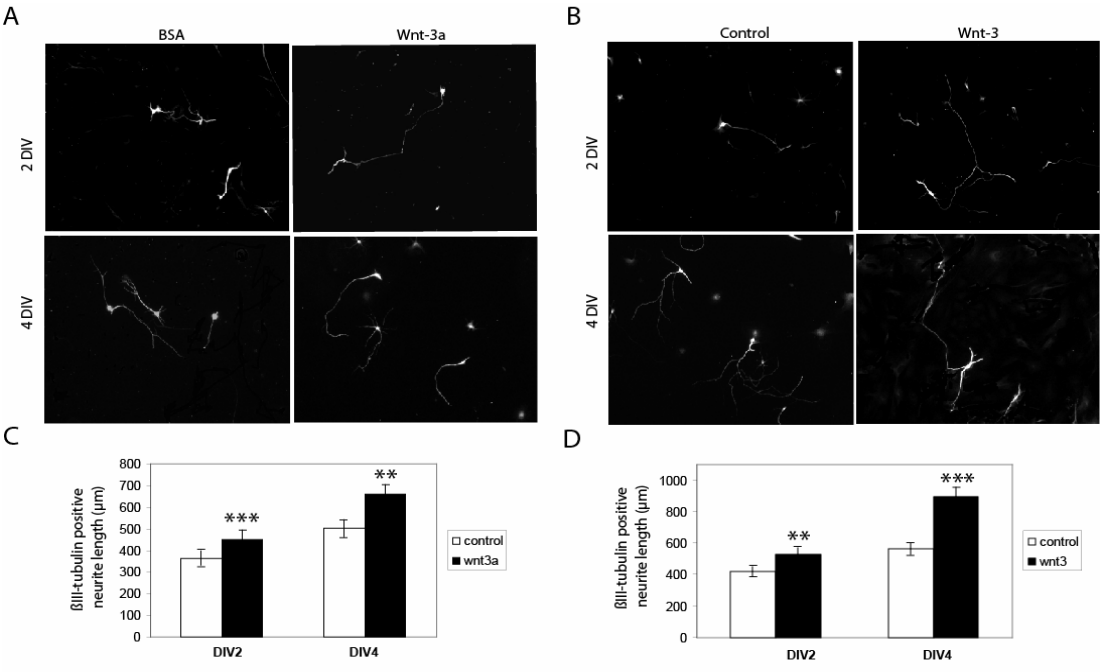

Figure 22. ***Wnt-3 and Wnt-3a induce neurite outgrowth in SCNP-derived neurons.***
Wnt-3a (A) and Wnt-3 (B) increase neurite extension compared to controls in SCNP cultures immunostained for βIII-tubulin at 2 and 4 DIV. Bar= 40 µm. C and D) Plots represent neurite length values for controls and Wnt-3a or Wnt-3-treated SCNP-derived neurons. Wnt-3a and Wnt-3 stimulate neurite extension in SCNPs compared to controls.

1.5. GSK-3β inhibition from plating or upon establishment of an adherent culture differently affects neurite outgrowth

To begin to understand through which signalling pathway Wnt-3a and Wnt-3 are acting to promote neurite outgrowth of SCNPs, we studied the consequences of inhibiting GSK-3β in this process. SCNPs were treated with the GSK-3β inhibitors, Bio and Kenpaullone, up to 3 DIV. Cultures treated with Wnt-3a or Wnt-3 from plating up to 3 DIV showed an increase in neurite length of about 42 % and 38 %, respectively (Fig. 23A).

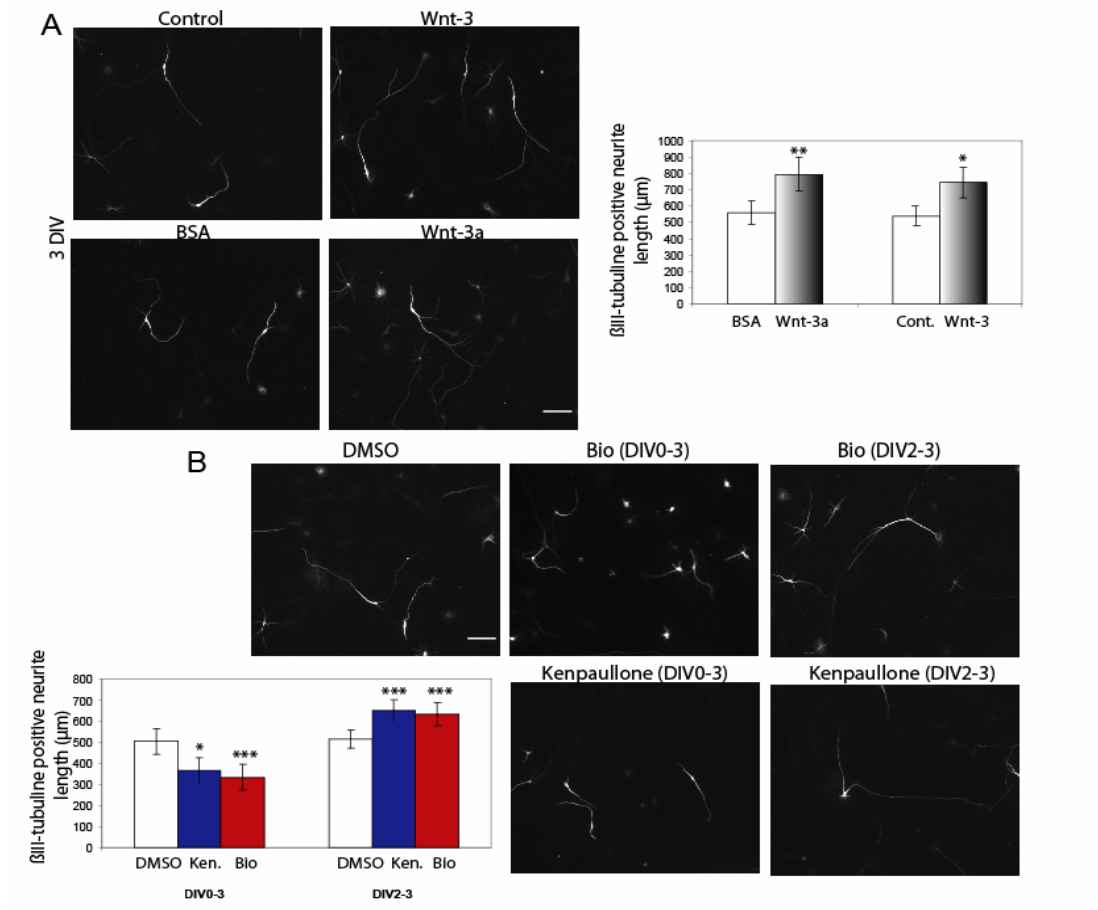

Figure 23. ***GSK-3β inhibition (DIV 0-3) prevents neurite outgrowth, whereas GSK-3β inhibition (DIV 2-3) mimics the Wnt-induced neurite outgrowth.*** *A) Wnt-3a and Wnt-3 increase neurite extension compared to controls in SCNP cultures immunostained for βIII-tubulin at 3 DIV. Plot represents neurite length values for controls and Wnt-3a or Wnt-3-treated SCNP-derived neurons at 3 DIV. B) SCNP cultures treated with the GSK-3β inhibitors Bio and Kenpaullone from plating up to 3 DIV or overnight on established adherent cultures (from 2 DIV to 3 DIV) and immunostained for βIII-tubulin. Neurite length values indicate that inhibition of GSK-3β upon plating results in shorter neurites than in control (DMSO-treated) cultures. Arrows point to the SCNPs with very short processes. In contrast, inhibition of GSK-3β from 2 DIV to 3 DIV stimulates neurite extension compared to control. Bars = 130 μm.*

Interestingly, treating SCNPs with Bio and Kenpaullone from plating resulted in the inhibition of neurite growth measured at 3 DIV (by 34 % and 28%) compared to controls (Fig. 23B). Nevertheless, incubating SCNPs with Bio or Kenpaullone from 2 to 3 DIV increased neurite outgrowth by 23% and 26 %, similar to Wnt treatment (Fig. 23B). findings indicate that Wnt-3a and Wnt-3 stimulate neurite outgrowth by inhibiting GSK-3β, suggesting activation of the canonical Wnt pathway. Furthermore, these results reveal opposite roles of GSK-3β during different stages of neurite outgrowth in SCNP cultures.

1.6. β-catenin and functional TCF-4 are required for neurite outgrowth of SCNPs promoted by Wnt-3a and Wnt-3

A canonical Wnt signalling pathway that diverges at the level of GSK-3β regulates the microtubule cytoskeleton and the axon behaviour (Ciani et al., 2004; Salinas, 2007; Purro et al., 2008) independent of β-catenin and transcriptional activation. To investigate the requirement of β-catenin in the Wnt-3a and Wnt-3-induced neuronal differentiation, SCNPs were transduced with lentivirus for the expression of β-catenin shRNAs. Using this method we obtained a reduction of β-catenin total levels of ~30% with shRNA1 (specific for mouse&rat β-catenin) and ~20% with shRNA3 (specific for mouse β-catenin) at 5 DIV compared to scrambled shRNA (Fig. 24A). SCNPs expressing control or β-catenin shRNAs were treated with or without Wnt-3a and Wnt-3 before neurite length was measured. Wnt-3a and Wnt-3 induced neurite outgrowth in SCNPs expressing scrambled shRNA. However, in SCNPs expressing the β-catenin shRNAs, the Wnt-induced stimulation of neurite growth was prevented (Fig. 24B-24C). These results indicate that β-catenin is required for the Wnt-3a- and Wnt-3-induced neurite outgrowth of SCNP-derived neurons.

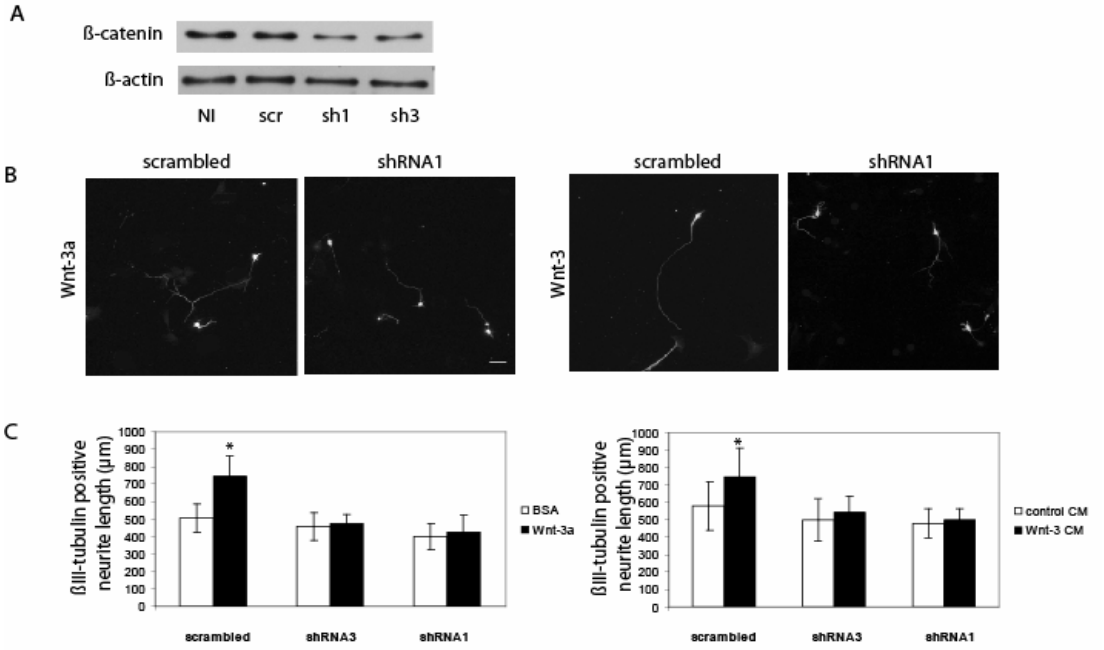

Figure 24. ***β-catenin is required for the Wnt-3a- and Wnt-3-induced neurite outgrowth of SCNP-derived neurons.*** *A) β-catenin western blot showing β-catenin levels in non-infected (NI) SCNP cultures or in SCNPs infected with lentivirus expressing scrambled (scr) or β-catenin shRNA (sh) #3 and #1 at 5 DIV. β-actin western blot was used as a loading control. B) SCNP cultures expressing scrambled (scr) or β-catenin shRNA3 treated with Wnt-3a or Wnt-3 and immunostained for βIII-tubulin. Bar = 40 μm. C) Plots represent neurite length values for SCNPs expressing scrambled (scr) or β-catenin shRNAs treated with BSA (control) or Wnt-3a (LEFt panel) or with control and Wnt-3 CM (right panel). Wnt-3a and Wnt-3 stimulate neurite extension in SCNPs expressing scrambled shRNA but not in SCNP cultures expressing β-catenin shRNA1 or shRNA3.*

Stabilized β-catenin translocates to the nucleus and regulates transcription by TCF/LEF transcription factors of Wnt target genes (Logan and Nusse, 2004; Ciani and Salinas, 2005).To investigate the involvement of gene transcription in the effects promoted by Wnt-3a and Wnt-3, we first treated SCNPs with Actinomycin D (ActD). We used a concentration of ActD (5 nM) that did not affect neurite growth in control SCNP cells (Fig. 25A). However, the neurite growth promoted by Wnt-3a and Wnt-3 was blocked in SCNPs treated with ActD (Fig. 25A). These results indicate that gene transcription is needed during the neurite growth induced by these Wnt factors. Next we tested whether expression of a dominant negative ΔN-TCF-4, whose promoter can not bind β-catenin (Roose et al., 1999), could block the neurite extension induced by Wnt-3a and Wnt-3 in SCNPs. In hippocampal neurons ΔN-TCF-4 blocks the axon growth induced by Wnt-3a (David et al., 2008). SCNPs

expressing EGFP and treated with Wnt-3a or Wnt-3 displayed neurites ~ 30% longer than controls (Fig. 25B). However, the increase in neurite length promoted by Wnt-3a and Wnt-3 was blocked in SCNPs expressing ΔN-TCF-4 (Fig. 25B).

