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Modulating Wnt signaling at the root: Porcupine and Wnt acylation

Viviana I. Torres [a,*], Juan A. Godoy [a], Nibaldo C. Inestrosa [a,b,c,*]

[a] Centro de Envejecimiento y Regeneración (CARE Chile-UC), Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
[b] Center for Healthy Brain Ageing, School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, Australia
[c] Centro de Excelencia en Biomedicina de Magallanes (CEBIMA), Universidad de Magallanes, Punta Arenas, Chile

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ABSTRACT

Communication between cells occurs through secreted molecules, among which Wnt ligands play a critical role in balancing cell proliferation, differentiation and cellular homeostasis. The action of Wnt signaling can be modulated at several levels, including posttranslational modification of the Wnt ligands, whose acylation is critical for biological activity. At least three enzymes are necessary for Wnt acylation/deacylation: stearoyl CoA desaturase (SCD), porcupine (PORCN) and Notum. At the endoplasmic reticulum (ER), SCD provides the monounsaturated fatty acid to PORCN, which adds it to the Wnt ligand; at the extracellular matrix, the fatty acid is removed by Notum. Obviously, the interplay between these enzymes will define Wnt signaling ligand secretion and activity. Excessive activation of Wnt signaling has been observed in a variety of solid tumors, which has led the pharmaceutical industry to develop specific inhibitors for this pathway that mainly target PORCN, some of which are in early clinical trials. In the central nervous system (CNS), Wnt signaling activation has been shown to have a neuroprotective effect, and conversely, its inhibition induces neurodegeneration, which implies that the inhibition of PORCN in cancer therapies should be used with caution, and the cognitive performance of the patient should be monitored during treatment. This review collects information about the PORCN enzyme in relation to its role in the Wnt pathway through the acylation of Wnt ligands, its inhibition by drugs in the treatment of some cancers, and its putative modulation in the treatment of neurodegenerative diseases.

Contents

Abbreviations: APCDD1, Membrane-bound glycoprotein adenomatous polyposis coli; APC, Adenomatous Polyposis Coli; ASD, Autistic spectrum disorder; CNS, Central nervous system; ECM, Extracellular matrix; ER, Endoplasmic reticulum; Fzd, Frizzled; GSK-3β, Glycogen synthase kinase-3beta; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; FDH, Focal dermal hypoplasia; Hhat, Hedgehog acyltransferase; IWP, Inhibitor of Wnt production; MBOAT, Membrane-bound O-acyltransferase; SWIM, Wg-Interacting molecule; PORCN, Porcupine; SCZ, Schizophrenia; SCD, Stearoyl CoA desaturase; Wg, Wingless; WIF, WNT-inhibitory factor; Wls, Wntless.

* Corresponding authors at: Centro de Envejecimiento y Regeneración (CARE Chile-UC), Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile.
E-mail addresses: ninestrosa@bio.puc.cl (N.C. Inestrosa), vtorresa@bio.puc.cl (V.I. Torres).

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1. Introduction

Wnt proteins were discovered in invertebrates and vertebrates in the 1980s by two groups. In 1980, Christiane Nüsslein-Volhard and Eric Wieschaus, in search of gene mutations affecting the segmental pattern of the *Drosophila* larva, discovered Wnt/Wingless (wg) (Nüsslein-Volhard & Wieschaus, 1980). Two years later, Nusse and Varmus, while exploring genomic sites where DNA integration might induce breast cancer in mice, discovered Int1 (Nusse & Varmus, 1982), which was later shown to be the same Wg gene previously described in *Drosophila* (Nüsslein-Volhard & Wieschaus, 1980). Therefore, the name Wnt is derived from a combination of Wg and Int (Nusse, 1991).

Wnt ligands are involved in a number of cellular processes during development, including cell fate, proliferation, polarity, cell migration, and the homeostasis of mature tissues (Nusse, 2012; Nusse & Varmus, 2012; Willert et al., 2003). In humans, 19 Wnt proteins have been described, and Wnt ligands are recognized by seven transmembrane-spanning receptors, including Frizzled (Fzd) and its coreceptor Low-density lipoprotein receptor-related protein 5/6 (LRP5/6), which have ten and two isoforms in humans, respectively. The ligand, its receptor and the scaffold protein Disheveled (Dvl) form a signalosome that transduces Wnt signaling intracellularly (Acebron & Niehrs, 2016; Bilic et al., 2007).

Wnt ligands are secreted short-and long-range action molecules that can trigger two intracellular pathways: the canonical and the noncanonical (Gordon & Nusse, 2006). In the canonical pathway, β-catenin is stabilized in the cytoplasm through inhibition of the β-catenin degradation complex. Then, β-catenin is free to enter the nucleus, where it activates Wnt-regulated genes through its interaction with T-cell factor (TCF) family of transcription factors. This pathway branches to trigger a variety of other processes that are independent of β-catenin (noncanonical Wnt signaling). Two noncanonical pathways that have been well characterized are (i) planar cell polarity (PCP) signaling, which leads to the activation of the small GTPases RAS homologue gene-family member A (RHOA) and RAC1, which in turn, activates the stress kinase Jun N-terminal kinase (JNK) and RHO-associated coiled-coil-containing protein kinase 1 (ROCK) to instigate remodeling of the cytoskeleton and changes in cell adhesion and motility, and (ii) the Wnt-calcium pathway, in which G proteins and phospholipases mediate a transient increase in cytoplasmic free calcium activating protein kinase C (PKC), calcium calmodulin mediated kinase II (CAMKII) and the phosphatase Calcineurin (Acebron & Niehrs, 2016; Habas, Kato, & He, 2001; Nusse, 2012; Rosso, Sussman, Wynshaw-Boris, & Salinas, 2005).

PORCN is a membrane-bound O-acyltransferase (MBOAT) that acylates Wnt molecules at specific sites, conferring functional activity on the Wnt protein family (Cho & Park, 2016; Clements, 2009; Hofmann, 2000). This acylation is critical for Wnt molecules to bind Wntless (Wls), a cargo receptor that allows transportation of the Wnt ligand from the Golgi apparatus to the cell surface (Bartscherer, Pelte, Ingelfinger, & Boutros, 2006; Galli, Zebarjadi, Li, Lingappa, & Burrus, 2016), and for efficient ligand binding to the Fzd receptor (Janda, Waghray, Levin, Thomas, & Garcia, 2012; Komekado, Yamamoto, Chiba, & Kikuchi, 2007)(Fig. 1). The presence of Wls in the Golgi apparatus of *Drosophila* Wg-producing cells is essential for Wnt secretion and activity (Port et al., 2008). Wls, traffics in a loop, i.e., it cycles between the Golgi and the plasma membrane; it returns to the Golgi by clathrin-mediated endocytosis in a mechanism that depends on the retromer (Fig. 1). In the *Drosophila* neuromuscular junction (NMJ), the release of Wnt1 and Wls occurs in association with exosome vesicles (Koles & Budnik, 2012). Interfering with this process impairs Wnt secretion (Bradley & Brown, 1990; Du et al., 2016; Herr & Basler, 2012; Papkoff & Schryver, 1990; Port et al., 2008).

Diverse drugs that recognize specific molecules involved in Wnt signaling have been used to decipher the operation of the Wnt pathway in normal and pathological conditions. For that purpose, drugs that act at three different levels of Wnt signaling have been designed: drugs that inhibit intracellular signaling at different molecular targets, drugs that interfere with the binding of the ligand to the receptor and drugs that inhibit the secretion of the Wnt ligand. The last group includes PORCN inhibitors (Blagodatski, Poteryaev, & Katanaev, 2014; Tapia-Rojas & Inestrosa, 2018a).

Here, we review the existing literature about Wnt acylation sites, PORCN isoforms, PORCN-specific inhibitors and their effects on neuronal health. We also present an open question about the potential of the modulation of Wnt signaling in the early treatment of neurodevelopmental and neurodegenerative diseases.

