REVIEW

Targeting mediators of Wnt signalling pathways by GnRH in gonadotropes

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Abstract

The binding of GnRH to its receptor on pituitary gonadotropes leads to the targeting of a diverse array of signalling mediators. These mediators drive multiple signal transduction pathways, which in turn regulate a variety of cellular processes, including the biosynthesis and secretion of the gonadotropins LH and FSH. Advances in our understanding of the mechanisms and signalling pathways that are recruited to regulate gonadotrope function are continually being made. This review will focus on the recent demonstration that key mediators of the canonical Wnt signalling pathway are targeted by GnRH in gonadotropes, and that these may play essential roles in regulating the expression of many of the key players in gonadotrope biology, including the GnRH receptor and the gonadotropins.

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Introduction

GnRH occupancy of GnRH receptors leads to the activation of multiple signal transduction pathways (Naor 1997, 2009, Naor *et al.* 2000, Millar *et al.* 2004, 2008, Caunt *et al.* 2006, Dobkin-Bekman *et al.* 2006). In gonadotropes, GnRH activates phospholipase-C β via coupling to G $_{q/11}$, resulting in the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacylglycerol, which respectively mobilise intracellular Ca $^{2+}$ and activate protein kinase C (PKC). The diverse mechanisms and intracellular signalling pathways that have been reported to contribute to the regulation of gonadotrope function in response to GnRH stimulation have been reviewed extensively (Millar *et al.* 2004, 2008, Pawson & McNeilly 2005, Naor 2009), and will not be discussed further here. Instead, this review will focus on the proposed roles of Wnt signalling mediators in regulating gonadotrope function in response to GnRH.

A 'rough guide' to Wnt signalling

Wnt signalling plays an important role in embryonic development influencing cell proliferation, survival

and differentiation (Huelsken & Behrens 2002, van Es *et al.* 2003, Moon *et al.* 2004, Nelson & Nusse 2004). Aberrant Wnt signalling can lead to a range of diseases, most notably cancer (Polakis 2000). The canonical Wnt/ β -catenin pathway is perhaps the best described Wnt signalling pathway. The key effector of this pathway is β -catenin. In the absence of Wnt ligand (19 family members in humans) stimulation of the Frizzled (FZD) family of receptors (10 family members in humans), cellular β -catenin levels are kept very low. This is because β -catenin is held in a destruction complex, which includes amongst others adenomatous polyposis coli, axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). GSK3 hyperphosphorylates β -catenin, thereby targeting it for ubiquitination and subsequent degradation via the proteasomal degradation pathway. When Wnts bind to and activate the FZD receptors, GSK3 activity is inhibited, allowing the levels of β -catenin to stabilise, and β -catenin translocates to the nucleus where it acts as a co-factor to T cell factor (TCF)/lymphoid enhancer factor transcription factors to promote the transcription of Wnt target genes, many of which are key in developmental processes (Huelsken & Behrens 2002, van Es *et al.* 2003, Moon *et al.* 2004).

Less well characterised are the so-called non-canonical Wnt pathways, several of which have been proposed over the past 20 years, including the Wnt/Ca²⁺ pathway and Wnt/c-Jun N-terminal kinase (JNK) pathway (Kuhl *et al.* 2000, van Es *et al.* 2003, Veeman *et al.* 2003, Kohn & Moon 2005). These pathways are also activated by Wnt ligands binding to FZD receptors, and are thought to influence processes such as cell polarity, cytoskeletal reorganisation and cell movement, and utilise a diverse array of signalling mediators and transcription factors to drive these events, including PKC, Ca²⁺/calmodulin (CaM), calcineurin (CaN), Ca²⁺/CaM-dependent kinase II, nuclear factor of activated T cells (NFAT), dishevelled, RhoA, Rac, Cdc42 and JNK (Kuhl *et al.* 2000, van Es *et al.* 2003, Veeman *et al.* 2003, Kohn & Moon 2005, Katoh & Katoh 2007).

