FUNCTIONAL CHARACTERIZATION OF WNT7A SIGNALLING IN PC12 CELLS: INTERACTION WITH A FZD5/LRP6 RECEPTOR COMPLEX AND MODULATION BY DKK PROTEINS.

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SUMMARY

WNT factors represent key mediators of many processes in animal development and homeostasis, and act through a receptor complex comprised of members of the Frizzled and Low Density Lipoprotein-related receptors (LRP). In mammals, 19 genes encoding WNTs, 10 encoding Frizzled and 2 encoding LRP proteins have been identified, but little is known of the identities of individual Frizzled-LRP combinations mediating the effects of specific WNT factors. Additionally, several secreted modulators of WNT signalling have been identified including at least three members of the Dickkopf family. WNT7A is a WNT family member expressed in the vertebrate CNS capable of modulating aspects of neuronal plasticity. Gene knock-out models in the mouse have revealed that WNT7A plays a role in cerebellar maturation, although its function in the development of distal limb structures and of the reproductive tract have been more intensely studied. In order to identify a receptor complex for this WNT family member, we have analysed the response of the rat pheochromocytoma cell line PC12 to WNT7A. We find that PC12 cells are capable of responding to WNT7A as measured by increased β-catenin stability and activation of a TCF-based luciferase reporter construct, and that these cells express three members of the Frizzled family (Frizzled-2, Frizzled-5 and Frizzled-7) and LRP6. Our functional analysis indicates that WNT7A can specifically act via a Frizzled-5/LRP6 receptor complex in PC12 cells, and that this activity can be antagonized by Dickkopf-1 and Dickkopf-3.
INTRODUCTION

Members of the WNT gene family encode structurally related secreted glycoprotein factors, modulating a vast array of processes during vertebrate and invertebrate embryonic development as well as several aspects of tissue homeostasis in the adult (1-4). In embryos, signalling by WNT factors controls the organization of the body plan during the early stages of development as well as organogenesis at later developmental stages. Post-natally, WNT signalling is involved in normal biological events such as tissue maturation and homeostasis and in several neoplastic pathologies (2;5-8). For example, in the mammalian central nervous system (CNS) WNT signal transduction is involved in neural induction and patterning in early embryogenesis (2;4) as well as in organogenesis and neuronal homeostasis at later stages (9). In the adult, WNTs play a role in the control of neuronal plasticity and are implicated in CNS neoplasias such as medulloblastoma (10-14). The analysis of the signalling events mediated by WNTs has uncovered at least three signal transduction pathways, each involved in the mediation of specific biological responses (2;15). The most studied and best understood signalling cascade elicited by WNTs involves an interaction with a receptor complex comprising members of the Frizzled (FZD) class of 7-transmembrane receptors (7-TM) and a member of the Low Density Lipoprotein Receptor (LRP) family of single-pass membrane proteins (16). WNT interaction with its receptor results in an increase in the stability of β-catenin, whose accumulation results in translocation to the nucleus where it can interact with members of the TCF class of transcription factors and therefore modulate gene expression. The stability of β-catenin is controlled by WNT through the modulation of a large cytoplasmic protein complex comprised of the proteins AXIN, APC, GBP/FRAT and GSK3β, this latter controlling directly the level of β-catenin phosphorylation and its consequent degradation by the proteasome pathway (2;17). WNT action can also be modulated at the extracellular level by several classes of secreted factors, including members of the Dickkopf, Cerberus and FRP protein families (2;18). The vast array of processes controlled by WNTs is reflected in the numerous mammalian WNT and Frizzled classes, numbering 19 and 10 members (respectively) in man (2). During development, many of
these genes are expressed in a temporally and spatially regulated fashion, often in overlapping domains, while others are more widely expressed (19-21). These observations suggest a degree of specificity of action of the individual ligands and of relative affinities in the ligand-receptor interactions. Given the wider expression patterns of LRP genes and the tissue-specificity of expression displayed by many of the more numerous FZD genes, the specificity of the cellular response to individual WNT ligands is likely to depend largely on the individual FZD proteins expressed on the cell membrane. In spite of the fact that a number of studies have investigated the association between individual Wnts and Frizzled proteins (22-28) and of the wealth of information on the biological responses elicited by WNTs in different models systems, relatively little information is available on the identity of the FZD and LRP components of the receptor complex transducing the signal of individual WNT factors. Much interest is presently focussing on WNT signalling in the CNS as many of the effects mediated by WNTs in neuronal tissue can be phenocopied by LiCl, a GSK3 inhibitor widely employed in the treatment of mood disorders which displays well characterized neuroprotective and neuromodulatory activities (29;30). In particular, several studies have characterized the modulation of neuronal plasticity by WNT7A and LiCl in primary neuronal cultures, identifying WNT7A as a modulator of axonal remodelling and synaptic differentiation. Interestingly, recent evidence suggests that modulation of GSK3 activity in growth cones contributes to the signalling mechanisms by which semaphorins modulate neuronal plasticity, thus further implicating components of the WNT pathway in this process (31). No information is available regarding the receptor complex involved in the mediation of WNT7A effects in neuronal cells in mammals. We have investigated WNT7A responses in a cell line widely employed as a neuronal model, and well known to respond to WNT factors, to characterize a receptor complex capable of transducing WNT7A signals and to analyze the modulation of WNT7A signalling by members of the Dickkopf family. Our data indicate that a FZD5/LRP6 receptor complex can transduce a WNT7A signal in PC12 cells and that DKK1 and DKK3 can antagonize this signal.
MATERIALS AND METHODS