2. Wnt synthesis, posttranslational modification and secretion

Wnt molecules perform key roles during early development and throughout the adult life of an organism. They participate in cell proliferation, cell differentiation, cell migration, synaptic maturation and polarity. Functionally active Wnt proteins need to be modified posttranslationally to enable the Wnt ligand to be secreted and to bind to the Fzd receptor (Herr, Hausmann, & Basler, 2012). In eukaryote cells, after protein synthesis occurs, most of the proteins are delivered to the ER, where they undergo chemical modifications to specific amino acid side chains, including glycosylation and acylation, and are then transported to the Golgi apparatus.

The role of acylation has been studied for several Wnt ligands in both invertebrates and vertebrates, and although advances have been made regarding the function of the posttranslational modifications of these proteins, many questions remain open.

2.1. Wnt glycosylation

Amino acid sequence analysis and biochemical approaches have suggested that Wnt proteins have N-linked glycosylation sites (Gavin, McMahon, & McMahon, 1990; Smolich, McMahon, McMahon, & Papkoff, 1993). The N-glycosylation of proteins can affect their stability, solubility and folding (Hanson et al., 2009; Shental-Bechor & Levy, 2008), and the role that this modification plays in Wnt proteins appears to vary depending on the ligand. Mouse Wnt1, for example, has four sites for asparagine-linked glycosylation, and none seem to be necessary for secretion or for paracrine and autocrine activity (Mason, Kitajewski, & Varmus, 1992). However, the functional activity measurements in the latter study were qualitative, so none the Wnt1 glycosylation sites can be definitively said not to be involved in the functional efficiency of the ligand. However, when asparagine 316 was mutated to glutamine, the mutant Wnt1 was hypersecreted, suggesting that this residue is necessary for efficient secretion (Mason et al., 1992).

Similarly, Wg, the main *Drosophila* Wnt member, which is devoid of all the N-glycosylation sites, does not present defects in secretion, and a reduced but still significant activity was observed for the double mutant N103Q and N414S (Tang et al., 2012), suggesting that glycosylation is not essential for Wg function. A different scenario was found for Wnt3a and Wnt5a, two studies showed that glycosylation is important for the secretion of these vertebrate Wnt ligands (Komekado et al., 2007; Kurayoshi, Yamamoto, Izumi, & Kikuchi, 2007) but not necessary for their activities, since unglycosylated Wnt3a and Wnt5a, added *in vitro* to cells, were able to trigger intracellular signaling (Komekado et al., 2007; Kurayoshi et al., 2007). Interestingly, differential glycosylation of Wnt ligands seem to regulate the direction of ligand secretion in polarized epithelial cells in culture (Yamamoto et al., 2013).

2.2. Wnt acylation

The acylation of secreted proteins has been detected only for three key signaling proteins: Ghrelin, Hedgehog and Wnt (Chang & Magee, 2009). Wnt ligands are highly hydrophobic proteins, a characteristic given by the fatty acylation they undergo during biosynthesis. The enzyme SCD, is an ER-resident protein that supplies the monounsaturated

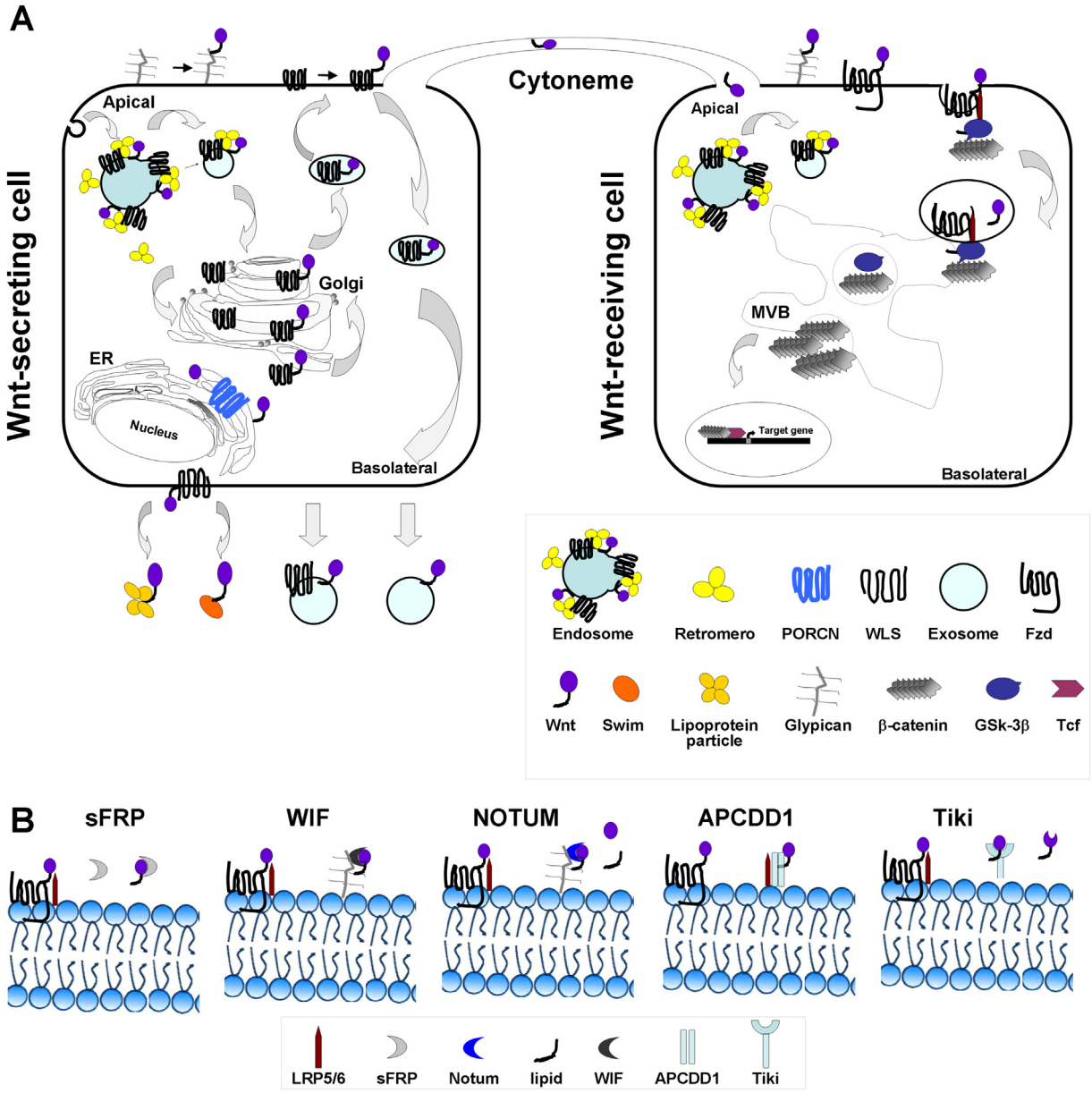

Fig. 1. Molecular and cellular mechanisms of Wnt synthesis and secretion. (A) After translation, Wnt proteins undergo a series of modifications as they transit through the secretory pathway. At the ER, Stearoyl CoA desaturase 1 (Δ-9-desaturase) (SCD1) provides the unsaturated fatty acid that PORCN adds to the Wnt ligand. At the Golgi apparatus, the transmembrane protein Wls binds the lipidated Wnt which is packed into vesicles that transit from the Golgi into the plasma membrane where is secreted to the ECM. It has been suggested that the retromer cargo adaptor binds Wls, and together undergoes retrograde transport from the Golgi to the ER. Once at the ECM, Wnt signals in neighboring cells associated either with lipoprotein particles, exosomes, micelles, or cytonemes which are cellular projections that contacts neighboring cells. (B) Molecules modulating Wnt activity. In addition, some factors act by binding to the receptors and/or the Wnt ligand. One of them, sFRPs have a domain that resembles the Wnt binding cysteine-rich domain (CRD) of Frizzled receptors and bind the Wnt ligand preventing its interaction with Fzd. A similar mechanism has been described for WIF-1. Notum is an enzyme that deacylates Wnts by hydrolyzing the carboxyester bond linking palmitoleic acid to the ligand. Adenomatosis polyposis coli downregulated 1 (APCDD1) binds Wnt and to the Frizzled coreceptor LRP5, inhibiting Wnt signaling, perhaps by titrating Wnts away from the signaling complex. Tiki acts as a protease that removes eight amino acids from the amino terminus of the processed mature Wnt ligand generating an inactive oxidized oligomer. (ER, endoplasmic reticulum; MVB, multivesicular bodies; Wls, wntless; SWIM, Wg-interacting molecule; GSK-3β, glycogen synthase kinase-3beta; sFRP, Secreted frizzled-related protein; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; WIF, WNT-inhibitory factor; APCDD1, Adenomatosis polyposis coli downregulated 1)