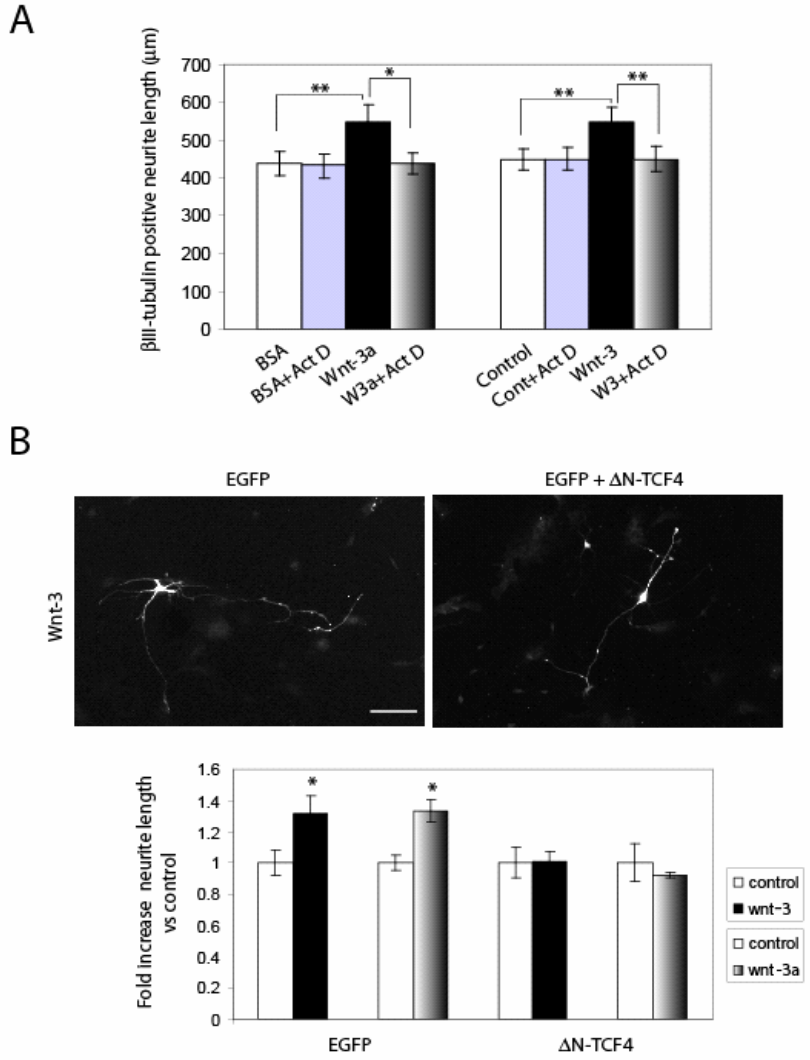

Figure 25. ***Gene transcription by TCF-4 is required for the Wnt-3a- and Wnt-3-induced neurite extension of SCNP-derived neurons.*** *A) SCNPs were treated with Actinomycin D (5 nM; Act D) together with BSA, Wnt-3a, control CM or Wnt-3 CM from plating to 2 DIV. Plot represent βIII-tubulin-positive neurite length values. Wnt-3a and Wnt-3 treatment increase neurite length and co-treatment with Actinomycin D blocks the Wnt-induced increase in neurite length. B) SCNPs expressing EGFP (control) or EGFP plus ΔN-TCF-4 treated with Wnt-3 and immunostained for EGFP. Bar = 65 µm. Plot represents neurite length values normalized to controls for SCNPs expressing EGFP or EGFP plus ΔN-TCF-4. Expression of dominant negative ΔN-TCF-4 blocks the Wnt-induced increase in neurite length observed in control EGFP-expressing cultures.*

These results demonstrate that transcription by TCF-4 is downstream of β-catenin in the Wnt-3a and Wnt-3-induced neurite outgrowth in SCNPs. We conclude that

Wnt-3a and Wnt-3 signalling by the Wnt/β-catenin canonical pathway promote the neurite outgrowth in SCNPs.

Objective 2

2. Signalling by neurotrophins and hepatocyte growth factor regulates axon morphogenesis by differential β-catenin phosphorylation

2.1. Trk interacts with β-catenin

β-catenin was shown to associate with and to be a substrate of EGFR (Hoschuetzky et al., 1994; Takahashi et al., 1997). Here we explored whether it interacts with other RTK such as Trk in neuronal cell lines and primary neurons. PC12 6/15 cells, a clone of PC12 cells stably expressing the non-neuronal form of human TrkA (Hempstead et al., 1992; Llovera et al., 2004) were treated with NGF. TrkA and β-catenin co-immunoprecipitated from PC12 6/15 lysates independently of NGF stimulation (Fig. 26A). To investigate whether this association extended to other Trk receptors we used hippocampal neurons, which express *TrkB* and *TrkC* (Martinez et al., 1998). Using anti-pan Trk antibodies we immunoprecipitated TrkB and TrkC from untreated and BDNF or NT-3-treated neurons. β-catenin co-immunoprecipitated with TrkB and TrkC from hippocampal neuron lysates (Fig. 26B). Thus, β-catenin interacts with Trk receptors in PC12 cells and developing neurons.

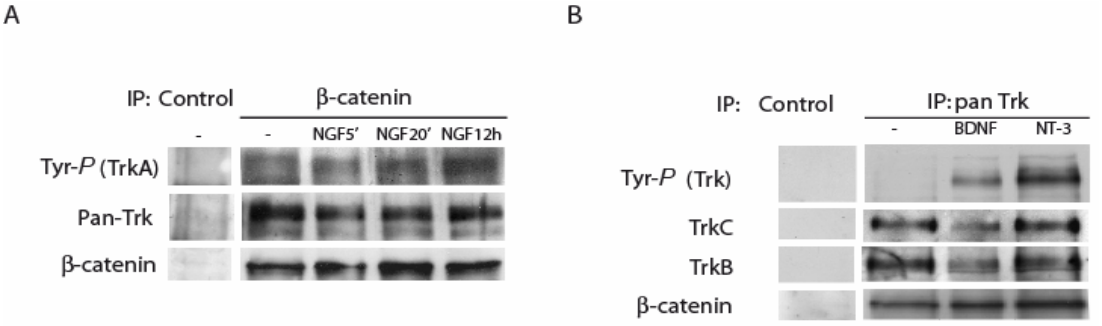

Figure 26. ***β-catenin interacts with TrkA in PC12 cells and with TrkB and TrkC in hippocampal neurons.*** *A) Control (Myc) or β-catenin immunoprecipitation from 6/15 PC12 cells starved or stimulated with NGF (100 ng/ml) for different times shows that TrkA (detected with panTrk antibodies: 140 kDa and 110 kDa forms) co-immunoprecipitates with β-catenin, independent of its phosphorylation state. B) Control (GFP) or pan-Trk immunoprecipitation from hippocampal neurons untreated or treated with BDNF or NT-3 (50 ng/ml; 5 min) shows that TrkB and TrkC co-immunoprecipitate with β-catenin.*

2.2. Trk phosphorylates β-catenin at Y654

The interaction of Trk receptors with β-catenin raised the possibility that β-catenin could be phosphorylated by these RTK. In *in-vitro* phosphorylation assays,

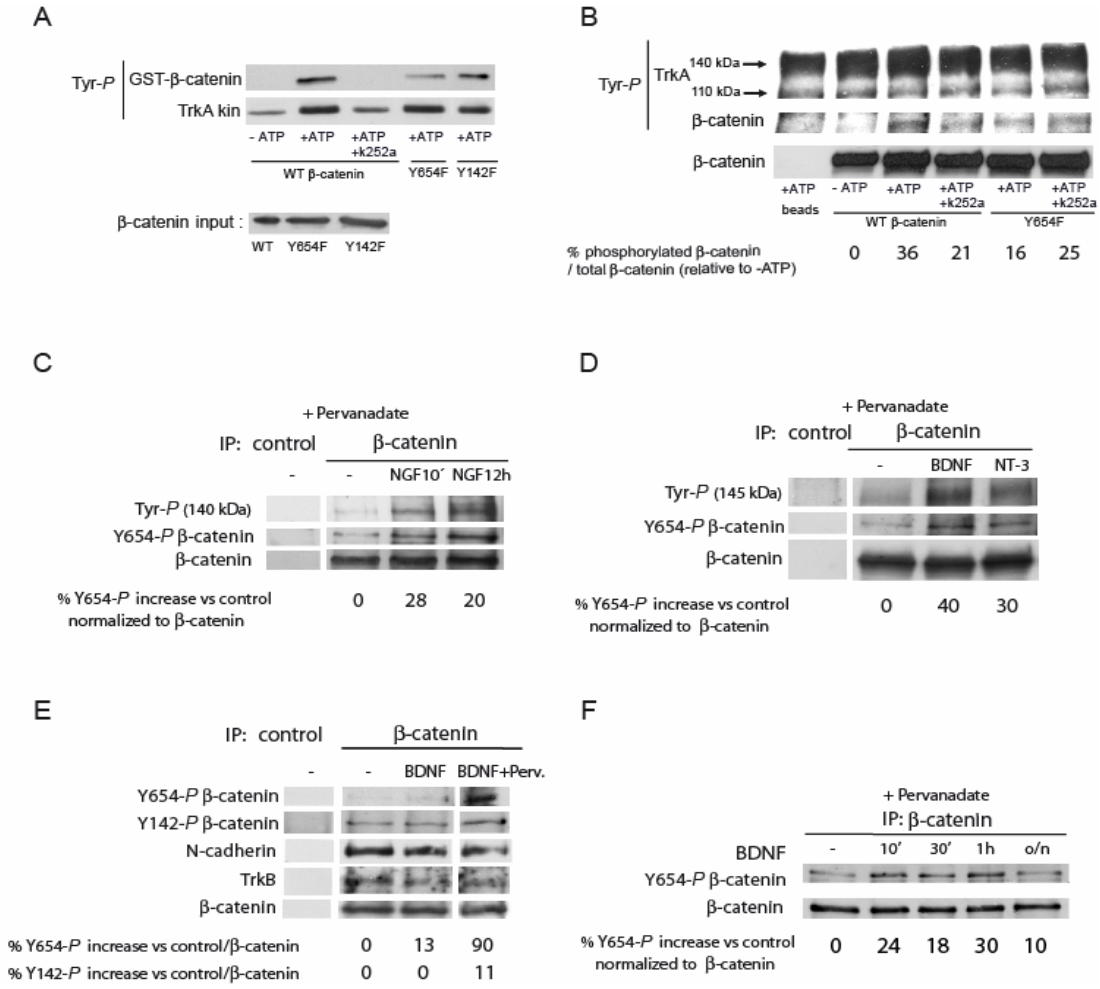

Figure 27. ***Trk phosphorylates β-catenin at Y654.*** *A) In vitro kinase assay using recombinant TrkA kinase and WT or mutant GST-β-catenin. Anti-P-Tyr Western blot shows phosphorylated TrkA kinase and GST-β-catenin. WT GST-β-catenin is phosphorylated by TrkA kinase upon addition of ATP, while K252a inhibits the TrkA induced-β-catenin phosphorylation. Phosphorylation of Y654F and Y142F GST-β-catenin by TrkA kinase is ~ 45% and 15% lower than that of WT GST-β-catenin. Panel below shows anti-β-catenin Western blot for the GST-β-catenin input in the kinase assay (0.8 μg). B) In vitro kinase assay using TrkA immunoprecipitated from 6/15 PC12 and recombinant WT and Y654F β-catenin. Anti-P-Tyr Western blot shows TrkA (140 kDa and 110 kDa bands) and the tyrosine phosphorylation of β-catenin. Phosphorylation of WT β-catenin is decreased by preincubation with K252a (100 nM), whereas phosphorylation of Y654F β-catenin is lower than that of WT β-catenin and not inhibited by K252a. In the first lane no recombinant β-catenin was added to control for phosphorylation of any endogenous β-catenin that may have been immunoprecipitated with TrkA. Quantification corresponds to % of the increase in the intensity of P-Tyr β-catenin relative to minus ATP/total β-catenin. C) Control (Myc) or β-catenin immunoprecipitation from 6/15 PC12 cells untreated or treated with NGF 100 ng/ml and pervanadate. Phosphorylation of β-catenin Y654 increases after NGF treatment in parallel with TrkA stimulation as detected by anti-P-Tyr Western blot. D) Control (His) or β-catenin immunoprecipitation from untreated hippocampal neurons or those treated with BDNF or NT-3 50 ng/ml (10min) in the presence of pervanadate. Upon NT stimulation, phosphorylation of β-catenin at Y654 increases parallel with phosphorylation of TrkB/C detected by anti-P-Tyr Western blot. E) Control or β-catenin immunoprecipitation from hippocampal neurons untreated or BDNF 50 ng/ml treated (10min) with or without*

pervanadate. β-catenin P-Y654 increases with BDNF treatments both with and without pervanadate, while phosphorylation at Y142 is maintained at basal level increasing only slightly with pervanadate. TrkB and N-cadherin co-immunoprecipitate with β-catenin and N-cadherin association shows a small decrease after pervanadate treatment. F) β-catenin immunoprecipitation from untreated neurons or those treated with BDNF 50 ng/ml for the indicated times in the presence of pervanadate. β-catenin P-Y654 peaks at 1 h and is reduced after overnight stimulation. Quantifications in C, D, E and F correspond to % of the increase in the intensity of the phosphospecific β-catenin (P-Y654 or P-Y142) band relative to the control normalized to total β-catenin.

recombinant TrkA kinase or TrkA immunoprecipitated from PC12 6/15 cells was incubated with recombinant WT or mutant β-catenin in the presence or absence of the Trk kinase inhibitor, K252a. Following the addition of ATP, TrkA kinase produced a robust tyrosine phosphorylation of WT GST-β-catenin (Fig. 27A). Preincubation of TrkA kinase with K252a reduced TrkA kinase activation to basal levels and blocked GST-β-catenin phosphorylation (Fig. 27A). Interestingly, phosphorylation of mutant Y654F GST-β-catenin by TrkA kinase was ~ 45% lower than that of WT GST-β-catenin, while Y142F GST-β-catenin phosphorylation was ~ 15% lower than phosphorylation of the WT protein (Fig. 27A). TrkA immunoprecipitated from PC12 6/15 cells also induced β-catenin tyrosine phosphorylation upon addition of ATP that was decreased by 30% by K252a (Fig. 27B). Furthermore, Y654F β-catenin phosphorylation was significantly lower (~ 40%) than that of WT β-catenin while the residual phosphorylation of Y654F β-catenin was not inhibited by K252a (Fig. 27B). Together, kinase assays indicate that TrkA phosphorylates β-catenin in vitro at Y654.