Cell culture and RNA isolation

PC12 cells were cultured in Dulbecco’s modified Eagle medium supplemented with Glutamine 2mM and 10% fetal bovine serum, and maintained at 37 °C in a 10% CO₂ humidified atmosphere. Total RNA was extracted from subconfluent monolayers as described (32).

Western Blotting

Western blot analysis was performed as previously described (33). Briefly, 24 hours post-transfection PC12 cells were lysed in TritonX lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride and 10 mM β-glycerophosphate) for 15 min. The cell lysates (PC12 cells cultured under standard conditions or in the presence of 10 mM LiCl for 24 hrs) were clarified by centrifugation (10,000 x g for 10 min). Protein cell lysates (80 μg) were separated by SDS-polyacrilamide gel electrophoresis, blotted onto nitrocellulose membranes and probed using polyclonal anti-β-catenin antibody (Santa Cruz, sc-1496) at 1 μg/ml dilution. Membranes were also probed with polyclonal anti-ERK1 antibody (Santa Cruz, sc-93) at 0.5 μg/ml to control equal protein loading. The immunoreactive bands were visualised by enhanced chemiluminescence (Amersham), using horseradish peroxidase-linked secondary antibodies. For the analysis of FZD expression constructs, cells were plated at a density of 2x10^6 cells/10mm petri dish the day before transfection. Cells were transfected using 8 ml of Lipofectamine 2000 (Invitrogen) and 10 μg of cDNA. 72 h post transfection, cells were rapidly rinsed in ice-cold PBS and lysed for 15 min in Triton X-100 lysis buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X-
100, 1 mM EDTA, 10% glycerol) added with protease and phosphatase inhibitors. The cell lysates were clarified by centrifugation (10,000 g for 10 min). Protein cell lysates were separated by SDS-PAGE (8% gel for LRP5 and LRP6 and 10% gel for FZD2, FZD5 and FZD 7) and blotted onto nitrocellulose. Membranes were probed using monoclonal anti-LRP5/6 (BioVision cat. 3801-106, 1:100 dilution) or polyclonal anti-pan Fzd (Santa Cruz cat. sc-9169, at 1:100 dilution). The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences) using horseradish peroxidase secondary antibodies.