fatty acid and catalyzes the D(9)-cis desaturation of a range of fatty acyl-CoA substrates added to Wnt by PORCN (Paton & Ntambi, 2009; Rios-Esteves & Resh, 2013) (Fig. 2). Kakugawa and cols. described in *Drosophila* the carboxylesterase Notum, a secreted enzyme that inactivates Wnt ligands by eliminating the acyl group, e.g., in *Drosophila*, Notum restricts Wg signaling by cleaving an essential palmitoleate moiety (Kakugawa et al., 2015). At the glutamatergic NMJ synapse, Notum secreted from the postsynaptic muscle strongly modulates synapse growth, structural architecture, development and functional differentiation (Kopke, Lima, Alexandre, & Broadie, 2017) (Fig. 2).

Two types of acylation have been described in Wnt ligands: O-acylation and S-acylation. The S-acylation of Wnt proteins is the addition of palmitate (C16:0) to a cysteine residue through thioester linkage. Wnt O-acylation occurs through the addition of monounsaturated fatty acid or palmitoleic acid (C16:1) through an oxyester linkage to a serine residue in the Wnt molecule (Hausmann & Basler, 2006; Smotrys & Linder, 2004; Takada et al., 2006). Table 1 shows the putative amino acid residues in Wnt ligands whose modification by O-acylation or S-acylation is experimentally documented (Hofmann, 2000).

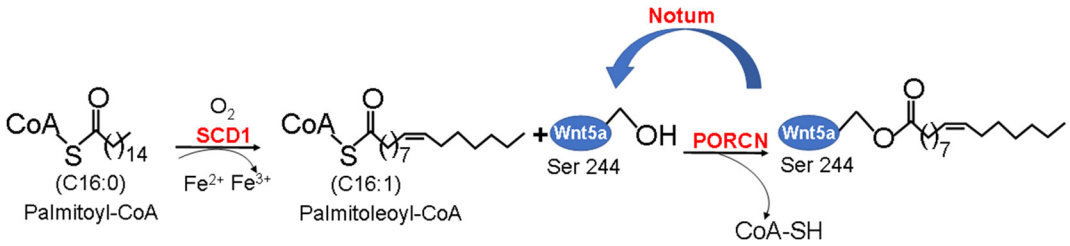

Fig. 2. Fatty acylation and deacylation of lipidated Wnt proteins. Stearoyl CoA desaturase 1 (Δ-9-desaturase) (SCD1) located at the endoplasmic reticulum catalyzes formation of palmitoleoyl-CoA from palmitoyl-CoA providing the lipid needed to modify the Wnts ligands. PORCN also at the endoplasmic reticulum takes palmitoleoyl-CoA and mediates O-fatty acylation of Wnt ligands. Once Wnt ligands are secreted to the extracellular medium their acyl moiety can be excised by Notum, a serine hydrolase, inactivating Wnt signaling activity.

Human Wnt3a was the first ligand of the Wnt family that was purified as a biologically active protein and was found to be highly hydrophobic (Willert et al., 2003). The mass of the ionized Wnt3a peptides was consistent with the presence of palmitate, and by liquid chromatography of proteolytic peptide fragments, the lipid binding site was mapped to C77 in mouse Wnt3a and to C51 in *Drosophila* Wnt8 (Table 1) (Willert et al., 2003). Wnt3a C77A was secreted at similar levels to those of the normal protein but had a lower activity rescued only by a high concentration of the mutant Wnt3a (Willert et al., 2003) suggesting that Wnt3 C77 is more important for Wnt signaling activity than for its secretion. A second acylation site was then found in Wnt1 and Wnt3a, corresponding to S224 and S209, respectively, where PORCN adds a monounsaturated fatty acid or palmitoleic acid (Galli, Barnes, Secrest, Kadowaki, & Burrus, 2007; Takada et al., 2006) (Table 1). Interestingly, the O-linked acylation of S209 and S229 was a prerequisite for the subsequent S-palmitoylation of Wnt1 C93 and Wnt3a C77, respectively (Doubravska et al., 2011). Double acylation was essential for the activity of Wnt1 but not for Wnt3a (Doubravska et al., 2011). The Wnt1 mutants C93A and S224A were trapped in the ER, impairing their traffic through the secretory pathway, which implied that both acylation sites are important for Wnt1 secretion. In the same study, a differential role of the acylation site in Wnt signaling was found: Wnt1 C93 activated a Wnt-independent pathway, whereas Wnt1 S224 activated the well-known β-catenin-dependent signaling (Galli & Burrus, 2011). Moreover, the inhibition of PORCN with Inhibitors of Wnt Production (IWP1) inhibited the β-catenin-dependent pathway, and PORCN acylated Wnt1 at S224 but not at C93. These findings are very compelling since they indicate that the acylation sites of Wnt molecules might determine the specific activated signaling pathway, and the selectivity of PORCN might play a critical role in this process (Galli & Burrus, 2011).

The physiological S-acylation of Wnt ligands is still controversial because a crystallography study of the *Xenopus* Wnt8 molecule showed that the putative cysteine is part of a disulfide bond; however, it cannot be ignored that this ligand might have specific characteristics (Janda et al., 2012).

In general, Wnt ligands are modified with lipids at one or two amino acid residues, and acylation plays a key role in their secretion, their biological activity and the formation of physiologically relevant extracellular concentration gradients (discussed later). In addition, differential Wnt acylation might determine which intracellular signaling pathway is activated according to the physiological cellular context.

Table 1
Acylation residues of Wnt ligands.

Wnt S-acylation				Wnt O-acylation			
Wg	88	LAISE**C**QHQFR	98	Wg	234	KCHGM**S**GSCTV	244
hWnt1	88	SAVRE**C**KWQFR	98	hWnt1	219	KCHGM**S**GSCTV	229
hWnt3a	72	IGIQE**C**QHQFR	82	hWnt3a	204	KCHGL**S**GSCEV	214
hWnt5a	99	TGIKE**C**QYQFR	109	hWnt5a	239	KCHGV**S**GSCSL	249
hWnt7a	68	MGLDE**C**QFQFR	78	hWnt7a	201	KCHGV**S**GSCTT	211

Amino acid region of *Drosophila* wingless (Wg) is compared with several human Wnt (hWnt) ligands. In bold are depicted the cysteine (C) and serine (S) residues that are modified by S- or O-acylation, respectively.