Although there are two isoforms (α and β) of GSK3, it is GSK3 β that phosphorylates β -catenin, and targets it for ubiquitination and proteolytic degradation in the inactive canonical Wnt/ β -catenin signalling pathway (Frame & Cohen 2001). Following activation of Wnt/ β -catenin signalling, GSK3 β is inhibited by a poorly defined mechanism, thereby allowing the stabilisation and accumulation of β -catenin levels. GSK3 was originally identified as a key mediator of insulin signalling, and is now thought to be involved in many other signalling pathways with a diverse array of proposed substrates, including Tau, CREB, NF κ B, MUC1, cyclin D1, MYC, NFAT and JUN (Frame & Cohen 2001, Grimes & Jope 2001). Multiple kinases have been implicated in the Ser⁹ and Ser²¹ phospho-inhibition of GSK3 β and GSK3 α respectively, including the phosphatidylinositol 3-kinase (PI3K)–Akt/protein kinase B (PKB) signalling axis (such as that occurring in classical insulin signalling), p90RSK, PKC, protein kinase A (PKA), p70 S6 kinase and extracellular signal-regulated protein kinase (ERK) (Stambolic & Woodgett 1994, Frame & Cohen 2001, Grimes & Jope 2001, Ding *et al.* 2005). It was initially thought that Ser⁹ GSK3 β phospho-inhibition was the mechanism of GSK3 β inhibition within the Wnt/ β -catenin pathway (Frame & Cohen 2001). However, a study using a mouse knock-in of a Ser⁹Ala GSK3 β mutation reported normal embryonic development, suggesting that the Ser⁹ phospho-inhibition of GSK3 β is not implicated in canonical Wnt signalling (McManus *et al.* 2005). Both isoforms of GSK3 are probably important for Wnt signalling because deletion of one has no effect, as long as the other is present, possibly suggesting isoform redundancy (Doble & Woodgett 2003). Furthermore, in the mouse knock-in analysis where the Ser^{9/21} residues of GSK3 β / α respectively were mutated to alanine residues, normal β -catenin accumulation was observed in response to Wnt3a stimulation, again confirming that Ser⁹ phosphorylation is not the mechanism for inhibiting GSK3 β in Wnt/ β -catenin

signalling (McManus *et al.* 2005). In addition, mutational studies of the ‘insulin pool’ of GSK3 suggest that insulin-induced Ser^{9/21} phospho-inhibition is not involved in the activation of the Wnt signalling pathway, and that Wnt signalling does not alter the glycogen synthase output of insulin signalling (Ding *et al.* 2000, Ng *et al.* 2009). The ‘insulin’ and Wnt pools of GSK3 β may therefore be considered to be functionally distinct.

A number of theories have arisen regarding the mechanism involved in GSK3 β inhibition in the Wnt/ β -catenin pathway. Some involve the GSK3 β -binding protein called frequently rearranged in advanced T-cell lymphoma (FRAT), which can block GSK3-induced phosphorylation of β -catenin without affecting glycogen synthase activity in the insulin signalling pathway (Thomas *et al.* 1999). Other theories involve the essential Wnt pathway protein dishevelled (Huelsken & Behrens 2002, van Es *et al.* 2003, Moon *et al.* 2004). It has also been demonstrated that Wnt signalling inhibits GSK3 through a PKC-mediated mechanism (Cook *et al.* 1996), suggesting that PKC may have a role in inhibiting GSK3 within the canonical Wnt/ β -catenin pathway, in addition to the phospho-inhibition at Ser^{9/21} such as in classical insulin signalling. CK1 can enhance GSK3 activity by acting as a priming kinase. However, CK1 also has an essential role in positively transducing the canonical Wnt signal, suggesting its involvement in GSK3 β inhibition (Doble & Woodgett 2003). Alternatively, different CK1 isoforms may positively and negatively regulate GSK3 β activity. Interestingly, GSK3 β not only has been implicated in canonical Wnt/ β -catenin signalling, but may also play a negative regulatory role in the non-canonical Wnt/Ca²⁺ pathway, in which it is thought to function as a nuclear export kinase, thus terminating NFAT transcriptional activity (Crabtree & Olson 2002, van Es *et al.* 2003, Katoh & Katoh 2007).