**cDNA synthesis and RT-PCR studies**

Synthesis of cDNA and RT-PCR analysis were carried out essentially as described (21;34;35). Briefly, human polyadenylated RNA samples from CNS and peripheral tissues were purchased from Clontech (Palo Alto, CA). cDNA was synthesised from 1µl of polyadenilated RNA using Superscript II reverse trascriptase (Life Tech. Inc., MD) and oligodT and random hexamer oligonucleotides (250 ng each) in a final volume of 20 µl, according to manufacturer’s instructions. Following first strand cDNA synthesis, the reaction volume was increased to 100 µl and 1 µl was used for each PCR reaction. Assuming a 50% efficiency in the reverse transcription reaction, approximately 5 ng of cDNA were employed in each PCR and TaqMan reaction. For the analysis of the expression of gene expression by RT-PCR reaction conditions were the following: 3 min at 94°C, 30 sec at 94°C, then 30 sec at 55°C and 30 sec at 72°C for 35 cycles. For β-actin amplification PCR conditions were the same except that primers were fwd 5’ - TGAACCCTAAGGCCAACCCTTG-3’, rev 5’ -GCTCATAGCTCTTCTCCAGGG-3’. For the analysis of expression of FZD genes in PC12 cells cDNA, PCR conditions were the same as above except that the primers used were those reported previously (21). RT-PCR primers for LRP-5 and LRP-6 were as follows: LRP-5 (fwd 5’-GCAAGAAGCTGTACTGGACG-3’ and rev 5’-
TGTTGCAGGCATGGATGGAG-3') and LRP-6 (fwd and rev 5’-GCATTTGGCTGCCTCTGGT-3’).

**PCR amplification employing a proofreading thermostable DNA polymerase**

PCR amplification was carried out employing the GeneAmp XL PCR kit, using human adult brain cDNA as a template, as described previously (21;34;35). Reactions conditions were according to manufacturer’s protocol, with a final Mg(OAc)₂ concentration of 0.8 mM. Primers sequence were as follow: fwd 5’-GGGCGGGCTATGTTGATTGC-3’, rev 5’-ACAAGCTCAGCATCCTGCCA-3’ for WNT7a; for 5’-GAGGAGAAGCGCAGTCAATCA-3’, rev 5’-GGTTCCGGTTGCAATTCTTG-3’ for WNT5a; fwd 5’-GGCCCCGCAGCAGCCTC-3’, rev 5’-CGTCCCTCACACGGTGTT-3’ for FZD2; fwd 5’-GGCGATGGCTCGGCCTGAC-3’, rev 5’-CCTCCTACACGTGCGACAG-3’ for FZD5; fwd 5’-GGCTGAGAGCACCGCTGCACT-3’, rev 5’-CTACCGTGCCCTCTTTTG-3’ for FZD7. PCR products were analysed by electrophoresis on a 1% agarose gel poured and run in 1X TAE buffer. Products were cloned using the TOPO TA Cloning system (Invitrogen BV, Groningen, NL). Plasmid DNA was recovered and subjected to automated DNA sequencing by standard protocols using an ABI377 machine (PE Biosystems, Branchburg, NJ).

**Plasmids**

Construction of plasmids was as follows. For the WNT7a expression construct, the WNT7a cDNA was amplified from human adult brain cDNA by using primers carrying EcoRV and XbaI restriction sites at the flanking ends. The amplified cDNA was sequenced and subcloned into the EcoRV and XbaI restriction enzyme sites of the eucariotic expression pCIN4 vector (35;36). For the WNT5a expression plasmid, the WNT5A cDNA was amplified from human adult brain cDNA by
using primers carrying SmaI and XbaI restriction sites at the flanking ends. The amplified cDNA was sequenced and subcloned into the EcoRV and XbaI restriction enzyme sites of pCIN4 vector. To prepare FZD2, FZD5 and FZD7 expression constructs, FZD2, FZD5 and FZD7 cDNAs were amplified from human adult brain. The amplified cDNAs were sequenced and subcloned into the EcoRI restriction enzyme site of pCIN4 vector in the sense (pCIN4/FZD2+, pCIN4/FZD5+ and pCIN4/FZD7+) and antisense direction (pCIN4/FZD2-, pCIN4/FZD5- and pCIN4/FZD7-). The FZD cDNAs were also subcloned in the pCIN4/WNT7a plasmid in order to obtain plasmids expressing a FZD receptor and WNT7a ligand spaced by an IRES sequence (pCIN4/FZD2/WNT7a, pCIN4/FZD5/WNT7a and pCIN4/FZD7/WNT7a). The construction details of expression plasmids for human LRP-5, LRP-6, and mouse DKK-1, DKK-2 and DKK-3 expression plasmids were kind gifts from Drs. C.Niehrs and M.Semenov, and were reported previously (16;27;37).