2.3. Mechanisms of Wnt activity modulation at the extracellular space

Once Wnts ligands reach the extracellular space, they could form short and long-range concentration gradients. Accordingly, Wnts might be transported bound to heparan sulfate proteoglycans (HSPGs) (Baeg, Lin, Khare, Baumgartner, & Perrimon, 2001), exosome like vesicles (Greco, Hannus, & Eaton, 2001), lipoprotein particles (Panáková, Sprong, Marois, Thiele, & Eaton, 2005) and through transcytosis mediated by retromer complex (Coudreuse, Roël, Betist, Destrée, & Korswagen, 2006). Furthermore, Wnt at the extracellular matrix will be assisted by several proteins (Fig. 1). For example, recently, the *Drosophila* secreted protein secreted Wg-interacting molecule (SWIM) was shown to bind Wg in a lipid-dependent manner facilitating Wg diffusion through the extracellular matrix (ECM) (Mulligan et al., 2012). Also, in *Drosophila* a less specific protein, Reggie-1/flotillin-2, a major component of membrane microdomains modulates the extracellular spreading of both Wnt and Hedgehog (Katanaev et al., 2008).

Other group of molecules postulated to regulate Wnt action at the ECM is the proteoglycans glypicans (Fig. 1). These are bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor (Filmus & Capurro, 2014). Glypican-3 (GPC3) interacts with Wnt and Frizzled stimulating endocytosis of the complex and stimulating the canonical Wnt signaling (Capurro, Martin, Shi, & Filmus, 2014). The Secreted frizzled-related proteins (sFRP) are five secreted glycoproteins that block Wnt signaling by binding to Wnt proteins (Kawano & Kypta, 2003) (Fig. 1). Similar to sFRP the secreted WNT-inhibitory factor (WIF) inhibits Wnt signaling by binding to the ligand (Surmann-Schmitt et al., 2009). The membrane-bound glycoprotein adenomatous polyposis coli (APCDD1) prevents binding of Wnt to its receptor complex (Shimomura et al., 2010) (Fig. 1). Another type of inactivation of the Wnt protein is mediated by Tiki, a transmembrane metalloprotease that binds to a conserved region within the Wnt molecule and cleaves the amino terminus inactivating specific Wnt ligands (Zhang et al., 2015) (Fig. 1).

All mentioned molecules are possible targets to inhibit or stimulate Wnt signaling, however, PORCN is at the center of this review, since appears to be the most specific molecule acting on Wnt ligands, its action occurs early during Wnt synthesis, and drugs that block its activity are currently being utilized in clinical studies to treat cancer.

3. Porcupine gene and protein

PORCN was first discovered in *Drosophila* during a screen of genes affecting patterning during development (van den Heuvel, Harryman-Samos, Klingensmith, Perrimon, & Nusse, 1993). PORCN belongs to a family of 16 evolutionarily conserved genes with predictable acyltransferase activity, the MBOAT family (Hofmann, 2000). It is located in the membrane of the ER and contains 11 predicted transmembrane domains (Fig. 3A) (Hofmann, 2000; Rios-Esteves, Haugen, & Resh, 2014) and a carboxy-terminal tail ending within the bilayer of the ER membrane (Fig. 3A). PORCN carries a catalytic site within the third loop

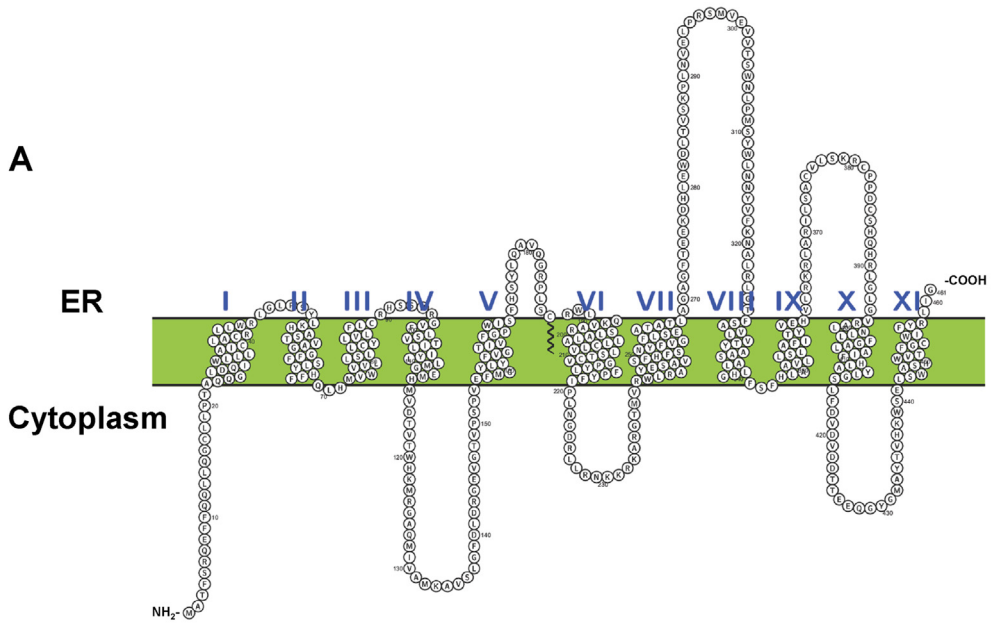

Fig. 3. Schematic diagram and topological model of human PORCN D. (A) Transmembrane domain prediction was performed by the MEMSAT-SVM server (Nugent & Jones, 2009), and the schematic model was created by Protter Server (Omasits, Ahrens, Müller, & Wollscheid, 2014) using the manual setting. (B) Multiple sequence alignment of human PORCN isoforms was performed using the Align tool of UniProt. The least conserved region within the PORCN isoforms is represented.

facing the ER lumen, close to the seventh transmembrane domain and a conserved histidine within its active site, which is essential for enzymatic activity (H341 in human PORCN isoform D) (Herr et al., 2012).

PORCN is highly conserved between species, enables the specific processing and secretion of Wnt molecules in invertebrates and vertebrates and is abundantly expressed in the CNS (Caricasole, Ferraro, Rimland, & Terstappen, 2002). Transcriptome analysis by the RNA sequencing of diverse brain cell types revealed that PORCN is expressed in neurons, astrocytes and oligodendrocytes (Zhang et al., 2014), which is consistent with the expression of Wnt ligands in these cells (Kerr et al., 2014; Richards et al., 2015). In flies, only one isoform has been found, but in other animal species, such as *Xenopus*, mice and humans, four isoforms (A-D) are produced by alternative splicing of exons 7 and 8 (Caricasole et al., 2002; Tanaka, Okabayashi, Asashima, Perrimon, & Kadowaki, 2000) (Fig. 3B). Messenger RNA (mRNA) expression analysis showed its presence in all the examined tissues, but the isoforms were differentially distributed (Table 2). For example, type A is highly expressed in the kidney, while isoform D is abundantly expressed in the brain, followed by muscle and heart (Caricasole et al., 2002).

Isoforms B, C and D have extra amino acids with a net positive charge provided mostly by arginine and lysine residues (Fig. 3B). These amino acids are predicted to be positioned towards the cytoplasmic site and are not required for activity (Rios-Esteves et al., 2014). The residues that are essential for enzymatic activity have been mapped to transmembrane domain 9 (Rios-Esteves et al., 2014). In humans, isoform D is the longest variant, with 461 amino acids (Caricasole et al., 2002). The specific function of each isoform is unknown, but they could have differential preferences for Wnt ligands. The ability of each isoform to restore Wnt/β-catenin signaling activity in PORCN-null HT1080 cells was examined in an *in vitro* assay (Proffitt et al., 2013). Interestingly, differences in activity were observed, with isoform A being less active. However, further studies are necessary to determine whether there each of the isoforms has a preference for a specific ligand.

The human PORCN gene is X-linked with a cytogenetic band location at Xp11.23. *PORCN* deletion causes *focal dermal hypoplasia* (FDH; Goltz syndrome) (Goltz, Peterson, Gorlin, & Ravits, 1962; Grzeschik et al., 2007; Wang et al., 2007) and *angioma serpiginosum* (Houge, Oeffner, & Grzeschik, 2008) in humans. FDH is a rare ectomesodermal X-linked dominant genetic disorder characterized by abnormalities in the skin, skeleton, face and eyes (Goltz et al., 1962; Wang et al., 2007). The condition occurs mostly in females who are heterozygous or have partial deletion of *PORCN* and more rarely in males with mosaic mutations (Clements, 2009; Grzeschik et al., 2007; Harmsen et al., 2009; Schaffer,

Table 2
Human porcupine isoforms.