To study further the role of Trk in β-catenin phosphorylation, β-catenin immunoprecipitation was performed on lysates from 6/15 PC12 cells and hippocampal neurons in the presence or absence of pervanadate (to inhibit tyrosine phosphatases) for subsequent Western blot analysis using phosphospecific antibodies. Phosphorylated-Y654 (P-Y654) β-catenin was detected in both PC12 and hippocampal cell lysates and levels of P-Y654 β-catenin increased following NGF, BDNF and NT-3 stimulation of PC12 cells and hippocampal neurons respectively, correlating with Trk phosphorylation (Fig. 27C, D). Stimulation with BDNF (50 ng/ml) for 10 min induced the phosphorylation of Y654, which peaks at 1 hour and was reduced after overnight treatment (Fig. 27E, F). In contrast, phosphorylation of Y142 was maintained at basal levels (Fig. 27E), indicating that

NT signalling specifically phosphorylates β-catenin Y654. In addition to TrkB, N-cadherin also co-immunoprecipitated with β-catenin (Fig. 27E), suggesting that β-catenin, N-cadherin and Trk interact in a big complex. Altogether these findings demonstrate that Trk receptors phosphorylate β-catenin Y654 in PC12 cells and in primary neurons.

2.3. Met interacts with β-catenin and phosphorylates β-catenin Y142

HGF and *Met*, its RTK, are both expressed in the hippocampus (Honda et al., 1995; Korhonen et al., 2000). This localization led us to examine whether Met, like Trk, interacts with and phosphorylates β-catenin.

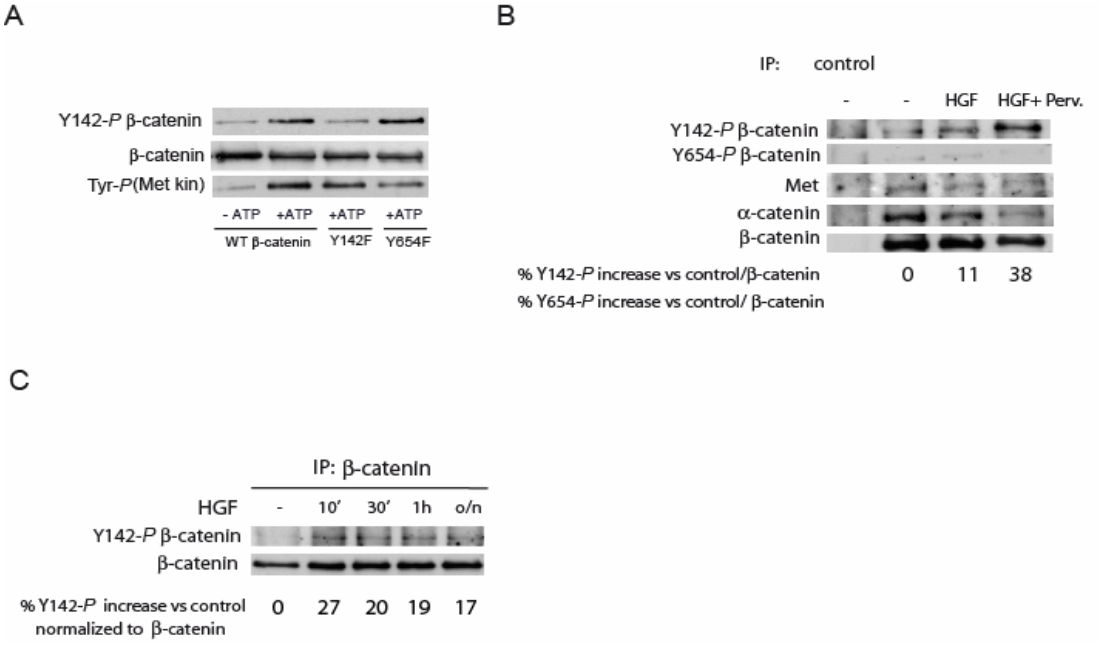

Figure 28. ***Met phosphorylates β-catenin at Y142.*** *A) In vitro kinase assay with recombinant Met kinase and WT or mutant GST-β-catenin. Anti-P-Tyr Western blot shows phosphorylated Met kinase upon addition of ATP and its basal activation (-ATP condition). Anti-P-Y142 Western blot indicates that WT and Y654F GST-β-catenin are phosphorylated by Met kinase at Y142, whereas phosphorylation of Y142F GST-β-catenin is as low as in basal condition (-ATP). B) Control (His) or β-catenin immunoprecipitation from untreated hippocampal neurons or those treated with HGF 50 ng/ml for 10 min with or without pervanadate. β-catenin P-Y142 increases with HGF treatments, whereas phosphorylation at Y654 is not stimulated by HGF signalling. Met and α-catenin co-immunoprecipitate with β-catenin, and the recovery of both proteins decreases upon phosphorylation of β-catenin at Y142. C) β-catenin immunoprecipitation from untreated neurons or those treated with HGF 50 ng/ml for the indicated times. β-catenin P-Y142 peaks at 10 min, is maintained at similar levels up to 1 hour and starts decaying slightly after overnight stimulation. Quantifications in B and C correspond to % of the increase in the intensity of the phosphospecific β-catenin band relative to the control normalized to total β-catenin.*

We found that Met and β-catenin co-immunoprecipitate from hippocampal neuron lysates and that this association decreases upon HGF treatment with or without pervanadate by 87.6 ± 3.3% and 85.6 ± 5.1%, compared to control (n≥3) (Fig. 28B). This interaction suggested that β-catenin may be phosphorylated by Met. Using antibodies specific for P-Y142, recombinant Met kinase was found to induce the phosphorylation of WT GST-β-catenin at Y142 upon addition of ATP. This decreased to basal levels when using mutant Y142F GST-β-catenin (Fig. 28A). In contrast, phosphorylation of Y654F GST-β-catenin was similar to that of the WT protein. Furthermore, prior treatment with HGF (50 ng/ml, 10 min) with or without pervanadate induced the phosphorylation of β-catenin Y142 in β-catenin immunoprecipitates isolated from hippocampal neurons (Fig. 28B). Following a peak phosphorylation at 10 min, P-Y142 was maintained up to 1 hour and started to decay slightly after overnight stimulation with HGF (Fig. 28C). Contrary to BDNF and NT-3 signalling, Y654 phosphorylation was not stimulated by HGF treatments (Fig. 28B) indicating that NT and HGF signalling phosphorylate different tyrosine residues. Interestingly, α-catenin co-immunoprecipitated with β-catenin (Fig. 28B) and the phosphorylation of β-catenin at Y142 resulted in decreased amounts of α-catenin in the β-catenin immunoprecipitates (88 ± 5% and 77 ± 3.4 % lower in HGF and HGF+pervanadate than in control; n≥3; Fig. 28B). Together these findings indicate that Met interacts with β-catenin engaged in the adhesion complex and phosphorylates Y142, resulting in the detachment of α-catenin from β-catenin.

2.4. β-catenin is involved in axon growth in hippocampal neurons

Next we investigated the possible role of β-catenin and its interactions with Trk and Met in axon growth because both types of molecules have been implicated in axon morphogenesis (Honda et al., 1995; Riehl et al., 1996; Huang and Reichardt, 2001, 2003). First, we analyzed the involvement of β-catenin in axon growth in the absence of neurotrophic factor signalling. In order to do this we designed scrambled and β-catenin shRNA vectors for lentiviral infection of hippocampal neurons. Quantification of Western blots of neurons expressing β-catenin shRNA#1 (specific for mouse&rat β-catenin) and #2 (specific for rat β-catenin) indicate a decrease of β-catenin total levels of about 25 and 20% at three days of infection compared to

neurons expressing scrambled shRNA (Fig. 29A).

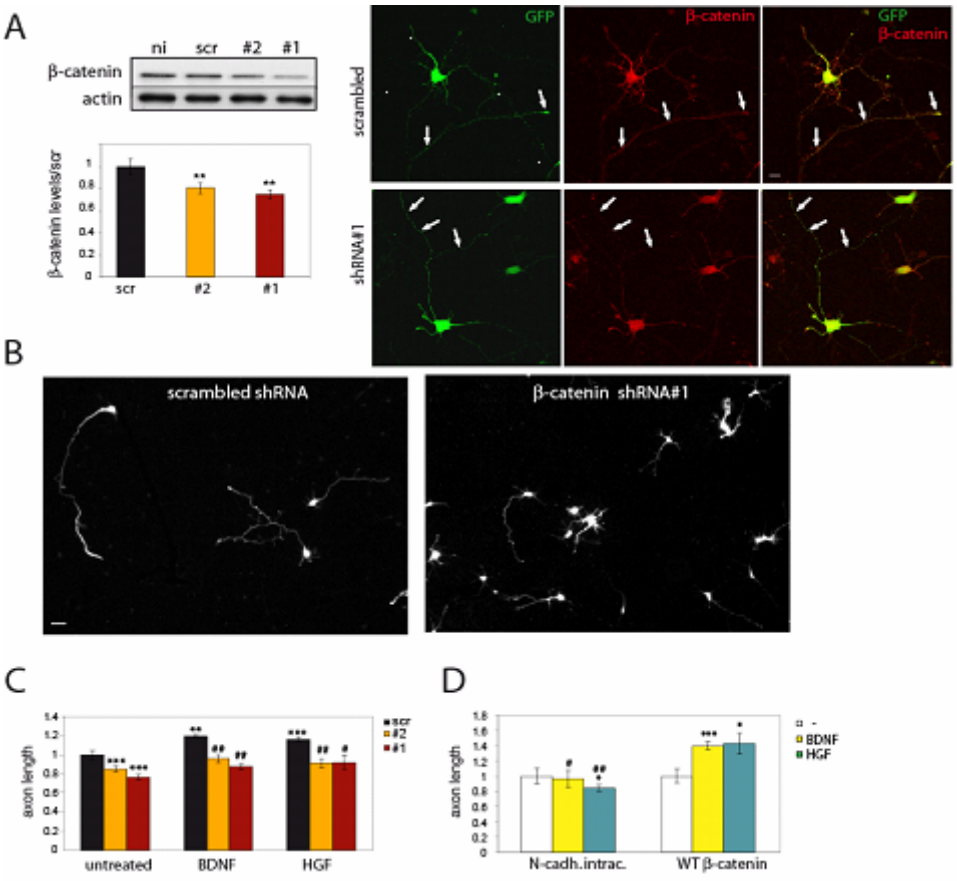

Figure 29. ***β-catenin is involved in axon growth downstream of BDNF and HGF signalling.*** *A) Western blot from hippocampal neurons infected with lentiviral-driven scrambled or β-catenin shRNA#1 and #2 shows the decrease in β-catenin expression by β-catenin shRNAs compared to scrambled shRNA (scr) or non-infected (ni) neurons. Actin was used as a loading control. Plot represents quantification of β-catenin corrected against the loading control (n ≥ 4 experiments). Panels show confocal images for anti-β-catenin and anti-GFP (indicating transduced neurons) immunostainings in neurons expressing scrambled or β-catenin shRNA#1. Note the lower levels of β-catenin especially along the axon in β-catenin shRNA#1-expressing neurons. Arrows indicate colocalization between GFP and β-catenin immunostainings in neurons expressing scrambled shRNA that is reduced in neurons expressing β-catenin shRNA#1. Bar= 20 µm. B) Hippocampal neurons expressing scrambled or β-catenin shRNA#1 fixed and immunostained against GFP (driven by the lentiviral vector) at 3 DIV show the decrease in axon length obtained by expressing shRNA#1. Bar=40 µm. C) Quantification of axon length of scrambled and β-catenin shRNA neurons, untreated or treated with BDNF or HGF (normalized to untreated neurons expressing scrambled shRNA). Neurons expressing β-catenin shRNA#1 and #2 display axons shorter than controls. Stimulation with BDNF or HGF (50 ng/ml) promotes axon growth in neurons expressing scrambled shRNA, but not in neurons expressing β-catenin shRNA#1 and #2 (n = 4-8 experiments). *p≤0.05, **, p≤0.01, *** p≤0.001 when comparing to the untreated neurons expressing scrambled shRNA; #p≤0.05, ## p≤0.01 when comparing to the BDNF or HGF-treated neurons expressing scrambled shRNA.D) Quantification of axon length in neurons expressing N-cadherin intracellular or β-catenin, either untreated or stimulated with BDNF or HGF (normalized to the respective untreated neurons). Neurons expressing β-catenin display axons longer than controls upon BDNF or HGF (50 ng/ml)*

*stimulation. In contrast, in neurons expressing intracellular N-cadherin, BDNF or HGF can not stimulate axon growth (n = 4 experiments). *p≤0.05, *** p≤0.001 when comparing to the corresponding untreated control; #p≤0.05, ## p≤0.01 when comparing to the BDNF or HGF-stimulated β-catenin-expressing neurons.*

Anti-β-catenin immunostaining indicates reduced β-catenin immunoreactivity, especially along the axon of GFP-positive neurons expressing β-catenin shRNA#1 (Fig. 29A). We measured axon length in neurons expressing scrambled or β-catenin shRNAs. In these experiments axon length was measured at 3 DIV because this was the earliest time point at which we observed a significant reduction in the levels of β-catenin and axon length was still easily measured. Neurons expressing β-catenin shRNAs exhibit significantly shorter axons than neurons expressing scrambled shRNA (Fig. 29B, C). Furthermore, stimulation of TrkB and Met by BDNF and HGF respectively, increases axon length in neurons expressing scrambled shRNA (Fig. 29C). However, in neurons expressing β-catenin shRNA#1 and #2, BDNF or HGF treatment can not stimulate axon growth (Fig. 29C). These results demonstrate that β-catenin is required for axon growth downstream of BDNF and HGF signalling.