Transient transfection assays for reporter studies

Transfections and reporter assays were carried out essentially as described (35). Transient transfections of PC12 cells were carried out in triplicate employing Lipofectamine 2000 (Life Tech. Inc.) according to manufacturer’s instructions. PC12 cells (~6 x 10⁵/well) were plated 1 day before transfection in collagen coated 96-well culture plates. A total of 0.32 µg DNA was transfected into each well, including luciferase reporter plasmid (100 ng), expression construct (100 ng of each expression construct, for up to 2 different plasmids), Renilla luciferase CMV-driven internal reporter (Promega; 20 ng) and carrier plasmid DNA (pGEM4Z, Promega; to 320 ng) as appropriate. For transfection sets involving more than 2 expression plasmids (i.e. those depicted in Figs. 6B and 6C), a total of 0,64 µg DNA was transfected, including luciferase reporter plasmid (100 ng), expression construct (100 ng of each expression construct), Renilla luciferase CMV-driven
internal reporter (Promega; 20 ng) and carrier plasmid DNA (pGEM4Z, Promega; to 640 ng) as appropriate. The luciferase reporter plasmid is the p4TCF, comprising 4 copies of a TCF responsive element upstream of a TATA element–luciferase coding sequence transcriptional unit (38). Luciferase activity was measured using the Promega’s Dual Luciferase Assay Reagent and read using a Berthold LUMAT LB3907 tube luminometer. Readings were from triplicate transfections and were automatically normalized relative to the internal standard (Renilla luciferase). For experiments involving LiCl, cells transfected with the reporter were in the presence of 10 mM LiCl for 24 hrs after transfection.

RESULTS

WNT7A signals via the canonical pathway in PC12 cells

PC12 cells are widely employed as a neuronal cell model, are known to activate the canonical WNT pathway in response to WNT1 (39-44) and do not express WNT7A ((45), Caricasole et al., unpublished). We therefore selected this cell model to assay the capacity of WNT7A to activate the canonical WNT pathway. This was performed by transiently transfecting a WNT7A expression construct (35) in PC12 cells followed by an analysis of the intracellular accumulation of β–catenin and the activation of a co-transfected TCF-luciferase construct, using culture in the presence of 10 mM LiCl as a positive control. As illustrated in Fig.1, expression of WNT7A in PC12 cells can induce the accumulation of β–catenin (Fig. 1A) to a degree comparable to that achieved by LiCl. Functional activation of the canonical WNT pathway by WNT7A in this cell model is demonstrated by the activation of a TCF-luciferase construct, an effect which is dependent on the amount of transfected WNT7A expression construct (Fig.2A). The effect is dependent on the integrity of the encoded WNT7A protein, as an expression construct encoding a truncated, non-functional WNT7A cDNA (WNT7Apx) does not result in reporter gene activation (Fig.2B). This latter mutation represents a human allele serendipitously isolated during the
amplification of full length WNT7A cDNA (35) analogous to that associated with the *postaxial hemimelia* mutation in the mouse, which was predicted to encode a non-functional protein (46). The data presented here demonstrate that the *px* WNT7A allele does indeed encode a non-functional product.

Interestingly, while WNT7A can activate the canonical pathway in PC12 cells, it is a rather inefficient inducer of a Ca++-dependent NFAT-reporter construct (data not shown), suggesting that it cannot stimulate the Ca++-dependent WNT pathway in this system.