Isoform	Transcript tissue expression	Amino acids	Mass (Da)	Accession number	Reference
A	Kidney = Uterus = Testis>Spleen = Lung>Brain	450	51,019	NP_073736.2	Caricasole et al., 2002;
B	Heart>Spinal cord>Substantia nigra>Lung	456	51,773	NP_982299.1	Caricasole et al., 2002
C	Heart>Spinal cord>Substantia nigra>Lung	455	51,564	NP_982300.1	Caricasole et al., 2002
D	Brain>Heart>Skeletal muscle>Testis	461	52,318	NP_982301.1	Caricasole et al., 2002

Four porcupine isoforms generated by alternative splicing have been described in human. Porcupine D is the largest isoform and is more abundant in brain.

Cantatore-Francis, Shin, & Rosenman, 2009; Wang et al., 2007). Characteristics similar to FDH and defects in Wnt secretion were observed in mice with inactive *PORCN* gene; these effects attributed to defective Wnt signaling in ectoderm- and mesenchyme-derived structures (Barrott, Cash, Smith, Barrow, & Murtaugh, 2011; Liu et al., 2012).

4. Role of porcupine acylation in Wnt ligand properties and mechanism of action

The role of PORCN in Wnt signaling was first suggested during a genetic screening in *Drosophila*, where it was found that the mutation of its gene produces a phenotype similar to those generated by Wg mutations (Kadowaki, Wilder, Klingensmith, Zachary, & Perrimon, 1996; Manoukian, Yoffe, Wilder, & Perrimon, 1995; van den Heuvel et al., 1993). In PORCN mutant flies, Wg accumulated in ligand-producing cells, suggesting that PORCN was necessary for the processing and/or secretion of Wg (Kadowaki et al., 1996). A bioinformatic study suggested that PORCN is an O-acyltransferase and not a *S*-acetyltransferase (Hofmann, 2000). Recently, a new experimental strategy involving fluorescence imaging gave clues about the specificity of the enzyme, showing that the substrate preference of PORCN is the addition of palmitoleic acid and other fatty acids 13 to 16 carbons in length to S209 and not to C77 of Wnt3a, confirming that PORCN is an O-acyltransferase (Gao & Hannoush, 2014).

Acylation is postulated to control the solubility and diffusion rate of Wnt ligands in the extracellular matrix. Wnt activity gradients participate in tissue patterning during development (Megason & McMahon, 2002). In fact, in the developing nervous system of invertebrates and vertebrates, Wnt ligands are expressed in gradients in the anterior-posterior axis of the spinal cord and the medial-lateral axis in the superior colliculus, and PORCN temporal/spatial expression is believed to regulate the generation of those gradients (Galli et al., 2007; Hollis & Zou, 2012). However, a recent study suggests that Wnt molecules do not diffuse far from their secreting cells, acting as short-range signals (Farin et al., 2016). In that study, Wnt3 secreted by gut Paneth cells was found to bind to Fzd receptors located in neighboring stem cells that behave as a reservoir of Wnt, and through stem cell proliferation, the complex Wnt3-Fzd was diluted, forming an epithelial Wnt gradient (Farin et al., 2016).

Therefore, although we cannot rule out the appearance of other players acting on Wnt proteins, we speculate that the intracellular interaction of SCD with PORCN along with the extracellular activity of SWIM, Reggie-1/flotillin-2, Notum, Tiki, APCDD1, WIF, sFRP modulate the extracellular concentration of Wnt, thus regulating their interaction with the ECM and Fzd receptors and consequently the concentration/activity gradients in different cellular systems (Fig. 1).

5. Specific drugs targeting PORCN

Dysregulation of the Wnt pathway has been associated with endocrine (Schinner, 2009), metabolic (Ackers & Malgor, 2018), inflammatory (Chilosi et al., 2003) and neurodegenerative diseases (Inestrosa & Arenas, 2010; Inestrosa, Montecinos-Oliva, & Fuenzalida, 2012; Inestrosa & Toledo, 2008). Moreover, Wnt signaling is known to be a critical pathway in oncogenic processes (Nusse & Varmus, 2012; Tsukamoto, Grosschedl, Guzman, Parslow, & Varmus, 1988; Zhan, Rindtorff, & Boutros, 2017), and dysregulation of β-catenin signaling and intracellular components of this pathway, including axin, adenomatous Polyposis Coli (APC), and β-catenin, are frequently mutated in a range of human tumors (Chartier et al., 2016) making it an interesting target for the development of anticancer drugs.

The fact that PORCN specifically acylates Wnt ligands has led to a search for drugs that specifically inhibit this enzyme as a possible treatment against cancerous processes in which Wnt signaling is altered (Ho & Keller, 2015).

5.1. IWP

During screening of a synthetic chemical library, several inhibitors of the Wnt pathway were found (Chen et al., 2009). One group inhibited the Wnt intracellular response (IWR), and a second group inhibited Wnt production (IWPs). IWRs block the destruction of Axin proteins, suppressors of Wnt/β-catenin pathway activity. Therefore, in the presence of IWR, the cytosolic pool of free β-catenin decreases, the levels of inactivated phosphorylated β-catenin increase, and β-catenin is consequently degraded by the proteasome (Chen et al., 2009). In the same study, IWPs were found to decrease the levels of lipidated Wnt ligands by inhibiting PORCN, and the effect was rescued by overexpressing PORCN (Chen et al., 2009; ten Berge et al., 2011)(Fig. 4). No other member of the MBOAT family was able to rescue the effect of IWP on the Wnt response, which supports a specific role of PORCN in Wnt acylation. Furthermore, a benzothiazole chemical group in the IWP molecule was found to be critical for the inhibition of PORCN by IWP2. Additionally, the effect of IWP was specific since it blocked the acylation of Wnt proteins but not the acylation of Hedgehog (Hh), which is modified by Hedgehog acyltransferase (Hhat) (Dodge et al., 2012). Moreover, IWP inhibited Wnt3a palmitoylation without affecting total cellular protein palmitoylation. Despite the above, IWPs functionally inhibit PORCN in an *in vitro* setting but not *in vivo* studies, suggesting low bioavailability of these molecules (Chen et al., 2009; Gao & Hannoush, 2014).

5.2. LGK974

During the chemical optimization of the compound GNF-1331, it was discovered that LGK974 could inhibit the Wnt pathway (Liu et al., 2013) (Fig. 4). Liu and colleagues cocultured the L-cell line overexpressing Wnt3a and the reporter cell line TM3 containing a luciferase reporter gene controlled by Wnt-responsive elements. In this *in vitro* assay, LGK974 inhibited Wnt signaling with an IC_{50} of 0.4 nM, and no toxicity was observed up to 20 μM. Comparable inhibitions were observed for several tested Wnt ligands (Liu et al., 2013). In addition, LKG974 was able to block the secretion of Wnt3a from HEK293 cells transfected with HA-tagged Wnt3a, validating PORCN as its target. In the *in vivo* studies, well-established xenografts from mammary tumor virus (MMTV)-driven Wnt1 transgenic mice implanted in nude mice were used to evaluate the pharmacodynamics and antitumor efficacy of Wnt inhibitors (Nusse & Varmus, 2012). A reduction in tumor size was observed, and no toxic effects were detected in histopathological studies when 3 mg/kg of LKG974 per day was used for 14 days. A loss of intestinal epithelium was observed only with concentrations of 20 mg/kg per day for 14 days, an important observation since Wnt signaling is key to the self-renewal of this tissue (Fevr, Robine, Louvard, & Huelsken, 2007). There are no *in vivo* studies addressing the effect of LGK974 on different types of tumors; however, a Phase I clinical trial sponsored by Novartis Pharmaceuticals has been running since 2011 with a completion date of 2020 in a group of adult patients with selected solid malignancies (Zhang & Hao, 2015).