To further test the requirement of β-catenin in axon growth, we also expressed the intracellular domain of N-cadherin, which was previously used to study dendritogenesis (Yu and Malenka, 2003). Intracellular N-cadherin sequesters β-catenin, inhibiting its degradation and transactivation capacity (Sadot et al., 1998). For these experiments neurons were co-transfected with EYFP and β-catenin or intracellular N-cadherin (1:4 ratio) (Yu and Malenka, 2003) and stimulated with either BDNF or HGF before axon length was measured. BDNF and HGF promoted axon growth in neurons expressing β-catenin (see below) but these effects were inhibited in neurons that express intracellular N-cadherin (Fig. 29D). Together with our results on β-catenin knockdown, these findings demonstrate that decreasing the availability of β-catenin inhibits the axon growth induced by BDNF and HGF signalling.

2.5. NT signalling regulates axon growth and branching by phosphorylating β-catenin Y654

We then looked at the physiological consequences of β-catenin phosphorylation by Trk, by studying axon growth induced by NTs in neurons co-expressing WT or

mutant β-catenin and EYFP.

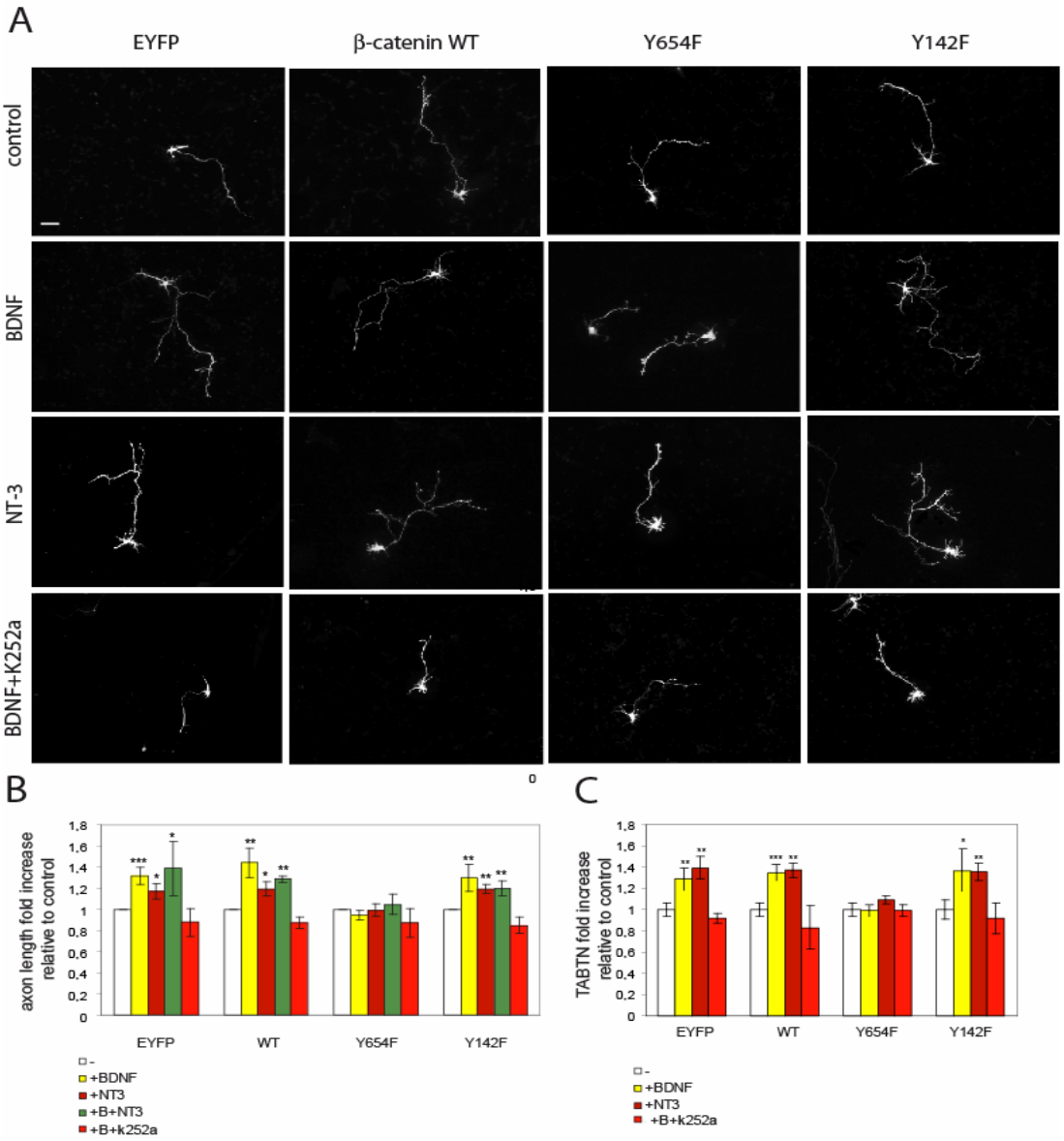

Figure 30. ***β-catenin phosphorylation at Y654 regulates axon growth and branching induced by NTs.*** *A) Hippocampal neurons transfected with EYFP alone (control) or with EYFP and WT or mutant (Y654F or Y142F) β-catenin. BDNF and NT-3 (50 ng/ml) induce axon growth and branching in control neurons and in neurons overexpressing WT or Y142F β-catenin, but the NT-induced axon growth is inhibited in neurons expressing the mutation Y654F. Treatment with BDNF plus K252a results in axons with a length and branching values similar to those of untreated neurons and of Y654F-expressing cells. Bar= 80 µm. B) Quantification of axon length normalized to the respective control. BDNF and NT-3 increase axon length in control EYFP neurons and in neurons expressing WT or Y142F β-catenin, but not in neurons expressing the Y654F mutant. Treatment with BDNF together with NT-3 does not result in a significant further increase. Treatment with K252a (100 nM) and BDNF abolishes the increase in axon length induced by BDNF. C) Quantification of axon branching (TABTN) shows that BDNF and NT-3 increase branching of axons expressing WT and Y142F β-catenin, but expression of Y654F abolishes the branching induced by NTs. K252a (100 nM) together with BDNF abolishes the BDNF-promoted branching. (*P≤0.05, ** P≤0.01, *** P≤0.001; n = 3-8 experiments).*

Neurons expressing EYFP alone or together with β-catenin did not show axon length values that were significantly different. Neurons were treated with BDNF, NT-3 or both and axon length was measured at 2 DIV. Stimulation with BDNF, NT-3 or both increased axon length in neurons expressing EYFP alone by 31%, 18% and 39% respectively (Fig. 30A, B). Neurons overexpressing β-catenin treated with BDNF, NT-3 or both exhibited axons 44%, 20%, and 29% longer respectively than untreated neurons (Fig. 30A, B). Thus, NT treatment increases axon growth in a similar manner in neurons overexpressing or not β-catenin. BDNF together with NT-3 did not increase axon length significantly more than BDNF or NT-3 alone, suggesting that signalling by only one NT was already maximal. Neurons expressing β-catenin treated with BDNF together with the pharmacological inhibitor of Trk activity K252a displayed axons with a length similar to that of controls (Fig. 30A, B). We found that in addition to increasing axon length, BDNF and NT-3 also increased axonal branching (measured as Total Axonal Branch Tip Number, TABTN) by a 35% and 37% respectively compared to control neurons. Furthermore, addition of K252a together with BDNF reduced branching to values similar to the control (Fig. 30A, C). K252a has recently been reported to inhibit Met (Morotti et al., 2002) as well as Trk. However, as BDNF binds to TrkB and K252a inhibited the effects of BDNF, our results strongly suggest that Trk receptors mediate the BDNF effects in axon growth and branching.

We also examined the role of β-catenin phosphorylation at Y654 in axon growth and branching, using mutant Y654F β-catenin which is not phosphorylable at Y654. Untreated neurons expressing Y654F β-catenin exhibited axons of similar length to those of neurons expressing WT β-catenin. Interestingly, we found that neurons expressing Y654F β-catenin treated with BDNF or NT-3 exhibited axon lengths similar to those of untreated neurons (Fig. 30A, B). We therefore concluded that expression of Y654F mutant blocks the NT-induced stimulation of axon growth. Expressing Y654F β-catenin also abolished the NT-induced axon branching (Fig. 30A, C). Conversely, expression of the Y142F β-catenin mutant did not affect the length or branching of the axon induced by NTs (Fig. 30). Altogether, these results demonstrate that phosphorylation at β-catenin Y654 (and not at Y142) is required for the axon extension and branching promoted by NTs.

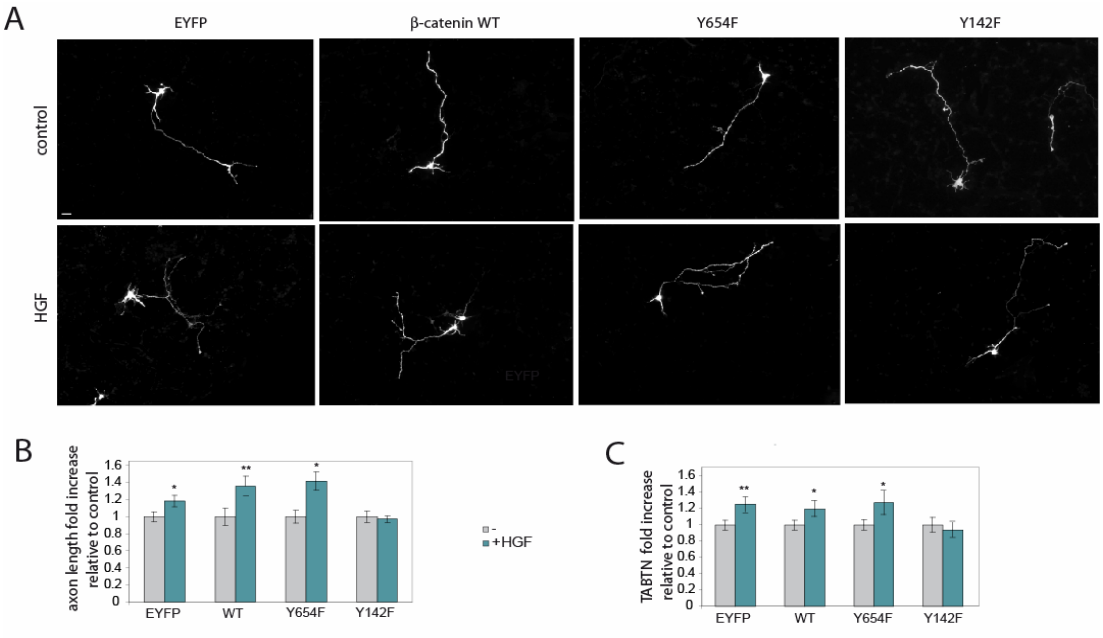

Figure 31. ***β-catenin phosphorylation at Y142 regulates axon growth induced by HGF.*** *A) Hippocampal neurons transfected with EYFP alone (control) or with EYFP and WT or mutant (Y654F or Y142F) β-catenin. HGF (50 ng/ml) induces axon growth in neurons expressing WT or Y654F β-catenin, but the HGF-induced axon growth is inhibited in neurons expressing the mutation Y142F. Bar = 40 µm. B) Quantification of axon length normalized to the respective control shows that HGF increases axon length in neurons expressing WT and Y654F β-catenin, but not in neurons expressing Y142F. C) Quantification of axon branching shows that HGF treatment induces axon branching that is blocked by the Y142F mutation (* P≤0.05, ** P≤0.01; n = 4-7 experiments).*

2.6. HGF signalling regulates axon growth and branching by phosphorylating β-catenin Y142

Treatment with HGF also increased axon growth of hippocampal neurons expressing EYFP (18 %) or EYFP and WT β-catenin (35 % compared to untreated neurons; Fig. 31A, B). Remarkably, neurons expressing Y142F β-catenin showed no increase in axon length compared to control cells upon HGF treatment (Fig. 31A, B). In contrast, neurons expressing Y654F β-catenin treated with HGF exhibited axons longer than untreated neurons (41% compared to control; Fig. 31A, B). Treatment with BDNF and HGF did not increase axon length further than BDNF or HGF alone (data not shown). Moreover, HGF signalling increased axon branching in neurons expressing EYFP, EYFP and WT β-catenin or EYFP and Y654F β-catenin (Fig.31, C). However, the axon branching induced by HGF was blocked when expressing Y142F β-catenin. These results demonstrate that phosphorylation of Y142 is required for the regulation of axon growth and branching induced by HGF. Dendrite length

and branching of EYFP-expressing neurons was not stimulated by BDNF or HGF treatments at 2 DIV suggesting that dendrite morphogenesis is not regulated by tyrosine phosphorylation of β-catenin at this stage of the hippocampal neuron development (Table 1).

Table 1**. **Dendrite morphogenesis is not regulated by tyrosine phosphorylation of β-catenin in 2 DIV hippocampal neurons.