**FZD5 can function as a component of the WNT7A receptor complex.**

WNT signalling through the canonical pathway involves the dismantling of the AXIN-APC-GSK3β macromolecular complex, stabilization of β-catenin and stimulation of TCF-mediated transcription through the activation of a receptor complex comprised of members of the FZD and LRP protein families (2). There are indications that distinct FZD proteins display different affinities for individual WNT ligands (23;25). Consequently, an appropriate combination of FZD-LRP proteins must be expressed in order for PC12 cells to respond to WNT7A. The expression of FZD genes in this system was therefore analysed by RT-PCR. The results (Fig.3A) indicate that FZD2, FZD5 and FZD7 are expressed in PC12 cells, in general agreement with a previous report where FZD expression in PC12 cells was analysed by Northern blotting (47). We reasoned that one or more of these FZD proteins would be responsible for mediating WNT7A signalling in PC12 cells. Therefore, the complete ORFs for FZD2, FZD5 and FZD7 were obtained and cloned into expression constructs either individually or in tandem with the WNT7A cDNA as dicistronic constructs. These plasmids were first tested by Western blotting for appropriate expression of the encoded FZD proteins in PC12 cells (Fig. 3B) and then co-transfected in the TCF-luciferase reporter assay in order to evaluate a possible synergistic effect with WNT7A on TCF-mediated transcription, indicative of functional interaction. The results (Fig. 3C, Fig. 4) indicate that the most substantial synergy with WNT7A in the stimulation of TCF-mediated transcription is achieved by
FZD5. While a synergistic stimulation of canonical Wnt signalling by the FZD7-Wnt7A combination was never observed, some activity was displayed by the FZD2-Wnt7A combination in most experiments, but only when FZD2 and Wnt7A were expressed from separate plasmids. On the other hand, the synergy between FZD5 and Wnt7A was observed independently of whether the FZD5 and WNT7A cDNAs were encoded by the same (dicistronic) construct (Figs 3C and also Fig.5) or by separate expression plasmids (Fig. 4). Importantly, transfected FZD5 cDNA was incapable of stimulating the TCF reporter in the absence of co-transfected WNT7A expression plasmid. Overall, this suggests that FZD5 is the major component of the highest affinity Wnt7A receptor for canonical Wnt signalling in PC12 cells.

**A FZD5/LRP6 complex can function as a WNT7A receptor.**

Two LRPs can function as co-receptors for FZD proteins, namely LRP5 and LRP6 (16). An RT-PCR analysis revealed that only LRP6 is expressed in PC12 cells (Fig. 5A), suggesting that the WNT7A receptor complex in PC12 cells may include FZD5 and LRP6. In order to analyze the specificity of LRP proteins in mediating WNT7A signalling in PC12 cells, experiments were carried out in which LRP5 or LRP6 expression plasmids were co-transfected together with WNT7A-FZD receptor combinations and the TCF-luciferase reporter. Western blotting analysis demonstrated that both LRP expression plasmids were capable of driving the expression of the corresponding LRP cDNA to comparable levels (Fig. 5B). The results of the reporter studies (Fig.5C) indicated that both LRP5 and LRP6 can synergize with WNT7A in inducing TCF-mediated transcription in PC12 cells, probably through endogenous FZD5 receptor expression. Additionally, both LRPs can synergize with exogenously provided FZD5, again confirming the functional role of FZD5 in mediating WNT7A signalling. Therefore, though both LRPs can in principle facilitate WNT7A signalling, the response to this WNT family member in PC12 cells is likely mediated by a FZD5/LRP6 receptor complex.
Modulation of WNT7A signalling by DKK proteins.

DKK proteins represent a novel family of modulators of WNT signalling (48). DKK1 in particular has been reported to antagonize WNT signalling via direct interaction with LRP5/6, resulting in rapid LRP co-receptor removal via Kremen proteins-mediated endocytosis and consequent inhibition of WNT signalling (49). As the activity of DKK proteins on WNT7A signalling is unknown, the effects of co-transfected expression plasmids encoding DKK1, DKK2 or DKK3 on WNT7A mediated stimulation of the TCF-luciferase reporter was analysed in PC12 cells. The results (Fig. 6A) indicate that DKK1 is the only tested Dickkopf family member capable of dramatic inhibition of WNT7A signalling in PC12 cells. A small but significant induction of reporter activity by DKK2 was noted, consistent with published data indicating DKK2 as a positive modulator of WNT signalling through an interaction with LRP6 (50). In order to analyze the specificity of DKK proteins for LRP5 and LRP6, a series of co-transfection experiments were carried out in which the activity of individual DKK proteins was examined on LRP5 or LRP6-mediated enhancement of WNT7A signalling. As illustrated in Fig. 6B and Fig. 6C, DKK1 and DKK3 can inhibit the enhancement of WNT7A signalling afforded by expression of an LRP5 or LRP6 cDNA, an effect which is not observed with DKK2. In fact, DKK2 overexpression can further induce TCF-mediated transcription when co-expressed with LRP5 (but not with LRP6). These results suggest that DKK3 can also act as a negative modulator of WNT signalling. Moreover, the data indicate that DKK2 can act as a positive modulator of WNT signalling (as previously reported), and that this activity may be mediated by LRP5. As for the other expression plasmids, the amount of protein produced by the three DKK expression constructs in PC12 cells was assayed by Western blotting using commercial antibodies specific for each DKK protein. Unfortunately, we were unable to detect any signal. As a functional response to DKK expression was observed, we believe the inability to detect DKK protein expression from the three plasmids was not due to lack of protein expression but rather to a technical problem associated with the use of the anti-DKK antibodies.
DISCUSSION