5.3. Wnt-C59

Another drug recently developed by Novartis is 2-(4-(2-methylpyridin-4-yl)phenyl)-N-(4-(pyridin-3-yl)phenyl) acetamide or Wnt–C59, which inhibits all isoforms of murine PORCN (Proffitt et al., 2013) (Fig. 4). Wnt-C59 appears to be more potent than LKG974 in a cell-based assay, showing an IC_{50} of 74 pmol/L. *In vivo* studies of tumor growth in MMTV-Wnt1 transgenic mice showed that oral doses of 5–10 mg/day for 17 days reversed nasopharyngeal carcinoma by inhibiting the growth of undifferentiated cells (Cheng et al., 2015). The reduced tumor size was accompanied by inhibition of the Wnt/β-catenin pathway. Interestingly, Wnt-C59 was unable to inhibit *Xenopus* PORCN, which is 77% identical to human PORCN, indicating a sequence-specific effect of Wnt-C59 on human PORCN (Cheng et al., 2015).

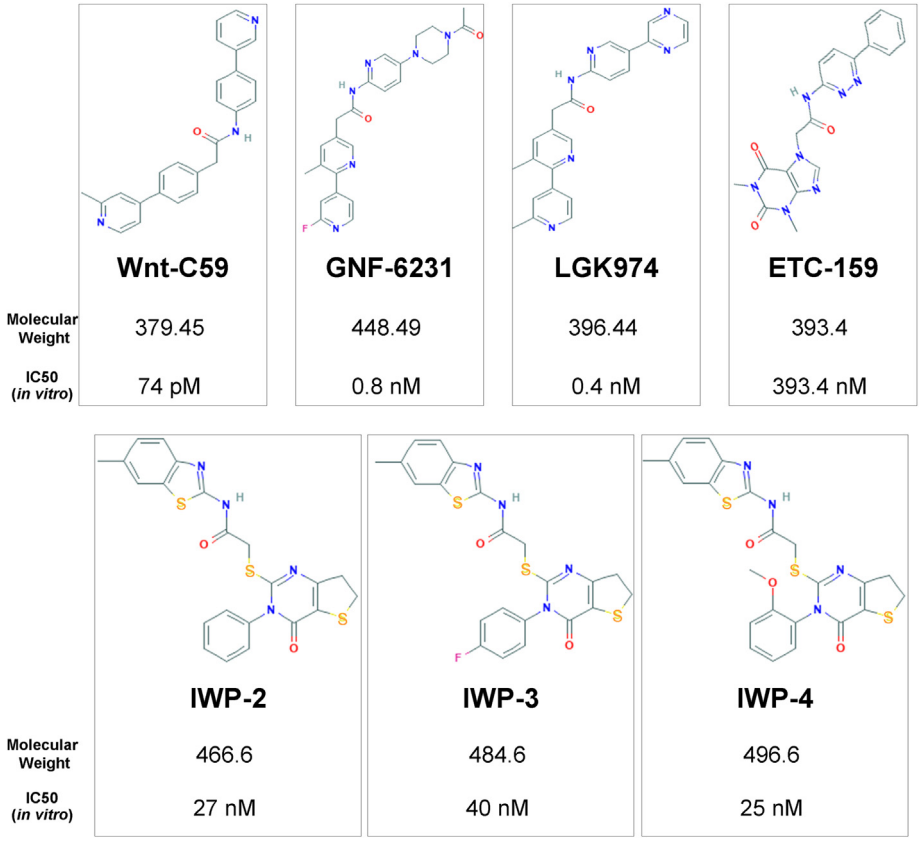

Fig. 4. Structures of the PORCN inhibitors, molecular weight and their IC_{50}'s *in vitro*. In the last few years, academic groups and pharmaceutical companies have discovered several small molecules that act on PORCN, a O-acyl transferase, preventing the secretion of the Wnt ligand and consequently its activity. PORCN has become a molecular target for the treatment of certain types of cancer and nowadays there are clinical studies in progress. The figure shows the structure of several PORCN inhibitors classified as Inhibitors of Wnt production (IWP) that have been identified from compound libraries.

5.4. ETC-159

The drug ETC-1922159, called ETC-159 (1,3-dimethyl-7-((6-phenylpyridazin-3-yl)glycyl)-3,4,5,7-tetrahydro-1H-purine-2,6-dione), was discovered during a cell-based assay screening of more than 200,000 small molecules (Madan et al., 2016)(Fig. 4). Similar to Wnt–C59, ETC-159 was more potent in inhibiting mouse PORCN than *Xenopus* PORCN, indicating the selectivity of the drug for the mammalian enzyme. No toxic effects were observed in mice with doses up to 100 mg/kg -day for 7 days. ETC-159 inhibited tumor growth in MMTV-Wnt1 transgenic mice, showing 92% tumor growth inhibition at 10 mg/kg/day.

Human teratocarcinoma mouse xenograft growth and the expression of the Wnt target gene AXIN2 were inhibited by ETC-159. The study went a step further by testing the effect of ETC-159 in patient-derived colon cancer xenografts with high R-spondin (RSPO) expression. The drug inhibited the growth and induced the differentiation of this type of tumor and total loss of the carcinoma after 28–30 days of treatment, and irreversible cellular differentiation was observed. These findings suggest that ETC-159 has potential as a drug for use in the treatment of specific cancers with RSPO translocation (Madan et al., 2016). An initial preliminary study in human patients with metastatic solid tumors showed that ETC-159 is well tolerated (Teneggi et al., 2016).

5.5. GNF-6231

GNF-6231 is the product of several chemical modifications of the compound GNF-1331 and was found after screening of a 2.4 million-compound library for effectiveness in blocking Wnt ligand secretion in a two-cell-based system, as mentioned above (Cheng et al., 2016) (Fig. 4). Specific inhibitors of PORCN were unable to block Hedgehog signaling and Wnt signaling when Wnt3a was added to the extracellular medium. GNF-6231 has better pharmacokinetic properties than GNF-1331, and at concentrations up to 10 μM, it does not affect 200 off-target genes and exhibits high plasma membrane permeability and good oral bioavailability in rodents and dogs. In the tumor model, 2 weeks of treatment with 0.3 mg/kg/day resulted in significant tumor reduction, and a complete tumor regression was obtained with concentrations of 1 and 3 mg/kg/day. Robust inhibition of Wnt signaling activity was observed without apparent toxic effects (Cheng et al., 2016).

6. Wnt signaling targeting during neurodevelopment and neurodegeneration

Wnt signaling plays a role in several biological processes both in prokaryotes and in eukaryotes. There is a body of evidence that the canonical and noncanonical Wnt signaling pathways participate in different physiological aspects of synaptic differentiation, strength, and synaptic plasticity in later stages of development and in the mature organism (Dickins & Salinas, 2013; Dinamarca, Di Luca, Godoy, & Inestrosa, 2015; Inestrosa & Arenas, 2010; Oliva, Montecinos-Oliva, & Inestrosa, 2018). During growth and cell patterning, Wnt is one of the main morphogens acting on neuronal induction and axis formation for axon pathfinding where its activity would be finely regulated. In this regard, its inhibition by Wnt-C59 was shown to be useful in the differentiation of pluripotent stem cells towards cortical neurons, where inhibition of both the canonical and noncanonical pathways could contribute to replacement therapy for motor neuron diseases or injury (Motono, Ioroi, Ogura, & Takahashi, 2016).

