WNT Signaling Affects Gene Expression in the Ventral Diencephalon and Pituitary Gland Growth

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We examined the role of WNT signaling in pituitary development by characterizing the pituitary phenotype of three WNT knockout mice and assessing the expression of WNT pathway components. Wnt5a mutants have expanded domains of Fgf10 and bone morphogenetic protein expression in the ventral diencephalon and a reduced domain of LHX3 expression in Rathke’s pouch. Wnt4 mutants have mildly reduced cell differentiation, reduced POU1F1 expression, and mild anterior lobe hypoplasia. Wnt4, Wnt5a double mutants exhibit an additive pituitary phenotype of dysmorphology and mild hypoplasia. Wnt6 mutants have no obvious pituitary phenotype. We surveyed WNT expression and identified transcripts for numerous Wnt, Frizzleds, and downstream pathway members in the pituitary and ventral diencephalon. These findings support the emerging model that WNT signaling affects the pituitary gland via effects on ventral diencephalon signaling, and suggest additional Wnt genes that are worthy of functional studies.


Key words: Wnt5a; Wnt4; Wnt6; Wnt11; Wnt16; mouse knockout; development

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INTRODUCTION

The pituitary gland is a central organ in the endocrine system of all vertebrates and is responsible for the production and regulation of peptide hormones necessary for growth and development; regulation of thyroid function, lactation, sexual maturation, and fertility; and the ability to respond to physiological stresses (Cushman and Camper, 2001). The mature gland is composed of three lobes in rodents: the posterior lobe, the intermediate lobe, and the anterior lobe, which is comprised of five major pituitary hormone-producing cell types (Japon et al., 1994).

The posterior lobe is derived from neural ectoderm, whereas the anterior and intermediate lobes of the pituitary gland arise from Rathke’s pouch, a primitive structure resulting from an invagination of the oral ectoderm beginning at embryonic day (E) 9.0 in the mouse. The pouch pinches off from the remaining oral ectoderm at approximately E11.5 and is characterized by a domain of apoptosis at the separation point (Charles et al., 2005). In early pituitary development, the dorsal aspect of the pouch undergoes extensive cell proliferation, and cells migrate out ventrally and rostrally from Rathke’s pouch and form the anterior lobe (Ikeda and Yoshimoto, 1991; Ward et al., 2005).

Pituitary development is mediated by the temporal and spatial expression of transcription factors in the pouch in response to bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signaling in the
surrounding tissues of the developing gland (Ericson et al., 1998; Danen et al., 2001; Davis and Camper, 2007). These factors include the POU-domain transcription factor Pit1, recently renamed Pou1f1, and its predecessor Prophet of Pit1 (Prop1); LIM homeodomain factors Isl1, Lhx3, and Lhx4; the pituitary homeobox genes Pitx1 and Pitx2; and the Rathke’s pouch homeobox gene Rpx (Hex1; Watkins-Chow and Camper, 1998; Cushman and Camper, 2001). Additionally, members of the SOX family of transcription factors have also been implicated in pituitary gland size and shape formation (Camper, 2004; Rizzoti and Lovell-Badge, 2005).

In addition to the various transcription factors involved in pituitary development, WNT signaling is emerging as an important contributor. Both the canonical and noncanonical WNT pathways are highly conserved throughout evolution and are essential for proper growth, development, and organogenesis in both vertebrate and invertebrate organisms (Rijswijk et al., 1987; Cadigan and Nusse, 1997). In the canonical pathway, a core set of proteins respond to WNT and prevent CTNNB1 (β-catenin) from being proteolyzed, thus, allowing β-catenin to activate target genes that modulate cell fate, proliferation, and apoptosis. In the noncanonical pathway, WNTs function independently of β-catenin and can activate CamKII and protein kinase C (PKC), GTP-binding proteins that in turn activate phospholipase C (PLC) and phosphodiesterase (PDE), and also the planar cell polarity (PCP) pathway that activates Jun-N-terminal kinase (JNK; reviewed in Kohn and Moon, 2005).