The FZD family of receptors are seven-transmembrane-spanning receptors that resemble G protein coupled receptor (GPCRs), and are now listed by the International Union of Pharmacology as a novel and separate family of GPCRs, the ‘Class FZD’ (Schulte & Bryja 2007). One of the first demonstrations that FZDs signal via G-protein coupling came from the early studies by Malbon *et al.* (Liu *et al.* 1999, 2001). It was demonstrated that β_2 -adrenergic receptor/FZD1 chimera bearing the cytoplasmic domains of rat FZD1 was able to stimulate β -catenin stabilisation and β -catenin/TCF transcriptional activity in response to isoprenaline stimulation, and that this was inhibited when certain α -protein α -subunits (G_{ao} and G_{aq}) were depleted by antisense RNA, or by pertussis toxin pretreatment (Liu *et al.* 1999, 2001). Importantly, these and subsequent studies appeared to suggest that non-FZD GPCRs could target β -catenin activity in response to stimulation by their cognate ligands (Malbon 2005).

Targeting Wnt signalling mediators by non-FZD GPCRs

A number of non-FZD GPCRs have been shown to target β -catenin/TCF activity in response to their cognate ligands, including the prostanoid receptors (Fujino & Regan 2001, Fujino *et al.* 2002), M1 muscarinic acetylcholine receptor (Farias *et al.* 2004), lysophosphatidic acid (LPA) receptor (Yang *et al.* 2005) and thromboxane A₂/TP_a receptor (Yan & Tai 2006). The first demonstration of non-FZD targeting of β -catenin/TCF-dependent signalling was through the FP_B prostanoid receptor (Fujino & Regan 2001). Stimulation of FP_B-expressing cells with prostaglandin F_{2 α} (PGF_{2 α}) led to reorganisation of β -catenin, a decrease in phosphorylation of cytoplasmic β -catenin and activation of β -catenin/TCF-dependent transcription. In addition to these findings, it was demonstrated that activation of the EP₂ and EP₄ prostanoid receptors by PGE₂ promoted β -catenin/TCF-dependent transcription (Fujino *et al.* 2002). In addition, the EP₂ and EP₄ receptors were demonstrated to target Ser⁹ GSK3 β phospho-inhibition by a PKA-dependent mechanism (Fujino *et al.* 2002). The M1 muscarinic acetylcholine receptor was demonstrated to inhibit GSK3 β activity, stabilise β -catenin levels and induce the expression of the Wnt target genes *en-1* and *cycD1* resulting in the protection of neurons from amyloid- β -peptide neurotoxicity in a rodent model of Alzheimer's disease (Farias *et al.* 2004). The LPA receptor was demonstrated to target β -catenin to induce colon cancer cell proliferation (Yang *et al.* 2005). LPA was shown to promote the nuclear translocation of β -catenin, activation of β -catenin/TCF-dependent transcription, phospho-inhibition of GSK3 β and activation of β -catenin/TCF target genes. Furthermore, all these LPA-induced events were apparently dependent on conventional PKC activity (Yang *et al.* 2005). The thromboxane A₂/TP_a receptor was demonstrated to activate β -catenin/TCF-dependent transcription apparently through the phospho-inhibition of GSK3 β (Yan & Tai 2006; Fig. 1).

The GnRH receptor targets GSK3 β phospho-inhibition and β -catenin/TCF transcriptional activity

The above studies suggested that further cross-talk between signalling mediators of the Wnt/ β -catenin pathway and those activated by GPCRs is likely. Indeed, several recent studies have demonstrated that GnRH, acting at the GnRH receptor, can target mediators of the Wnt/ β -catenin signalling pathway in both a heterologous HEK293 model cell line and the L β T2 gonadotrope cell line (Gardner *et al.* 2007, Salisbury *et al.* 2007, 2008, 2009, Gardner & Pawson 2009).