Dendrite length fold increase compared to control

Treatment	*Average±s.e.m.*	*Significance(n)*
Control	1 ± 0.12	N.S.* (n=11)
BDNF	1.16 ± 0.13	N.S. (n=9)
HGF	1.00 ± 0.07	N.S (n=6)

Dendrite branching (Total Dendrite Branch Tip Number, TDBTN) compared to control

Treatment	*Average±s.e.m.*	*Significance(n)*
Control	3.37 ± 0.29	N.S. (n=11)
BDNF	3.51 ± 0.48	N.S. (n=9)
HGF	3.49 ± 0.79	N.S (n=6)

*N.S. = not significant

In summary, our data indicates that β-catenin is specifically phosphorylated at two different sites, Y654 and Y142, by NTs and HGF signalling to regulate axon growth and branching.

2.7. P-Y654 β-catenin colocalizes with the neuronal cytoskeleton, whereas P-Y142 is found in the nucleus

To begin to elucidate the downstream events resulting from β-catenin tyrosine phosphorylation at Y654 and Y142 sites, we examined the subcellular localization of the phosphorylated forms, P-Y654 and P-Y142 in hippocampal neurons. Hippocampal neuron cultures were fixed using PFA or detergent fixation. Detergent staining allowed a better preservation of microtubule structure and the visualization of microtubule bundling at growth cones (Fig. 34).

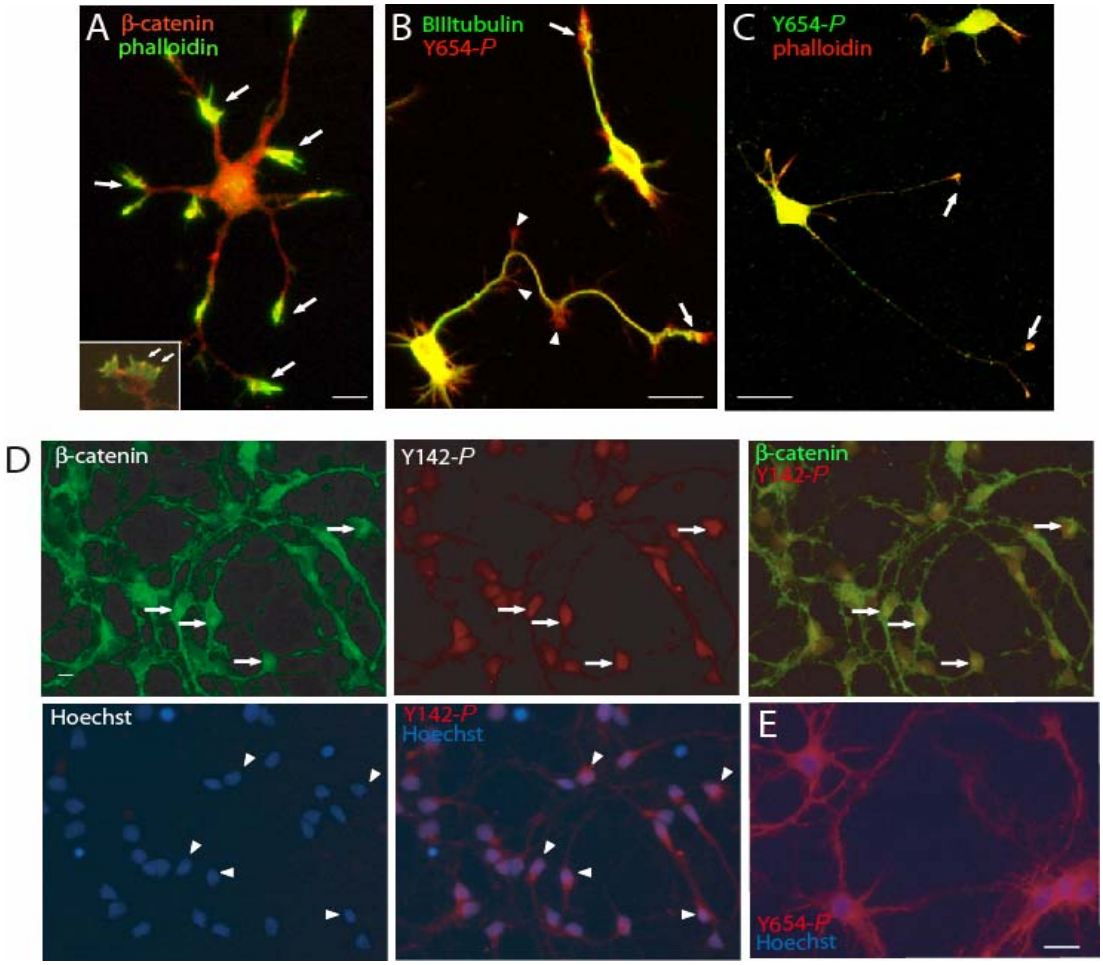

Figure 32. ***P-Y654 β-catenin colocalizes with the neuronal cytoskeleton and P-Y142 is found in the nucleus.*** *A) Double immunostaining for endogenous β-catenin and F-actin (phalloidin) in hippocampal neurons shows that β-catenin and F-actin partially colocalize (arrows) at neurite tips and lamella (inset). B) Confocal image of hippocampal neurons fixed using detergent fixation shows the double immunostaining for P-Y654 and βIII-tubulin colocalizating at enlarged growth cones where microtubules unbundle and along the axon (arrows). P-Y654 immunostaining can also be observed along filopodial-like processes and axonal spread areas devoid of βIII-tubulin (arrowheads). C) Confocal image of neurons fixed by detergent fixation shows the double immunostaining for P-Y654 and F-actin (phalloidin). Similar to anti-β-catenin antibodies, anti-P-Y654 antibodies stain the cell body and neurite tips where P-Y654 and F-actin colocalize (arrows). D) β-catenin and P-Y142 colocalize within the cytoplasm of neurons (arrows), whereas colocalization of Hoestch and anti-P-Y142 immunostaining shows that P-Y142 β-catenin is found in the nucleus (arrowheads). E) Anti-P-Y654 antibodies stain the cell membrane at cell contacts and the cytoplasm, but the nucleus (stained by Hoestch) is negative. Bars = 15 µm (A), 20 µm (B and C) and 45 µm (D and E).*

β-catenin P-Y654 is present in the cytoplasm, along the axon and at neurite tips and growth cones, where it colocalizes with actin and βIII-tubulin (Fig. 32A-C). In contrast, β-catenin P-Y142 localizes to the cytoplasm and nucleus of neurons (Fig. 32D).

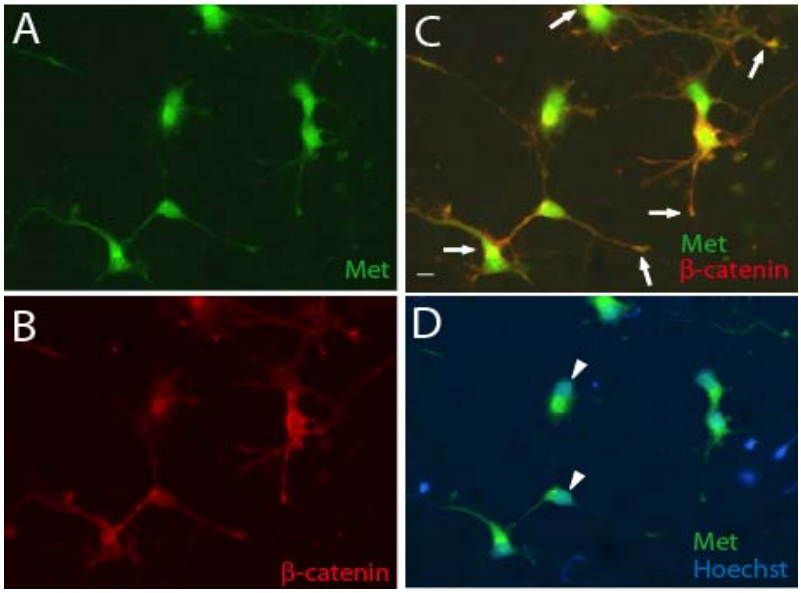

Figure 33. ***Cellular localization of Met and β-catenin.*** *Met A) and β-catenin B) immunostainings colocalize intracellularly and at neurite tips (C; arrows). D) Met immunostaining does not significantly colocalize with Hoechst nuclear staining (arrowheads). Bar = 45 µm*

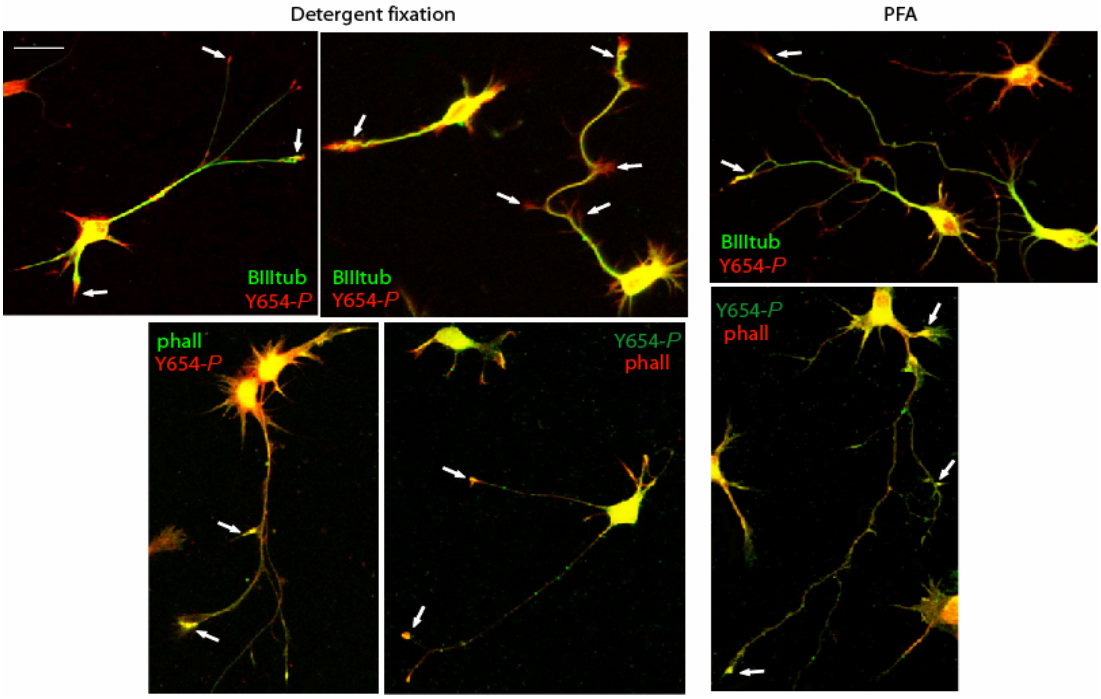

Figure 34***.Comparative confocal images of PFA- or detergent-fixed hippocampal neurons stainedfor actin or βIII-tubulin and P-Y654 β-catenin.*** *P-Y654 is present in the cytoplasm, along the axon and at neurite tips and growth cones, where it colocalizes with actin and βIII-tubulin.Detergent fixation allowed the visualization of microtubules bundling at growth cones.*

Anti-Met and anti-β-catenin immunostainings also colocalize along neurites, at tips and in the cytoplasm (Fig. 33). However, anti-Met and Hoestch staining do not significantly colocalize (Fig. 33), suggesting that Met does not translocate to the nucleus to maintain Y142 phosphorylation. Thus, phosphorylation at Y654 and Y142 targets β-catenin to different subcellular compartments, suggesting that these two phosphorylated forms of β-catenin may involve distinct mechanisms to regulate axon morphogenesis.

2.8. Dominant negative ΔN-TCF-4 blocks the effect of HGF in axon growth

In canonical Wnt signalling, nuclear β-catenin binds to the promoter of TCF/LEF to regulate gene transcription (Logan and Nusse, 2004). Consequently TCF factors are likely candidates in the response activated by nuclear β-catenin. Since P-Y142 β-catenin was observed in the nucleus, we investigated whether dominant negative ΔN-TCF-4 that can not bind β-catenin (Roose et al., 1999) was able to modify the NT or HGF effects on axon growth and branching. As a positive control, we treated ΔN-TCF-4 expressing hippocampal neurons with Wnt-3a, which signals through the canonical Wnt pathway in dorsal root ganglia neurite outgrowth (Lu et al., 2004). Wnt-3a-treated neurons overexpressing EYFP alone or together with WT β-catenin exhibited axons 47 % or 46% longer than controls. Expression of ΔN-TCF-4 abolished the stimulation of axon growth induced by Wnt-3a (Fig. 35B), indicating that ΔN-TCF-4 blocks β-catenin nuclear signalling and that in hippocampal neurons Wnt-3a acts through the canonical pathway to increase axon growth. Interestingly, the HGF-induced axon growth and branching was also prevented in neurons expressing ΔN-TCF-4, whereas the BDNF and NT-3 effects were not altered (Fig. 35). These results indicate that HGF signals through TCF-4 in axon growth and branching. In summary, our results indicate that HGF signalling phosphorylates β-catenin at Y142, inducing its nuclear translocation and TCF-4-mediated gene transcription to regulate axon morphogenesis. In contrast, NT signalling promotes axon growth and branching by phosphorylating β-catenin Y654 independent of TCF-4 function.