WNT factors have been functionally classified into “transforming” (e.g. WNT1, WNT3A and WNT7A) and “non-transforming” (e.g. WNT5A), based on their capacity to induce oncogenic transformation (4). The “transforming activity” is ascribed to the β-catenin/signaling pathway (see above), which appears to be specifically activated by WNT7A (51;52). Accordingly, many actions of WNT7A are phenocopied by lithium ions, which inhibit GSK3β thus stabilizing β-catenin (12;53;54). We have examined the molecular determinants of WNT7A signaling in PC12 cells, which originate from the neural crest and are known to respond to WNT1 (another member of the “transforming” WNT class). PC12 cells do not express WNT7A mRNA (see (45), and our own unpublished data) and are therefore particularly suitable for the study of WNT7A responses. Because WNT proteins associate with glycosaminoglycans in the extracellular matrix and are tightly bound to the cell surface, it is often difficult to collect WNT from the culture medium (reviewed in (35)). For this reason, we have transfected PC12 cells to study the autocrine/paracrine action of endogenously secreted WNT7A. Using this model, WNT7A was able to stimulate the canonical WNT/β -catenin pathway, as reflected by the increased transcription of a TCF-responsive luciferase reporter gene. The action of WNT7A was mimicked by lithium, which shared with WNT7A the ability to enhance the stability of β-catenin. Thus, transfected PC12 cells provide a powerful tool for the study of WNT7A. Searching for the FZD receptor subtype mediating the action of WNT7A, we found that PC12 cells expressed the transcript encoding for FZD2, FZD5 and FZD7 receptors. This is consistent with previous reports showing that PC12 express FZD2 and –5 receptors (47). In the absence of subtype-selective antagonist, the involvement of a specific receptor can be examined by knocking-down or overexpressing the receptor protein. We adopted the latter strategy and examined the ability of transfected FZD2, -5, and -7 to amplify the response to WNT7A. None of the three receptors had any effect per se (i.e. without WNT7A) on TCF-mediated transcription, suggesting that either no ligands are present or that endogenous FZDs maximally
respond to the WNT proteins constitutively secreted by PC12 cells. In contrast, FZD5 was able to amplify the response to co-transfected WNT7A, whereas no substantial changes were induced by FZD2 or FZD7. This suggests the following: (i) FZD5 is the specific receptor subtype mediating the activation of the β-catenin pathway by WNT7A in PC12 cells; (ii) the FZD5 receptors that are constitutively expressed by PC12 cells do not saturate the response to transfected WNT7A; and (iii) FZD2 and FZD7 do not mediate the activation of the β-catenin pathway by WNT7A, unless the molecules that convey the FZD signal to β-catenin are saturated by constitutive FZD2 and -7 receptors. The specificity of FZD5 for the WNT7A/β-catenin pathway is further supported by a number of reports showing that FZD2 and -7 are rather coupled to the WNT/PKC pathway (51;55;56). FZD receptors form a ternary complex with WNT and LRP5 or –6 in the induction of the β-catenin signaling pathway (16). Although both transfected LRP5 and –6 were effective in amplifying WNT7A/FZD5 responses, only LRP6 was endogenously expressed by PC12 cells. It is therefore reasonable to assume that, at least in this particular cell type, WNT7A interacts with FZD5 and LRP6 in stimulating the β-catenin signaling pathway. Interestingly, LRP6 knock-out mice display a phenotype which is a composite of phenotypes observed in several WNT knock-out models (57), while LRP5 null mice display fairly specific bone and eye defects (58). This suggests that LRP6 may function as a coreceptor for WNTs in most of the organs including the CNS, and that LRP5 has a specific role in mediating the action of WNT in bone formation and eye vascularization.