Figure 1 Targeting Wnt signalling mediators by GnRH in gonadotropes. GnRH activates PLC- β via coupling to G $_{\alpha/11}$, resulting in the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG), which respectively mobilise intracellular Ca²⁺ and activate protein kinase C (PKC). GnRH targets Ser⁹ phospho-inhibition of GSK3 β through a PKC-mediated mechanism, which may in part be responsible for the stabilisation and accumulation of β -catenin levels. β -catenin translocates to the nucleus where it acts as a co-factor to TCF, to increase the transcription of TCF target genes, including *Fra1*, *Jun* and *Myc*. β -catenin also interacts with SF1, which together with other DNA-binding proteins (including EGR1, JUN and ATF3) increases transcription of *Lhb*, and possibly of *Cga*, *Fshb* and *Gnhr*. In addition, β -catenin may act as a co-factor to regulate members of the FoxO family and the androgen receptor (AR). GSK3 β may target the activities of other transcription factors in gonadotropes, including CREB and NFAT, which may therefore be subject to an additional level of regulation by GnRH, since GnRH inhibits GSK3 β activity. See text for details.

The initial studies reported GnRH-mediated nuclear accumulation of β -catenin, activation of β -catenin/TCF-dependent transcription (using a TCF-dependent luciferase reporter plasmid) and the up-regulation of several β -catenin/TCF target genes including *Jun*, *Fra1* and *Myc* (Gardner *et al.* 2007). In addition, it was demonstrated that GnRH targets Ser⁹ GSK3 β phospho-inhibition (Gardner *et al.* 2007). As discussed above, the accumulation of β -catenin in response to GnRH may be independent of the Ser⁹ phospho-inhibition of GSK3 β since the Wnt pool of GSK3 β is probably functionally

distinct from the pool of GSK3 β that GnRH targets. The mechanism whereby GnRH targets β -catenin accumulation and Ser⁹ GSK3 β phospho-inhibition was shown to be mediated via G_{q/11} coupling, and is most likely PKC dependent (Gardner *et al.* 2007). Furthermore, GnRH does not signal through PI3K–Akt/PKB to target GSK3 β phospho-inhibition, as is the case in classical insulin signalling, since pharmacological inhibition of PI3K failed to block β -catenin/TCF transcriptional activity in response to GnRH (Gardner *et al.* 2007).

Apart from its role as a TCF co-factor in the canonical Wnt signalling pathway, β -catenin can function as a co-factor to a number of other transcription factors relevant to gonadotrope biology, including SF1 and JUN (Angel *et al.* 1988, Desclozeaux *et al.* 2002, Shah *et al.* 2002, Gummow *et al.* 2003, Mizusaki *et al.* 2003, Parakh *et al.* 2006). Thus, the ability of GnRH to stimulate the expression of β -catenin/TCF target genes suggests that SF1- and JUN-responsive genes may also be targets of GnRH-dependent β -catenin/TCF transcriptional activity. This is because functional cross-talk between SF1, JUN and β -catenin/TCF signalling has been demonstrated in several studies (Shah *et al.* 2002, Gummow *et al.* 2003, Mizusaki *et al.* 2003, Veeman *et al.* 2003, Le Floch *et al.* 2005). Furthermore, β -catenin acts as a co-factor of SF1 through a direct interaction (Gummow *et al.* 2003, Mizusaki *et al.* 2003, Parakh *et al.* 2006), while JUN can interact co-operatively with TCF in the β -catenin/TCF complex at JUN promoter sites (Nateri *et al.* 2005).