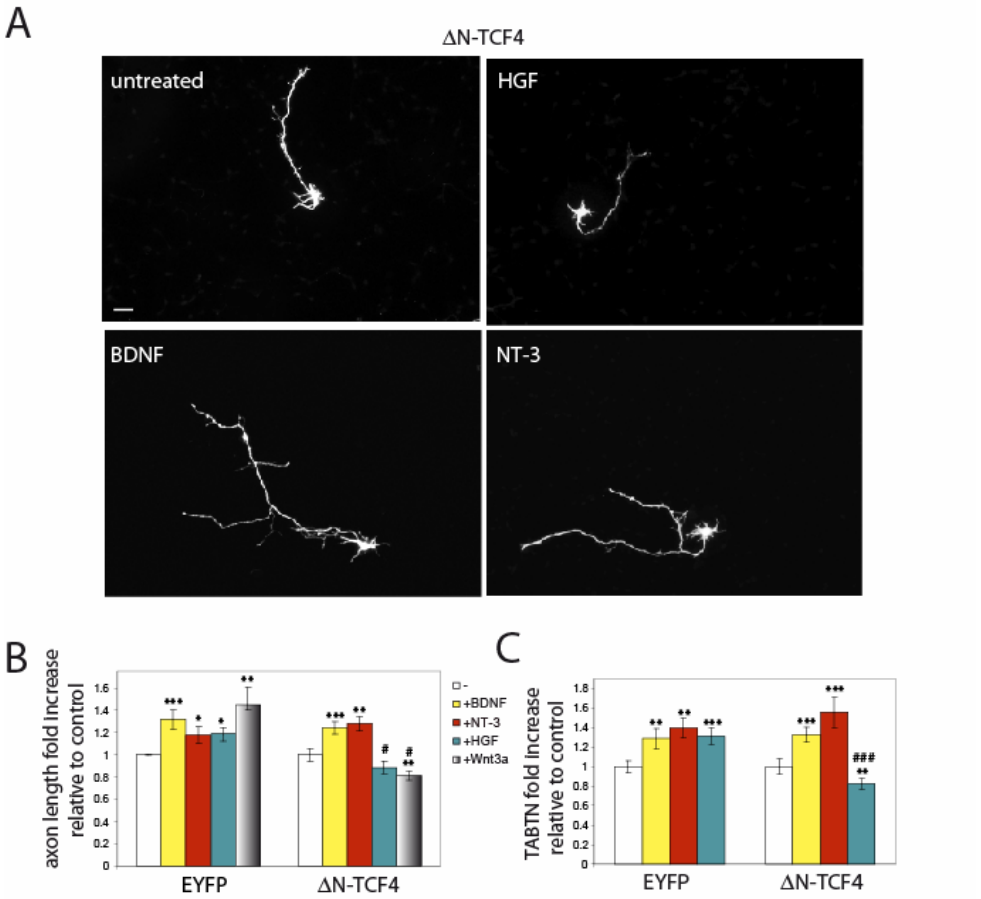

Figure 35. Δ***N-TCF-4 abolishes the effect of HGF in axon growth and branching.***
*A) Hippocampal neurons transfected with EYFP and ΔN-TCF-4, untreated or treated with BDNF, NT-3 and HGF (50 ng/ml). ΔN-TCF-4 abolishes the axon growth induced by HGF, but not by BDNF or NT-3. Bar = 40 µm. B) Quantification of axon length in EYFP- or EYFP plus ΔN-TCF-4-expressing neurons normalized to the respective untreated control shows that HGF and Wnt-3a (used as positive control; 50 ng/ml) do not increase axon length in neurons expressing ΔN-TCF-4, whereas NTs similarly stimulate axon growth in ΔN-TCF-4-expressing or control neurons. C) Quantification of axon branching in neurons expressing EYFP alone or EYFP and ΔN-TCF-4 shows that the HGF-induced branching is inhibited by ΔN-TCF-4 expression, whereas the BDNF and NT-3-induced effects are not affected. (n=5-7 experiments) * P≤0.05, ** P≤0.01, ***P≤0.001, when comparing to the corresponding untreated controls; # P≤0.05, ## P≤0.01, ###P≤0.001, when comparing to the corresponding stimulated control.*

Discussion

Proper development of the nervous system requires a precise control of neurogenesis and neuronal morphogenesis. A variety of extracellular signals regulate neuronal differentiation, a complex process including cell cycle exit, neuronal fate specification, migration to the final location, acquisition of neuronal morphology and synapse formation). Wnt factors are well-known secreted molecules that regulate proliferation of neural precursors, neurogenesis, terminal neuronal differentiation and synaptogenesis (Castelo-Branco et al., 2003; Arenas, 2005; Ciani and Salinas, 2005). Classical Wnt signalling involves stabilization of cytoplasmic β-catenin and regulation of transcription by LEF/TCF transcription factors (Logan and Nusse, 2004; Ciani and Salinas, 2005). Here we demonstrated that Wnt-3a and Wnt-3 signalling by activation of the canonical Wnt/β-catenin pathway regulate neuronal differentiation (neurogenesis and neuritogenesis) from SCNPs.

The establishment of brain networks relays on the correct development of neuronal projections. Different trophic factors act as regulators of neurite outgrowth in postmitotic neurons. Especifically, NTs and HGF that belong to different growth factor families that signal through RTK, regulate survival and neurite outgrowth (Ebens et al., 1996; Thompson et al., 2004; Lu et al., 2005; Reichardt, 2006). In this work we have shown the involvement of β-catenin downstream of NT and HGF signalling during the regulation of axon morphogenesis.

Role of Wnts in neural precursor development

Wnt signalling plays a prominent role regulating neurogenesis (Muroyama et al., 2002; Castelo-Branco et al., 2003; Hirabayashi et al., 2004; Lee et al., 2004; Lie et al., 2005; Michaelidis and Lie, 2008) and the terminal differentiation of distinct neuronal populations (Krylova et al., 2002; Lu et al., 2004; Ciani and Salinas, 2005). In the first part of this work we have studied the function of Wnt-3a and Wnt-3, two *Wnts* expressed by the developing spinal cord (Krylova et al. 2002; Megason and McMahon 2002), in the development and neuronal differentiation of SCNPs. The broad term of neuronal differentiation is used here as inclusive for neurogenesis and neuritogenesis. Despite high sequence homology, distinct expression patterns for Wnt-3a and Wnt-3 suggest different functions (Roelink and Nusse, 1991; Megason

and McMahon, 2002). We found that Wnt-3a and Wnt-3 stimulated proliferation of SCNPs. Interestingly, whereas Wnt-3a induced a sustained increase in SCNP proliferation, Wnt-3 stimulated SCNP proliferation only transiently. Nevertheless, Wnt-3 signalling increased neurogenesis from SCNPs. Both Wnt-3a and Wnt-3 also stimulated neurite outgrowth of SCNP-derived neurons. A molecular dissection of the pathway involved implicates the Wnt/β-catenin canonical pathway in the neurogenesis from SCNPs and subsequent neurite development. Our results indicate a different control of the mechanisms that govern cell cycle entry/exit by Wnt-3a and Wnt-3 signalling and demonstrate the role of Wnt-3a and Wnt-3 supporting neurite outgrowth in SCNP-derived neurons.

Previous studies illustrated a role for various Wnt factors in the specification and neurogenesis of embryonic and adult progenitor populations. For example, Wnt-3 derived from hippocampal astrocytes stimulate Wnt/β-catenin signalling to regulate neurogenesis in the adult hippocampus (Lie et al. 2005) and canonical Wnt-3a enhances proliferation without increasing neuron generation from dopaminergic midbrain precursors (Castelo-Branco et al. 2003). In the spinal cord, Wnt-3a collaborates with Wnt-1 in the generation of dorsal interneurons (Muroyama et al. 2002; Muroyama et al. 2004). Dorsal Wnt-1/Wnt-3a canonical signalling, by controlling the expression of Gli3 repressor, supress the ventral program instructed by a Shh gradient (Alvarez-Medina et al., 2008). We demonstrated increased SCNP proliferation by Wnt-3a and Wnt-3 signalling by two means: increased percentages of SCNPs in S phase and enhanced BrdU incorporation. In agreement with a mitogenic activity of Wnt-3a (Megason and McMahon, 2002), the effect of Wnt-3a on SCNP proliferation was sustained, suggesting that Wnt-3a maintains a pool of SCNPs cycling. In contrast, Wnt-3 induced a transient elevation of SCNP proliferation that resulted in an increased proportion of neurons immunoreactive for the neuronal marker βIII-tubulin at 4 DIV. What determines cell cycle entry or cell cycle exit and differentiation by different Wnt factors remains to be investigated. An attractive possibility, considering the implication of the same main Wnt/β-catenin pathway components, would be the usage by Wnt-3a and Wnt-3 signalling of different transcriptional co-activators (Miyabayashi et al., 2007) or changes in the target genes to regulate proliferation vs. differentiation. Future work should

investigate which are the target genes regulated by Wnt-3 during the stimulation of neurogenesis from SCNPs. Alternatively, additional pathways may contribute to the Wnts response. The Wnt receptor Ryk is expressed in neural progenitor cells of the developing spinal cord showing different expression levels in distinct spinal cord neuronal population (Kamitori et al., 2002). Ryk is required for the Wnt-3a induced neurite outgrowth of dorsal root ganglion neurons (Lu et al., 2004). Recent studies indicate that Ryk protein is cleaved and its intracellular domain translocates into the nucleus in response to Wnt-3 stimulation to regulate cortical neurogenesis (Lyu et al., 2008). It would be interesting to investigate the role of Ryk in Wnt-3a/Wnt-3 signalling in SCNPs.

Both Wnt-3a and Wnt-3 readily promoted neurite growth, a characteristic of the differentiating neuron. Especially in the case of Wnt-3a, this effect was observed at time points of the culture (4 DIV) at which the effect on proliferation was highest. These results suggest that Wnts signal in different populations of SCNP to stimulate proliferation or neurite outgrowth.

Wnt/β-catenin canonical pathway induces the neuronal differentiation and neurite outgrowth of SCNPs

Dissection of the Wnt pathway involved in the events promoted by Wnt-3a and Wnt-3 in SCNP cultures revealed the implication of the Wnt/β-catenin canonical pathway. Interestingly, GSK-3β inhibitors (added for one night on established cultures) mimicked the Wnt-3 induced neuronal differentiation, by increasing the proportion of βIII-tubulin-immunoreactive neurons and neurite development. However, inhibition of GSK-3β from cell plating prevented neurite growth. These findings highlight divergent roles of GSK-3β in initial/late stages of neurite outgrowth in agreement with a function in neuronal polarity and axon formation (Kim et al., 2006; Garrido et al., 2007). Our results differ from those obtained upon early application of GSK-3β inhibitors in ventral mesencephalon precursors (Castelo-Branco et al., 2004), suggesting different GSK-3β regulation in specific cell types. Furthermore, β-catenin was required for the neurite growth induced by Wnt-3a and Wnt-3 signalling as revealed by shRNA experiments. Although the total β-catenin levels decreased by ~30 % in SCNPs expressing the β-catenin shRNAs, the Wnt-induced neurite

extension was completely prevented. These results suggest that the β-catenin nuclear signaling pool was mainly affected in SCNP-derived neurons and that remaining β-catenin is likely contributed by the cadherin-β-catenin and/or glial β-catenin pools. Finally, ActD experiments and expression of ΔN-TCF-4 demonstrated that gene transcription by TCF-4 is needed for the Wnt-3a- and Wnt-3-promoted neurite extension in SCNP-derived neurons.

Several studies demonstrate the role of the proneural transcription factors Neurogenins (Ngn1 and Ngn2) instructing a neuronal fate in the CNS (Fode et al., 2000; Nieto et al., 2001). Ngn promotes cell cycle exit and neuronal differentiation (Farah et al., 2000; Mizuguchi et al., 2001; Lo et al., 2002). β-catenin signalling has been shown to upregulate Ngn1 (Hari et al., 2002; Hirabayashi et al., 2004). These evidences suggest that Ngn might be a target of Wnt-3 to induce neurogenesis. In addition, recent studies demonstrate that phosphorylated Ngn2 facilitates the interaction with LIM-homeodomain transcription factors and promotes motoneuron specification in response to Shh (Ma et al., 2008). GSK-3 phosphorylates Ngn2 (Ma et al., 2008), raising the possibility that Wnt signalling could regulate neuronal specification. Together, our results indicate that Wnt-3a and Wnt-3 signalling in SCNPs involve the stabilization of cytoplasmatic β-catenin and transcriptional regulation of target genes to promote neurogenesis and neurite growth. *In vivo* expression of stabilized β-catenin forms or focal β-catenin elimination showed the role of β-catenin-mediated transcriptional activation in the control of cell cycle progression (at least in part by regulating cyclin D1 and cyclin D2 expression), thus regulating the expansion of progenitor populations and brain growth (Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003; Woodhead et al., 2006). However, a later role for β-catenin in neuronal differentiation by controling the expression of neurogenin was also highlighted in the cerebral cortex (Hirabayashi et al., 2004; Hirabayashi and Gotoh, 2005; Woodhead et al., 2006). In agreement with these findings and with previous studies identifying a function for Wnt signalling in the neuronal differentiation of neural precursor populations (Castelo-Branco et al., 2003; Castelo-Branco et al., 2004; Hirabayashi et al., 2004; Muroyama et al., 2004; Lie et al., 2005; Hirsch et al., 2007), we report the role of Wnt-3a and Wnt-3 signalling in SCNP-derived neuronal differentiation (neurogenesis and neurite

outgrowth). Wnts regulate multiple aspects of neurite development during the establishment of neuronal connections (Ciani and Salinas, 2005). Wnt-3 induces growth cone enlargement, axon branching and stops axon extension during the terminal differentiation of NT-3-dependent sensory axons (Krylova et al., 2002). Wnt-3a, which displays an axon remodeling activity identical to that of Wnt-3 on this neuronal population, was shown to signal by divergent Wnt/β-catenin pathway, independently of transcription during axon remodeling (Purro et al., 2008). Nevertheless, our results indicate the requirement of TCF-4 function for the Wnt-3a and Wnt-3-dependent neurite extension of SCNP-derived neurons. Therefore, it appears that using different signalling pathways Wnts can induce different behaviors on neurites (neurite outgrowth or growth cone pausing and remodeling).

Wnt mutant mice

Wnt-3a mutant mice showed the requirement for Wnt-3a signalling in hippocampus formation (Lee et al., 2000b) and, together with Wnt-1, in the expansion of neural crest and CNS progenitors (Ikeya et al., 1997). Importantly however, ectopic expression of Wnt-3a in the vertebrate spinal cord revealed that a gradient of dorsal midline Wnt-3a controls its size and shape by positively regulating cell cycle progression (Megason and McMahon, 2002). In contrast, expression of Wnt-3 that displays a dorsal broad domain of expression, did not significantly affect neural precursor proliferation but increased neuronal differentiation (Megason and McMahon, 2002). These results support our findings on Wnt-3a and Wnt-3 in the proliferation and neuronal differentiation of SCNP cultures.