WNT signaling can be modulated by a number of secreted factors, which include members of the DKK family (48). Of the four known DKK family members (59), only DKK1 is described as a negative modulator of WNT signaling. DKK1 interacts with LRP6 and the transmembrane protein Kremen2, thus inducing the rapid internalization of the WNT co-receptor, LRP6 (37). Our finding that DKK1 negatively modulates the WNT7A signaling provides further support to the hypothesis that LRP6 is an essential component of the WNT7A receptor complex. Interestingly, however,
DKK1 could also modulate the amplification of WNT7A signaling induced by LRP5, suggesting that DKK1 interacts with both LRP isoforms, at least when they are overexpressed in PC12 cells. It is worth noting that DKK3 and DKK2 were able to modulate WNT7A signaling only when LRP5 or –6 were co-transfected with WNT7A. DKK3 negatively modulated WNT7A signaling in the presence of both LRP5 and –6, whereas DKK2 amplified WNT7A signaling exclusively in the presence of LRP5. With the assumption that the three DKK expression plasmids express their encoded proteins to similar extents we suggest that, as opposed to DKK1, the modulation of WNT7A by DKK2 and –3 is context-dependent, as being critically regulated by the extent of LRP expression.

Present results break the ground for further studies aimed at establishing whether WNT7A, FZD5 and LRP6 are co-localized and functionally interact in developing and adult CNS neurons. As targeted deletion of WNT7A in mice produces defects in cerebellar maturation (in addition to skeletal and urogenital abnormalities) (12;46;60;61), we expect that a similar phenotype can be produced by knocking-down FZD5 at critical stages of development. This cannot be addressed in FZD5 knock-out mice because these animals die in utero (10.5 dpc) due to abnormal placentation (62). The identification of FZD5 and LRP6 as WNT7A co-receptors in the CNS might gain new insights into the physiology of cerebellar development and might provide new targets for the search of molecules involved in the pathophysiology of inherited cerebellar dysfunctions.

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Reference List


FIGURE LEGENDS

**Fig. 1.** The canonical WNT signalling pathway is activated in PC12 cells in response to WNT7A. Western blotting analysis of total cellular protein from PC12 cells transfected with empty vector or with an expression plasmid encoding WNT7A. Levels of β-catenin (A) and of ERK1-2 (B; loading controls) in PC12 cells transfected with empty expression plasmid and cultured in the presence or absence of 10 mM LiCl for 24hrs, or transfected with a WNT7A expression plasmid.

**Fig. 2.** PC12 cells respond specifically in a TCF-reporter assay to WNT7A in transient transfection assays. **A.** Effects of culture in the presence of 10 mM LiCl or of transfection with expression plasmids encoding WNT7A, a truncated non-functional WNT7A allele (WNT7Apx) or WNT5A on TCF-mediated transcriptional activity, employing the TCF-luciferase reporter. **B.** The TCF-reporter response to WNT7A is dependent on the dose of transfected WNT7A expression plasmid. Increasing amounts of WNT7A expression plasmid (70 ng to 440 ng) result in increasing TCF-mediated transcriptional activity, while transfection of the WNT7Apx expression plasmid does not result in comparable reporter activation. The total amount of DNA transfected was maintained equivalent in all samples.

**Fig. 3.** Expression of FZD family members in PC12 cells, and identification of FZD5 as a WNT7A receptor. **A.** RT-PCR analysis of expression of the 10 FZD family members in cDNA derived from PC12 cells, indicating expression of FZD2, FZD5 and FZD7 in this cell line. A β-actin reaction serves as positive control. The same reactions carried out on PC12 cell RNA did not yield amplification products (data not shown). **B.** Western blot of PC12 cells and of PC12 cells transfected for 24 hrs with the dicistronic expression plasmids encoding human Wnt7A and FZD2, FZD5 or FZD7, probed with a commercial Pan-FZD antibody. **C.** Effects of FZD2, FZD5 or FZD7 co-expression with WNT7A (employing a dicistronic expression plasmid) on the activity of the
TCF-reporter, indicating that FZD5 is the only FZD expressed in PC12 cells that can synergize with WNT7A to induce TCF-mediated transcriptional responses.