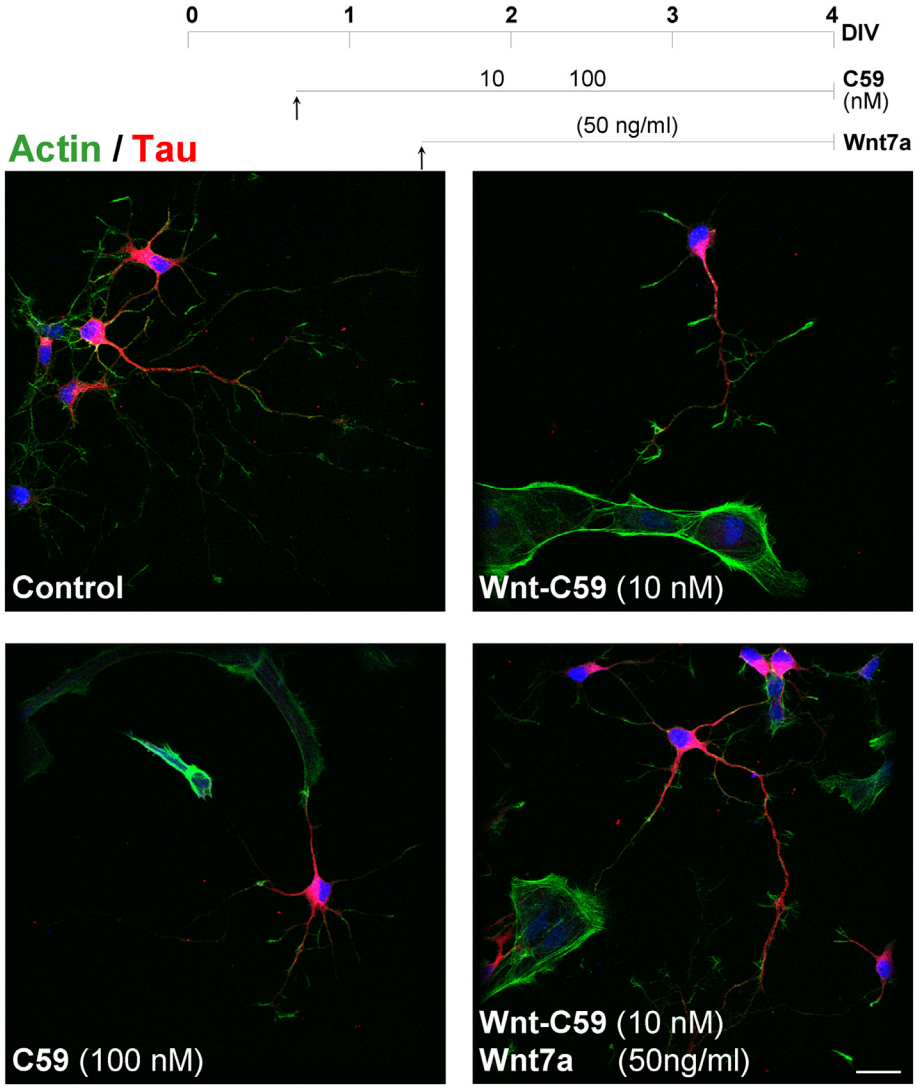

Fig. 5. Hippocampal neurons treated with PORCN inhibitors. Cultured Hippocampal neurons were treated with 10 and 100 nM of the PORCN inhibitor Wnt-C59 from the day of plating until 4 days *in vitro* (DIV). Wnt-C59 inhibited the development of neurites (fever and shorter branches, see representative images). The dendritic tree complexity was re-established partially when hippocampal neurons were treated with Wnt-C59 but in the presence of recombinant Wnt7a (see lower left image). Scale bar: 10 μm.

Wnt signaling also participates in synapse formation, synapse activity and plasticity (Inestrosa & Arenas, 2010; Rosso & Inestrosa, 2013; Torres & Inestrosa, 2017). In primary hippocampal neurons, Wnt7a/b and Wnt3a ligands have been shown to stimulate the formation of presynaptic sites and the pool of recycling synaptic vesicles (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Wnt7a induces the clustering of α7-nicotinic acetylcholine receptor (α7-nAChR), and nicotine acting through α7-nAChR prevents the loss of β-catenin induced by amyloid β (Aβ) peptide (Farías et al., 2007; Inestrosa et al., 2013). Also, acting through the canonical pathway, Wnt7a modulates dendritic spine morphogenesis and postsynaptic density protein 95 (PSD-95) gene expression (Ramos-Fernandez, Tapia-Rojas, Ramirez, & Inestrosa, 2018). Wnt5a, a noncanonical ligand, induces short-term changes in the clustering of PSD-95 without affecting its total levels, promoting recruitment of PSD-95 from a diffuse dendritic cytoplasmic pool to form new PSD-95 clusters in dendritic spines. Moreover, Wnt5a, acting as a noncanonical ligand, regulates PSD-95 distribution through a JNK-dependent signaling pathway, indicating that the Wnt5a/JNK pathway and Wnt7a modulate the postsynaptic region of mammalian synapses (Farías et al., 2009; Ramos-Fernandez et al., 2018).

In some neurodegenerative diseases, the loss and dysfunction of the synapses may explain the early cognitive defects observed in patients (Torres, Vallejo, & Inestrosa, 2017). In Alzheimer's disease, it is believed that the accumulation of the Aβ peptide inhibits the canonical Wnt pathway through its binding to the Fzd receptor (Magdesian et al., 2008), which would cause the loss of synapses (DeKosky & Scheff, 1990; DeKosky, Scheff, & Styren, 1996). Supporting evidence is the fact that the activation of Wnt signaling rescues the neurodegeneration observed in animal models of Alzheimer's disease (De Ferrari et al., 2003), and the action of the canonical ligand Wnt3a on the Frizzled-1 receptor protects against Aβ neurotoxicity (Chacón, Varela-Nallar, & Inestrosa, 2008). In addition, noncanonical Wnt/Ca^{2+} signaling protects against the changes in mitochondria function induced by Aβ oligomers (Silva-Alvarez, Arrázola, Godoy, Ordenes, & Inestrosa, 2013).

Interestingly, a recent report from our laboratory suggests that the inhibition of Wnt signaling could be detrimental for both normal and Alzheimer brains. In that work, the inhibition of the Wnt pathway by several drugs, including IWP-2, a PORCN inhibitor, accelerated the neurodegeneration of a murine model of Alzheimer's disease and induced the loss of cognitive abilities in wild-type animals (Tapia-Rojas & Inestrosa, 2018a). These data suggest that the regulated activation of Wnt signaling could be a strategy for neuroprotection (Inestrosa & Varela-Nallar, 2014; Tapia-Rojas, Burgos, & Inestrosa, 2016; Tapia-Rojas & Inestrosa, 2018b)

Canonical Wnt/β-catenin signaling also plays a critical role in mediating the development of dopaminergic neurons (Inestrosa & Arenas,

2010); hence, a protective role against the neurodegeneration in Parkinson's disease is expected (Berwick & Harvey, 2012). Interestingly, the PORCN inhibitor LGK974 was able to attenuate paraquat (1,1′-dimethyl-4,4′-bipyridinium, PQ)-induced apoptosis in human neuroblastoma SH-SY5Y cells (Yang et al., 2018).

As mentioned above, Wnt signaling plays a key role during the development of the CNS, so it is not unexpected to find a link between Wnt signaling and neurodevelopmental disorders. In autistic spectrum disorder (ASD) it is believed that the dysregulation of the canonical Wnt pathway during a critical period of embryogenesis could be the triggering factor for the development of ASD (Packer, 2016). ASD is generally detected no earlier than 18–24 months of age, when clinical signs become evident, suggesting that early detection and intervention might improve the outcome of children prone to developing autism. Schizophrenia (SCZ) and bipolar disorder (BD) are devastating mental illnesses that present structural and functional neuronal abnormalities suggesting disrupted neurodevelopment. An aberrant Wnt signaling pathway has been documented in SCZ and BD patients (Cotter et al., 1998; Kozlovsky, Belmaker, & Agam, 2002; O'Shea & McInnis, 2016). In SCZ, reduced β-catenin (Cotter et al., 1998) and glycogen synthase kinase-3beta (GSK-3β) (Beasley et al., 2001), and increased Wnt1 (Miyaoka, Seno, & Ishino, 1999) have been documented. Additionally, several polymorphisms in Wnt signaling molecule genes were found in large genome-wide association studies of SCZ and BD patients, suggesting a genetic component (Tabarés-Seisdedos & Rubenstein, 2009). Strong direct evidence for a link between BD and Wnt signaling lies in the longstanding pharmacological use of lithium, an inhibitor of GSK-3β (Sani, Perugi, & Tondo, 2017).