No studies using mutant mice have so far analyzed the role of Wnt signalling *in vivo* in the developing spinal cord. *Wnt-3* mutant mice are embryonic lethal at early stage of development (Liu et al., 1999). *Wnt-3a* mutant mice die at E16.5 (Takada et al., 1994). Future work should investigate the role of Wnt-3 and Wnt-3a signalling in neurogenesis *in vivo* in the spinal cord using *Dvl* or *Wnt-3a* knockout mice embryos.

In summary, in the first part of this work we demonstrated different roles of Wnt-3a signalling, which maintains the proliferation of a SCNP pool, and Wnt-3 signalling that increases neurogenesis from SCNP (Fig. 36). We also report on the

stimulation of neurite outgrowth shared by Wnt-3a and Wnt-3 in SCNP-derived neurons (Fig. 36). We conclude that Wnts, signalling through the Wnt/β-catenin canonical pathway, regulate the neuronal differentiation of SCNP at different levels. Our findings on the increased neurogenesis and neuritogenesis by Wnt/β-catenin signalling provide the basis to examine the use of Wnt/β-catenin pathway components as therapeutic targets in the treatment of neurodegenerative diseases and nerve lesions.

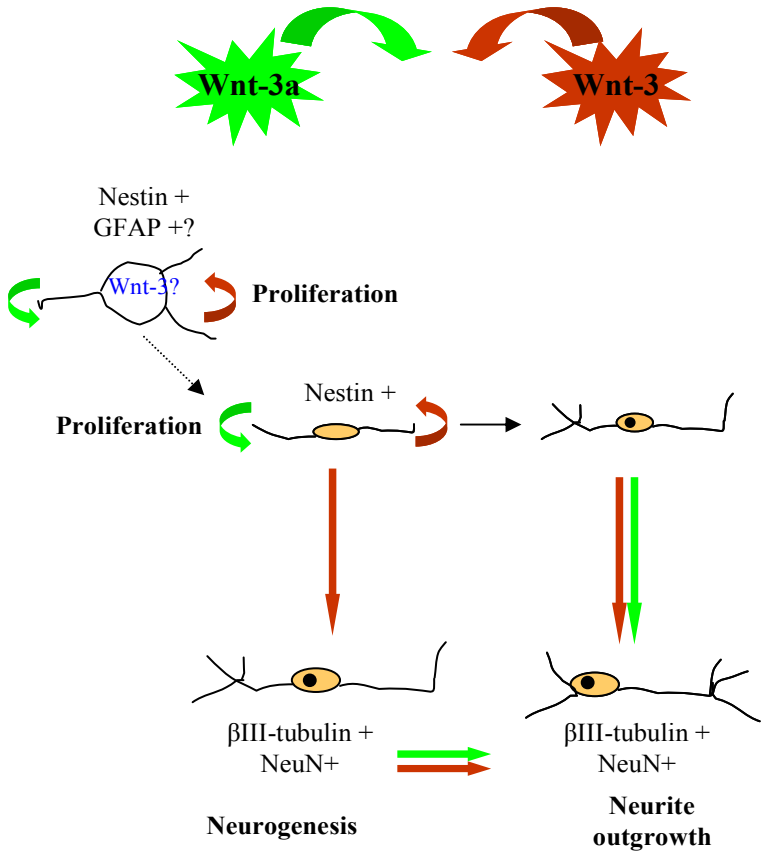

Figure 36. ***Model for the role of Wnt-3a and Wnt-3 signalling on SCNPs cells.*** *Nestin immunocytochemistry of SCNP cultures reveals the presence of two main cell types: cells with a diameter of 30-40 µm that could correspond to GFAP-immunoreactive precursors (data not shown) and small neuroblasts of about 15 µm of diameter typically showing small neuritic processes. GFAP-positive SCNP could be a source of endogenous Wnt-3 (Lie et al., 2005). Exogenously added Wnt-3a induces a sustained proliferation of SCNPs, whereas Wnt-3 stimulates SCNP proliferation transiently (total SCNPs and small neuroblasts in both cases) The relationship between the two types of precursors in unclear (dashed arrow). SCNPs show a basal tendency to differentiate into neurons on adherent conditions (black arrow). We propose that small neuroblasts give rise to neurons by effect of Wnt-3. Wnt-3a and Wnt-3 promote neurite outgrowth on post-mitotic neurons (green arrows illustrate Wnt-3a effects, red arrows show Wnt-3 effects).*

Growth factor/RTK signalling phosphorylates β-catenin to regulate axon morphogenesis

In the second part of this work we have studied the relationship between β-catenin and growth factor/RTK signalling, leading to β-catenin tyrosine phosphorylation, and its physiological consequences in the establishment of the morphology of axonal arbors in primary neurons.

During development neurons extend their axons under the influence of many signalling molecules (Huang and Reichardt, 2001; Ciani and Salinas, 2005; Ayala et al., 2007; Dalva et al., 2007) that results in the unique morphology of axon arbors and the correct neuronal connections. In neurons, β-catenin tyrosine phosphorylation was shown to regulate synapse formation and function (Murase et al., 2002; Bamji et al., 2006). We demonstrated that differential β-catenin tyrosine phosphorylation by growth factor (NTs and HGF) signalling regulates axon morphogenesis. First, by expressing lentiviral-driven β-catenin shRNAs or sequestering β-catenin with intracellular N-cadherin, we demonstrated the requirement for β-catenin in axon outgrowth downstream of growth factor signalling. Second, we showed that neuronal RTK Trk and Met phosphorylate β-catenin. We have identified Y654 and Y142 as the sites phosphorylated upon NT and HGF stimulation, respectively. We have also demonstrated the key role that Y654 and Y142 phosphorylations play in the regulation of axon growth and branching elicited by either family of growth factors. Our data indicates that although NT and HGF signalling converge on β-catenin, the pathways that control the growth and arborization of the axon diverge downstream of β-catenin Y654 and Y142 phosphorylations. The NT-stimulated form P-Y654 β-catenin is found at growth cones, whereas P-Y142 β-catenin translocates to the nucleus. Furthermore, the transcription factor TCF-4, a central player of canonical Wnt signalling, is needed for HGF but not for NT signalling during axon growth and branching. Therefore, components of the Wnt pathway are shared by the signalling cascades activated by growth factors during axon morphogenesis. We conclude that HGF and NTs, by activating their RTK, phosphorylate β-catenin at different sites, thus involving transcription-dependent and independent mechanisms that result in the fine tuning of axon morphogenesis.

Relationship between β-catenin and RTKs

We presented evidence for the interaction of β-catenin with Trk and Met and demonstrated for the first time the involvement of β-catenin phosphorylation downstream of both RTK in the regulation of axon growth and branching. Previous studies illustrated the relationship between β-catenin phosphorylation and RTK such as EGFR, Met and PDGF receptor in epithelial and tumoral cell migration (Hoschuetzky et al., 1994; Hiscox and Jiang, 1999; Brembeck et al., 2004; Theisen et al., 2007). We have shown that β-catenin is associated with TrkA, TrkB and TrkC in PC12 cells and hippocampal neurons. The interaction of β-catenin with Trk was independent of ligand stimulation, as described for EGFR (Hoschuetzky et al., 1994). In addition, β-catenin is a direct substrate of TrkA kinase in vitro. Substitution of Y654 by a phenylalanine results in a significant reduction of β-catenin phosphorylation by TrkA, whereas an Y142F mutation affected β-catenin phosphorylation to a much lesser extent. These experiments demonstrate that TrkA phosphorylates Y654 in vitro. Using TrkA immunoprecipitated from PC12 cells, the residual tyrosine phosphorylation of mutant Y654F β-catenin was not prevented by a Trk inhibitor. TrkB was reported to associate with the non-receptor tyrosine kinase Fyn (Iwasaki et al., 1998; Pereira and Chao, 2007), which phosphorylates β-catenin at Y142 (Piedra et al., 2003). Our results also suggest that TrkA or a kinase that co-immunoprecipitates with TrkA, possibly Fyn, phosphorylates Y654F β-catenin in vitro. More importantly, β-catenin is phosphorylated at Y654 following activation of all three Trk receptors in PC12 cells and primary neurons. Phosphorylation of Y142 was not induced by BDNF, suggesting that NT signalling specifically targets β-catenin Y654 in a cellular context. Moreover, we have shown that β-catenin also associates with neuronal Met. β-catenin was phosphorylated by Met at Y142 in vitro and in neurons following HGF stimulation. In contrast, phosphorylation of mutant Y654F was similar to that of WT β-catenin and HGF treatment did not stimulate phosphorylation of Y654 in neurons, indicating a selectivity of HGF/Met signalling for β-catenin Y142. Finally, we observed a decreased association of Met and β-catenin upon Y142 phosphorylation. Together, these findings extend to a neuronal context our knowledge about HGF signalling in non-neural cells (Monga et al., 2002; Brembeck et al., 2004; Rasola et al., 2007).

NT and HGF signalling require β-catenin phospho-Y654 and phospho-Y142 respectively, to promote axon growth and branching

BDNF, NT-3 and HGF all induce axon growth and branching in hippocampal neurons (Vicario-Abejon et al., 1998; Korhonen et al., 2000; Labelle and Leclerc, 2000). By expressing β-catenin single point mutants, we demonstrated that phosphorylation of β-catenin Y654 is required for the axon growth and branching induced by BDNF or NT-3. Conversely, phosphorylation of β-catenin Y142 is required for HGF signalling in axon morphogenesis. BDNF and HGF effects on axon growth were not additive, suggesting maximal signalling by each growth factor alone. Our results demonstrated that different growth factors target β-catenin to partially stimulate the growth and arborization of the axon, thus contributing to the complex network of pathways that direct this process. In agreement with this conclusion, regulating the availability of β-catenin by either shRNA-mediated β-catenin silencing or by sequestering it by expressing intracellular N-cadherin (Yu and Malenka, 2003) affected axon growth both in the presence and absence of exogenous growth factors. The ability of intracellular N-cadherin to block β-catenin transactivation (Sadot et al., 1998) and to place β-catenin away from the RTK could explain the inhibition of the axon growth that is otherwise promoted by BDNF and HGF. Together, these results indicate that β-catenin is required for axon growth downstream of growth factor signalling. Dendritic morphogenesis was not stimulated by BDNF or HGF signalling, suggesting that it is not regulated by tyrosine phosphorylation of β-catenin at the initial stages of the hippocampal neuron development analyzed. These results do not rule out a possible role of β-catenin phosphorylation on the maturation of the dendritic tree, in agreement with the implication of β-catenin in this later process (Yu and Malenka, 2003; Gao et al., 2007).

Mechanisms activated by NT and HGF signalling to regulate axon growth and branching

What are the mechanisms by which NT and HGF signalling regulate axon extension and branching? Tyrosine phosphorylation of β-catenin was proposed to

regulate cell migration and gene transcription by detaching β-catenin from the cadherin-catenins complex (Roura et al., 1999; Huber and Weis, 2001; Nelson and Nusse, 2004; Lilien and Balsamo, 2005). In keeping with this motion, Bamji et al. (2006) showed that tyrosine phosphorylation of β-catenin upon BDNF treatment reduces β-catenin affinity for N-cadherin and mobilizes synaptic vesicles during synapse formation. Likewise, Murase et al. (2002) reported that depolarization, which mimics the effect of a tyrosine kinase inhibitor on β-catenin redistribution to dendritic spines, increases β-catenin and cadherin association in mature hippocampal cultures. In contrast, we were able to observe the β-catenin detachment from N-cadherin in our younger neurons only using pervanadate (Ozawa and Kemler, 1998) (Fig. 27E). This result indicates that it is necessary to inhibit tyrosine phosphatases, which are working in consonance with tyrosine kinases (Kypta et al., 1996; Balsamo et al., 1998; Nelson and Nusse, 2004), to detect the detachment of β-catenin from N-cadherin in 2 DIV hippocampal neurons. This result also suggests that a phosphatase that is developmentally regulated may dephosphorylate Y654 β-catenin. In addition, β-catenin and cadherins change their location before and after synapse formation, showing a synaptic distribution as neurons mature (Uchida et al., 1996; Benson and Tanaka, 1998; Bamji et al., 2003; Bamji et al., 2006). Thus, the dynamic localization of cadherin-catenin complexes may result in the immunoprecipitation of adhesion complexes predominantly assembled at "local pools" (i.e., synapses) in older neurons, where the phosphorylation and detachment of β-catenin could mainly take place.

P-Y654 and P-Y142 β-catenin were found in different subcellular locations in hippocampal neurons. P-Y654 β-catenin is present at neurite tips and growth cones where it colocalizes with cytoskeletal components. This observation suggests that NTs by phosphorylating Y654 β-catenin stimulate axon growth and branching by locally regulating the cytoskeleton. Guidance cues regulate growth cone motility and behaviour by eliciting local synthesis of proteins (Piper and Holt, 2004) and extensively affecting the actin and microtubule cytoskeleton (Kalil and Dent, 2005). While pathways activated by NTs may involve signalling to the nucleus (Huang and Reichardt, 2001; Chao, 2003; Sole et al., 2004), NTs can also transduce their signals locally and affect the cytoskeleton to regulate motility, the neuronal morphology and

axon growth (Ye et al., 2003; Gehler et al., 2004; Zhou et al., 2004; Chen et al., 2006; Miyamoto et al., 2006). NGF induces growth of dorsal root ganglia neuron axons through inactivation of GSK-3β and regulation of the microtubule plus end binding protein APC at neurite tips (Zhou et al., 2004; Arevalo and Chao, 2005). BDNF increases filopodial length and number, a structure considered the first step in the formation of axonal branches (Gallo and Letourneau, 2004), by activating Actin Depolymerazing Factor /Cofilin (Gehler et al., 2004; Chen et al., 2006) and decreasing RhoA signalling (Yamashita et al., 1999). Indeed, members of the Rho family of small GTPases are key regulators of neuronal morphogenesis (Luo, 2000; Govek et al., 2005b). The HGF- or BDNF-induced motile phenotypes and neurite outgrowth are mediated by Rho GTPases (Royal et al., 2000; Pante et al., 2005; Bosse et al., 2007; Zhou et al., 2007). Constitutive active RhoA reduces dendrite branching, suggesting that interactions with the actin cytoskeleton are critical for the β-catenin effects in dendritic morphogenesis (Yu and Malenka, 2003). Future work should help to clarify the mechanisms mediating β-catenin phosphorylation and the growth cone response to NTs in axon morphogenesis.