Fig. 4. FZD5, but not FZD2 or FZD7 can synergize with WNT7A to potentiate activation of the TCF reporter. Expression plasmids comprising individual FZD cDNAs or their antisense sequences were co-transfected with the WNT7A expression plasmid (thus, ligand and receptor are encoded on separate plasmids). Only the expression of FZD5 cDNA can synergize with the WNT7A expression plasmid to increase TCF-mediated transcriptional activity. A. Absolute reporter activity for each sample. B. Fold induction levels for panel A.

Fig. 5. Expression of LRP genes in PC12 cells, and identification of LRP5/6 as a WNT7A co-receptors. A. RT-PCR analysis of expression of the LRP5 and LRP6 in cDNA derived from PC12 cells, indicating that only LRP6 is expressed in this cell line. A β-actin reaction serves as positive control. The same reactions carried out on PC12 cell RNA did not yield amplification products (data not shown). B. Western blot of PC12 cells and of PC12 cells transfected for 24 hrs with the expression plasmids encoding human LRP5 or LRP6, probed with a commercial anti-LRP5/6 antibody. C. Effects of LRP5 and LRP6 co-transfection with a WNT7A expression plasmid or with a dicistronic expression plasmid encoding WNT7A and FZD2, FZD5 or FZD7 on the activity of the TCF-reporter, indicating that both LRPs can synergize with WNT7A and FZD5 to activate the canonical WNT pathway.

Fig. 6. DKK1 family members modulate WNT7A signalling. A. Modulation of WNT7A signalling by DKK expression plasmids, measured using a TCF-reporter in transient transfection assays. DKK1 is a negative modulator of WNT7A signalling, while DKK2 and DKK3 expression plasmids have only a marginal influence on WNT7A-mediated reporter activation. B. Modulation of WNT7A signalling by DKK expression plasmids in the presence of co-transfected LRP5. In this context, co-
transfection of DKK1 or DKK3 expression plasmids inhibits TCF activity induced by WNT7A and LRP5, while DKK2 can further stimulate WNT7A-LRP5 signalling. C. Modulation of WNT7A signalling by DKK expression plasmids in the presence of co-transfected LRP6. Co-transfection of DKK1 or DKK3 expression plasmids inhibits TCF activity induced by WNT7A and LRP6. No stimulation of TCF activity by DKK2 is observed.
Fig. 1

A

β-catenin

B

ERK1  
ERK2  

-  
LiCl  
pCIN4  
WNT7A
Fig. 2

A

B
Fig. 3

A

B

Untransfected  +Wnt7A-FZD2
Untransfected  +Wnt7A-FZD5
Untransfected  +Wnt7A-FZD7

C

- 66 KDa

RLU

Samples

no DN  A  pT  CF  pTC  F+LiCl  pTCF  +W7A  pTC  F+W 7A  +FZD2  pTC  F+W 7A  +FZD5  pTC  F+W 7A  +FZD7

66 KDa

Untransfected  +Wnt7A-FZD2
Untransfected  +Wnt7A-FZD5
Untransfected  +Wnt7A-FZD7
Fig. 4

A

B
Fig. 5

A

B

C

Untransfected +LRP5

Untransfected +LRP6

250 KDa

165 KDa

RLU

Samples

pT CF

+LRP5

+LRP6 +W7A

+LRP5 +W7A +L

+LRP6 +W7A +F ZD2

+LRP5 +W7A +FZD2

+LRP6 +W7A +FZD5

+LRP5 +W7A +FZD7 +LRP6

+LRP6 +W7A +FZD7

+LRP5 +W7A +FZD7 +LRP6
Fig. 6
Functional characterization of WNT7A signalling in PC12 cells: Interaction with a FZD5/LRP6 receptor complex and modulation by DKK proteins
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