In recent years, the pharmaceutical industry has developed drugs that inhibit the PORCN enzyme as a therapy for treatments against some non-CNS Wnt-driven cancers (Fig. 3). In general, PORCN-targeting drugs are tested for their ability to inhibit the secretion of Wnt ligands and the palmitoylation of ligands both *in vitro* and in animal models. Considering that the Wnt pathway is involved in cellular processes throughout the organism, including the CNS, it is expected that specific cell/tissue mechanisms exist that modulate the pathway activity, preserving cellular homeostasis. As inhibitors of PORCN are being used for tumor treatments in early clinical trials, it will be pertinent to design drugs that specifically target the PORCN isoform expressed in the tumor under treatment and have no effect on isoform D, the major PORCN isoform expressed in the human brain. On the other hand, pathological CNS conditions with an abnormal increase in the Wnt signaling pathway could be treated with an inhibitor specific to the brain PORCN isoform. Conversely, in Alzheimer's disease, stimulation of PORCN at the hippocampus is predicted to increase Wnt signaling and improve the cognitive features of the patient. To our knowledge, no pharmacological activator of PORCN has been described thus far; therefore, the search for specific PORCN activators in drug libraries is something to consider.

Many signals participate in neurite outgrowth, a process wherein developing neurons produce new projections as they grow. Wnt ligands are among those signals (Montcouquiol, Crenshaw, & Kelley, 2006; Rosso & Inestrosa, 2013), however, more studies are necessary to decipher the contribution of the canonical and noncanonical pathways in the context of different neuronal types. Cultured neurons provide an easy way to dissect the role of each ligand on neuron polarization. Fig. 5 shows hippocampal neurons treated *in vitro* with nanomolar concentrations of Wnt-C59 from day 0 to day 4. In the presence of the drug, neurons showed abnormal polarization, but the normal morphology was rescued when neurons were coincubated with recombinant Wnt7a ligand and Wnt–C59.

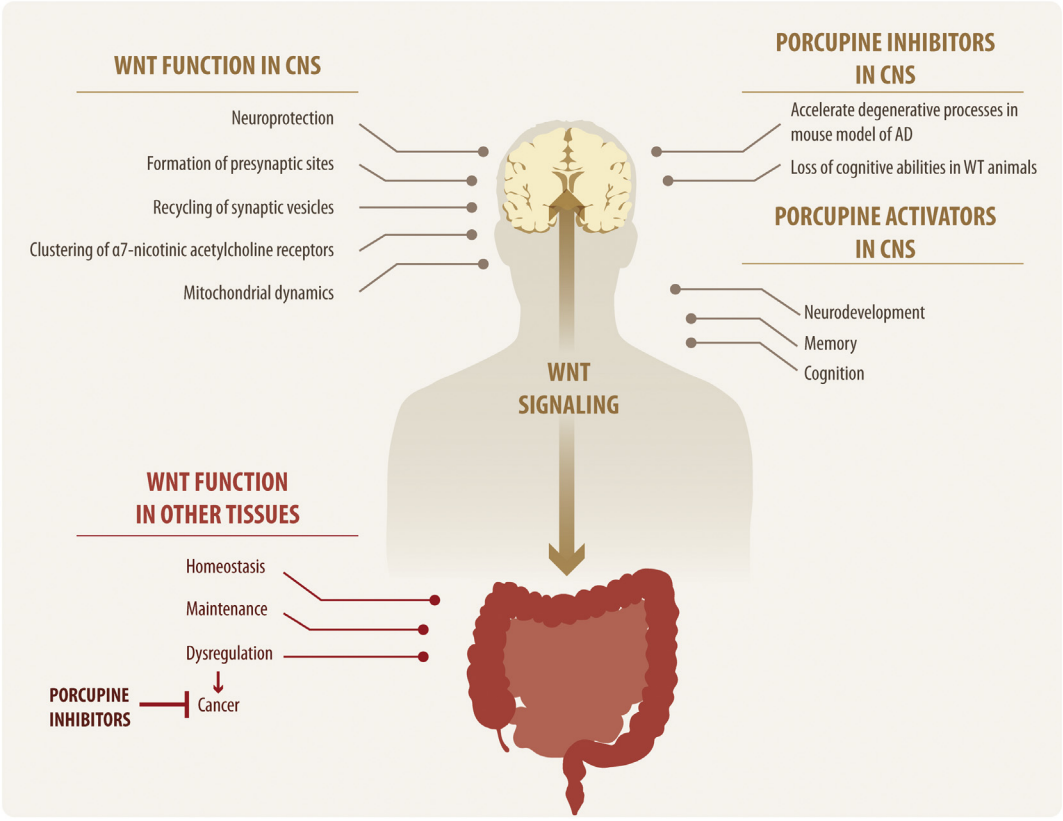

Fig. 6. Modulation of Wnt signaling using inhibitors and activators of PORCN to treat human diseases. Wnt signaling pathway participates in general cellular homeostasis and dysregulation of the pathway is implicated in numerous malignancies of non-neuronal tissues. PORCN inhibitors are being used in clinical trials in certain cancers and are a promising strategy for the recovery of cortical motor neurons after injury or neurodegeneration. In the CNS, Wnt ligands play a fundamental role in neuronal health and survival, and specific activators of PORCN could allow the recovery of Wnt signaling levels in neurodegenerative and neurodevelopmental diseases.

7. Concluding remarks

It is widely accepted that Wnt signaling plays diverse roles in organ development and during animal adulthood, and its dysregulation is associated with certain cancers, neurodegenerative diseases, osteoporosis, and fibrosis (Fig. 6). It is also being considered for regenerative medicine because Wnt ligands provide a mechanism for signaling to modulate synaptic plasticity and brain function in later stages of development and in the mature organism, turning this pathway into an interesting therapeutic target (Kahn, 2014; Serafino et al., 2017; Tai et al., 2015).

The Wnt ligand, after its synthesis, is acylated by the PORCN enzyme in the ER, and then brought through a Wls-dependent pathway to the surface of the cell from where it is released into the ECM. Once the ligand is released into the extracellular space, it must achieve an optimal concentration to act on the receptor of the target cell, where several molecular actors probably cooperate depending on the cellular context. Each of the stages of the Wnt pathway could be a pharmacological target.

LGK974 is the first molecule targeting PORCN to have entered a clinical trial, in 2011. As PORCN action is at the root of Wnt signaling, its inhibition will block both canonical and noncanonical Wnt pathways, suggesting that the use of PORCN inhibitors for cancer treatment must be controlled to avoid detrimental effects in normal tissue homeostasis (Lum & Clevers, 2012).

It would be interesting to delve into the structural-functional differences among PORCN isoforms for a better isoform-specific drug design that could inhibit or stimulate its activity at the specific target tissue. Additionally, we need to learn more about the expression of PORCN isoforms during CNS development. The latter will help in the future design of specific temporal-spatial treatments of neurodegenerative and neurodevelopmental CNS diseases.

In summary, the inhibition of PORCN can be an effective treatment in a disease as devastating as cancer, especially when it is known that the Wnt pathway is overexpressed; however, many more efforts are necessary to fully understand the Wnt pathway during tissue homeostasis and disease, and pharmacological approaches targeting Wnt signaling specifically at the diseased tissue remain to be developed.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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