Phosphorylated Y142 β-catenin was reported to decrease the interaction of α-catenin-β-catenin and to increase the interaction with BCL9-2/Legless allowing its nuclear translocation and transcription of β-catenin target genes (Brembeck et al., 2004). We found that in neurons α-catenin-β-catenin dissociate in response to the HGF-induced β-catenin phosphorylation at Y142. The α-catenin-β-catenin dissociation was observed on β-catenin immunocomplexes also containing Met, suggesting that RTKs interact with and phosphorylates a pool of β-catenin bound to the adhesion complex. P-Y142 β-catenin, contrary to P-Y654, was found in the nucleus in neurons. Consistent with this finding, we have shown that the HGF-induced axon growth and branching requires TCF-4-transcriptional activation. This dependence on TCF-4 function is also shared by Wnt-3a signalling in axon growth (our results) and for the Robo-Abl-Cables-N-cadherin-β-catenin regulation of axon guidance mediated by β-catenin Y489 phosphorylation (Rhee et al., 2007). In contrast, the activity-induced dendritic arborization does not require TCF-transcriptional activation but rather availability of the cadherin-catenin complex (Yu and Malenka, 2003). On the other hand, phosphorylation of Y654 stimulates the

association of β-catenin to the basal transcription factor TATA-binding protein and increases TCF-4-mediated transcription in non-neural cell lines (Hecht et al., 1999; Piedra et al., 2001). It has been suggested that Bcr-Abl phosphorylation of β-catenin Y86 and/or Y654 regulates nuclear β-catenin signalling in chronic myeloid leukemia (Coluccia et al., 2007). Our results in developing neurons however indicate that NTs modulate axon growth and branching by phosphorylating β-catenin Y654 independently of TCF-4 function, possibly by affecting the cytoskeleton.

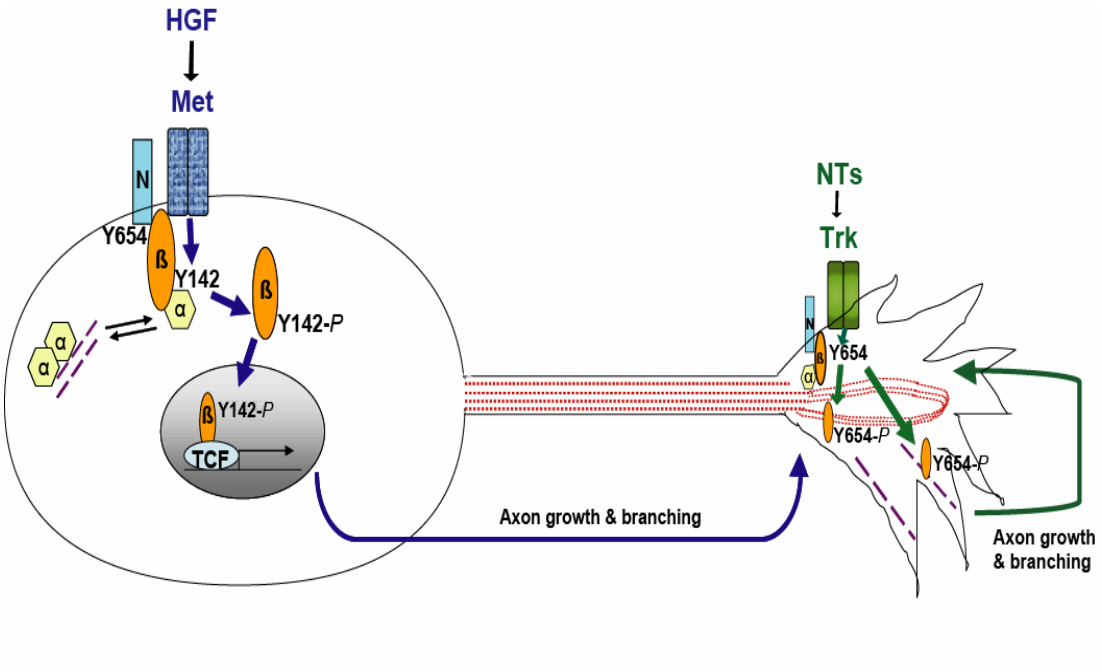

Figure 37. ***Model for the role of β-catenin tyrosine phosphorylation in axon growth and branching.*** *Adhesion complexes are assembled at the plasma membrane of the cell body, axon and growth cone. β-catenin residue Y654 is found at the region that binds to N-cadherin, whereas β-catenin Y142 is in the α-catenin binding region (Aberle et al., 1996; Lilien and Balsamo, 2005). α-catenin also binds to actin (Drees et al., 2005; Yamada et al., 2005). Trk and Met associate to a pool of β-catenin bound to N-cadherin and α-catenin. β-catenin phosphorylation by HGF/Met signalling at β-catenin Y142 targets β-catenin to the nucleus, where it activates transcription by TCF-4 to increase axon growth and branching. The detachment of P-Y142 β-catenin from the adhesion complex may imply dynamic regulation of Y654 phosphorylation, perhaps by Src family tyrosine kinases (Roura et al., 1999) and its dephosphorylation before nuclear translocation of P-Y142 β-catenin (according to the lack of nuclear immunostaining for P-Y654). β-catenin phosphorylation by NT/Trk signalling at β-catenin Y654 detaches β-catenin from N-cadherin (Roura et al., 1999; Huber and Weis, 2001; Lilien and Balsamo, 2005; Bamji et al., 2006) and P-Y654 β-catenin associates with actin and microtubules at the growth cone, possibly regulating the cytoskeleton to promote axon growth and branching. Note that whereas we demonstrate that β-catenin/α-catenin dissociate upon phosphorylation of β-catenin Y142, the depicted β-catenin dissociation from N-cadherin upon Y654 phosphorylation is based on the references above. For simplicity, Met has been located at the cell body and Trk at the growth cone plasma membrane (N, N-cadherin; β, β-catenin; α, α-catenin; ‒ ‒, F-actin; ⋯, microtubules).*

β-catenin mutant mice

Because β-catenin null mice die during early development, the function of β-catenin in the embryonic period has not been tested *in vivo*. Using conditional knock-out animals, a role of β-catenin on dendrite development of postnatally born hippocampal dentate gyrus neurons was identified (Gao et al., 2007). β-catenin conditional knock-out animals also showed a mild phenotype in mood- and anxiety-related behavioral models (Gould et al., 2008). However axon morphogenesis of hippocampal neurons has not been analyzed during the embryonic development of these animals. Expression of β-catenin deletion mutants in retinal ganglion cells of live *Xenopus* tadpoles (Elul et al., 2003) resulted in altered axonal arborization, suggesting that interactions with the N- and C-terminal domains of β-catenin (containing either Y142 or Y654) are required to shape these axons in vivo. Investigating the impact of β-catenin phosphorylation in axon morphogenesis during mouse development will require the generation of Y654F and Y142F knock-in mice.

Our results place β-catenin at a convergence point of both NT/Trk and HGF/Met signalling pathways in the regulation of axon growth and branching. Thus our findings illustrate the complex networks operating in axon morphogenesis, as stimulation of different RTK results in the activation of TCF-4 transcription-dependent and independent mechanisms downstream of β-catenin (Fig. 37). As future work, it would be interesting to investigate which are the TCF-4 target genes responsible for the axon growth and branching promoted by HGF. Preliminar results suggest that HGF stimulation could in turn induce the expression of chemokins in this process. In summary, the second part of this work highlights a novel role played by β-catenin in integrating trophic signals that emanate from RTK during axon morphogenesis.

Thus a global view of this work demonstrates the multiple roles of β-catenin during neuronal development. We studied the function of β-catenin in neurogenesis and neuritogenesis in SCNPs and postmitotic hippocampal neurons, downstream of both Wnt and growth factor signalling.

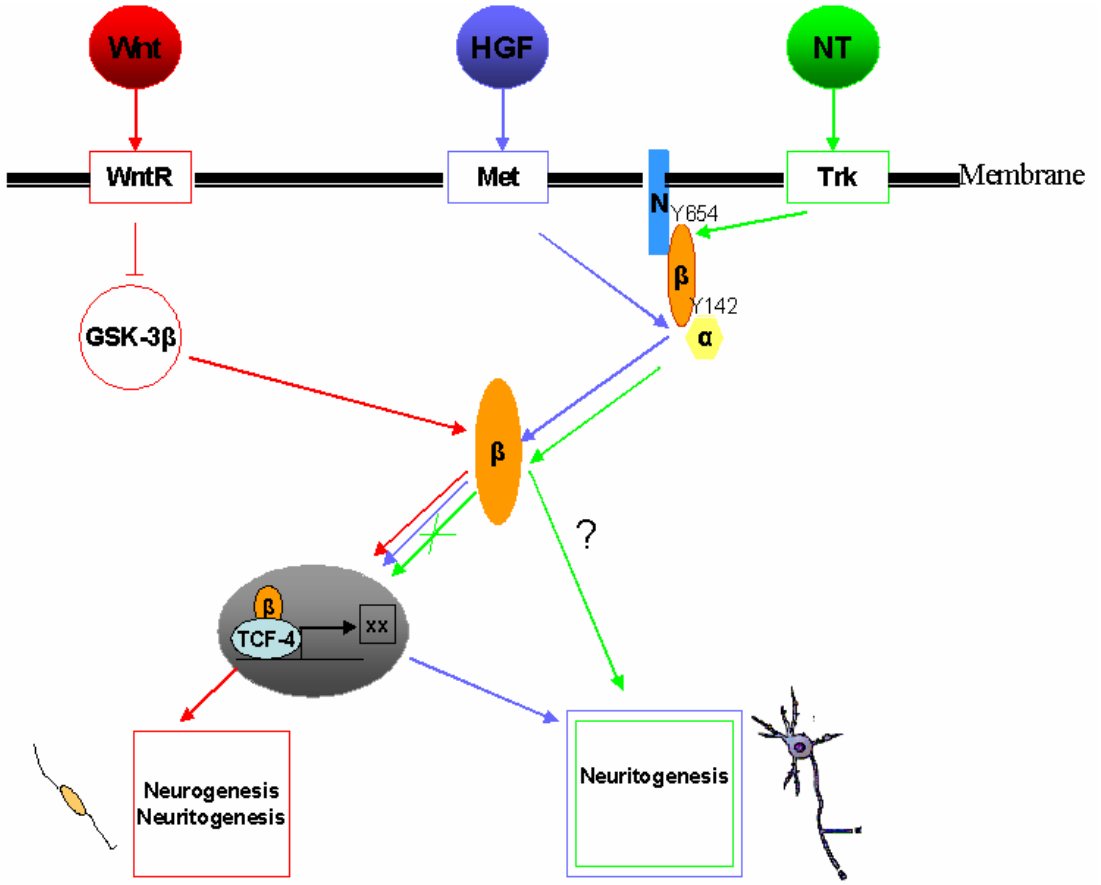

Figure 38. *β-catenin is a component of both the Wnt canonical pathway and the cell-cell adhesion complex. Binding of Wnts to their receptor(s) inhibits GSK-3β resulting in β-catenin nuclear translocation and activation of TCF/LEF-driven gene transcription. This pathway in involved in Wnt-3 and Wnt-3a-mediated neuritogenesis and probably in the Wnt-3-induced neurogenesis in neural precursors. RTKs Met and Trk phosphorylate β-catenin releasing β-catenin from the adhesion complex. Following HGF signalling, phospho-Y142 β-catenin is also translocated to the nucleus, where it regulates transcription of TCF target genes to promote axon growth and branching. In contrast, axon morphogenesis induced by NT signalling requires phospho-Y654 β-catenin, which signals independently of TCF-4 function probably by regulation of the cytoskeleton at growth cones (N, N-cadherin; β, β-catenin; α, α-catenin*).

As illustrated in Figure 38 our results place β-catenin at the intersection of the Wnt/β-catenin pathway and the pathways activated by growth factor/RTK signalling. β-catenin signalling then involves the activation of both transcription-dependent (in the response to canonical Wnt and HGF) and transcription-independent mechanisms (in NT signalling). This work emphatizes the role of β-catenin, acting downstream of different extracellular cues and from distinct cellular compartments, in different aspects of the neuronal development.

Conclusions

Conclusions

1. Wnt-3a induces a sustained increase in SCNPs proliferation.
2. Wnt-3 stimulates SCNPs proliferation transiently and increases neurogenesis by inhibiting GSK-3β.
3. Wnt-3a and Wnt-3 stimulate neurite outgrowth of SCNP-derived neurons through the Wnt/β-catenin canonical pathway.
4. β-catenin interacts with Trk receptors and is phosphorylated at Y654 *in vitro*.
5. β-catenin interacts with Met, which phosphorylates β-catenin Y142 *in vitro*.
6. β-catenin is required for axon growth downstream of BDNF and HGF signalling.
7. NT and HGF signalling stimulate the phosphorylation of β-catenin at Y654 and Y142 respectively to promote axon outgrowth.
8. Phospho-Y654 β-catenin associates with the cytoskeleton, whereas phospho-Y142 β-catenin translocates to the nucleus.
9. HGF signalling through phospho-Y142 β-catenin requires TCF-4 function to regulate axon morphogenesis.

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