Targeting β -catenin/TCF transcriptional activity in gonadotropes

The ability of GnRH to stimulate the expression of β -catenin/TCF target genes, and the finding that both SF1- and JUN-responsive genes may also be targets of GnRH-dependent β -catenin/TCF transcriptional activity, has important implications for gonadotrope function, including the expression of *Lhb*, *Cga*, *Fshb* and *Gnrhr* genes. An important study highlighting the role of β -catenin as a member of a transcription factor complex that drives maximal activity of the *Lhb* subunit promoter in response to GnRH was published (Salisbury *et al.* 2007). This study by Nilson *et al.* demonstrates the co-localisation of β -catenin with SF1 and EGR1 on the promoter of the *Lhb* subunit gene in response to GnRH, and suggests that endogenous SF1 and β -catenin can physically associate in L β T2 cells (Salisbury *et al.* 2007). A role for GnRH targeting of β -catenin/TCF activity to regulate the expression of JUN-responsive genes including *Cga*, *Fshb* and *Gnrhr*, which additionally require SF1, is yet to be demonstrated. What is clear though is that β -catenin has an important role to play, and that it regulates gonadotrope responsiveness to GnRH.

Concluding remarks and future direction

The ability of GnRH to impinge on the activity of Wnt signalling mediators has several implications for further understanding key processes in gonadotrope biology. For example, CREB has a well-known role in regulating *Gnrhr* expression by targeting CRE sites within the *Gnrhr* promoter in response to GnRH stimulation (Cheng & Leung 2001, Maya-Nunez & Conn 2001). GSK3 has been proposed as one of a number of kinases that regulate CREB activity (Frame & Cohen 2001, Grimes & Joje 2001, Doble & Woodgett 2003). Thus, by promoting the Ser⁹ phospho-inhibition of GSK3 β , GnRH signal transduction may provide an additional level of complexity in the regulation of CREB activity and its ability to function optimally at the *Gnrhr* promoter. GSK3 can also target the activity of the Ca²⁺-sensitive transcription factor NFAT by functioning as a nuclear export kinase, thereby terminating NFAT transcriptional activity (Crabtree & Olson 2002). GnRH has been shown to mediate the derepression of the *Fshb* gene in the α T3-1 gonadotrope cell line through the activation of the Ca²⁺/CaN pathway leading to NFAT-driven expression of *Nur77* (Lim *et al.* 2007). Furthermore, a recent study has reported that GnRH-mediated Ca²⁺/NFAT signalling does not act as the GnRH pulse frequency decoder in L β T2 cells (Armstrong *et al.* 2009). It will be interesting to determine if there is a role for GnRH-mediated Ser⁹ phospho-inhibition of GSK3 β in regulating NFAT nuclear residency and how this impacts on gonadotrope function with regard to these studies. As a multifunctional kinase with a diverse array of proposed targets, it is likely that many more roles for GSK3 in the gonadotropes will emerge.

Additional roles for β -catenin are also likely, including the regulation of androgen receptor (AR) activity. As an AR co-factor, β -catenin functions to both inhibit and stimulate AR target gene expression in a variety of cell types and tissues (Cheshire & Isaacs 2003, Mulholland *et al.* 2005, Terry *et al.* 2006, Robinson *et al.* 2008). Expression of *Cga* (α -polypeptide glycoprotein hormones), *Lhb* and *Fshb* subunit gene is widely reported to involve a component of androgen regulation in gonadotropes; however, possible roles for β -catenin in modulating AR activity at this level (in response to GnRH) have not been reported yet (Curtin *et al.* 2001, Jorgensen & Nilson 2001a,b, Curtin *et al.* 2004, Spady *et al.* 2004, Thackray *et al.* 2006, Burger *et al.* 2007, Thackray & Mellon 2008). Furthermore, roles for FOXO transcription factors, which employ β -catenin as a co-factor, have been proposed (Essers *et al.* 2005, Malbon 2005, Hoogeboom *et al.* 2008, Jin *et al.* 2008, Hoogeboom & Burgering 2009, Stavrou *et al.* 2009). Although clearly speculative, elucidating potential roles for GSK3 β and β -catenin would

enhance our understanding of the complexity of the regulation of gonadotropin subunit expression and gonadotrope function.

In conclusion, this review has highlighted both published and putative roles for mediators of the Wnt signalling pathways in targeting important biological processes in gonadotrope biology. We suggest that new avenues of research will continue to emerge in order to advance our understanding of the targeting of Wnt signalling mediators by GnRH to in turn regulate gonadotrope function. Indeed, such studies are already well underway.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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