Several lines of investigation support roles for WNT signaling in pituitary gland organogenesis. For example, β-catenin can regulate the activity of three transcription factors with roles in pituitary development: Pitx2, Nrs5a1 (Sf1), and Tcf7l2 (Tcf4; Kioussi et al., 2002; Brinkmeier et al., 2003, 2007; Gummow et al., 2003). Downstream factors in the WNT pathway are also important for proper pituitary development. Tcf4−/− embryos exhibit severe pituitary overgrowth, with a threefold increase in anterior lobe volume (Brinkmeier et al., 2003). The mechanism underlying the overgrowth appears to involve expanded BMP and FGF expression (Brinkmeier et al., 2007). Pitx2 is expressed in many tissues where WNT signaling is active, and in the presence of LiCl, which artificially activates downstream WNT signaling, an increase in Pitx2 expression is detected in the developing Rathke’s pouch at E10.5, as well as in cultured pituitary cells at this time point (Kioussi et al., 2002). Pitx2 activity is also increased in the presence of a constitutively active form of β-catenin expressed in the gonadotrope-like αT3-1 pituitary cell line. Wnt11 has been implicated as a target of Pitx2 and β-catenin in cardiac development (Zhou et al., 2007), but the WNT(s) responsible for the β-catenin–mediated activation of Pitx2 in the pituitary have yet to be identified. Moreover, the presence of activated β-catenin has not been demonstrated in the developing anterior pituitary. In addition, nuclear accumulation of β-catenin and subsequent activation of TCF/LEF transcription factors can occur after gonadotropin-releasing hormone (GnRH) stimulation in mouse pituitary gonadotrope-like cells (Gardner et al., 2007). Because GnRH receptor, like other G-protein coupled receptors can activate the canonical WNT signaling pathway, β-catenin activation of Pitx2 or other critical transcription factors could be independent of a WNT signal.

Direct evidence for WNT signaling in pituitary development stems from pituitary abnormalities arising from disruption of Wnt5a and Wnt4 (Treier et al., 1998; Cha et al., 2004). Wnt5a mRNA expression has been detected in the ventral diencephalon adjacent to the pituitary and in the pituitary primordium beginning at E9.5 (Treier et al., 1998). Wnt5a mutant embryos exhibit abnormal branching and looping of the developing pituitary, although all hormone-producing cell types are generated (Cha et al., 2004). Wnt4 is expressed from E9.5 onward in Rathke’s pouch and in the oral ectoderm. Expression becomes restricted to the dorsal aspect of the pouch by E14.5. Mice deficient in Wnt4 reportedly have a reduced population of cells producing growth hormone (GH), thyroid-stimulating hormone (TSH), and the alpha subunit common to luteinizing hormone (LH), follicle stimulating hormone, and TSH (alpha glycoprotein hormone subunit = aGSU or choric gonadotropin alpha = CGA) at E17.5 (Treier et al., 1998). The mechanisms underlying the defects in Wnt5a and Wnt4 mutants have not been elucidated.

Here, we examine the role of WNT signaling in modulating ventral diencephalon gene expression and pituitary gland organogenesis. In the absence of Wnt5a, we show that FGF and BMP expression patterns are perturbed in the ventral diencephalon, supporting the idea that WNT and FGF signaling pathways interact in pituitary development (Wang and Shackleford, 1996; Brinkmeier et al., 2007). We confirmed that mice deficient in Wnt4 alone exhibit reduced pituitary growth, although the effect on cell type specification is less dramatic than previously suggested. Using a classic genetic double mutant analysis, we tested for functional redundancy between Wnt5a and Wnt4 and found evidence that the mutant phenotypes are additive in the pituitary gland. Wnt6 is expressed near the pituitary gland during critical times in development; however, examination of embryos deficient in Wnt6 showed no obvious pituitary malformation. Because the effects of deficiencies of Wnt4, 5a, or 6 are unlikely to account for the consequences of deficiencies in the known, critical, β-catenin–regulated transcription factors in the pituitary gland, we conducted a gene expression survey and identified several WNTs, FZDs, and WNT pathway molecules expressed in the pituitary and/or neighboring tissues in early development (E12.5–E14.5). We cataloged their spatial and temporal expression in the developing and adult pituitary gland, producing several candidate genes for future studies. In conclusion, our data suggest that the WNT signaling pathway regulates pituitary development, in part, through functional intersection with other signaling pathways. The identification of additional WNT pathway components in the pituitary and ventral diencephalon provides additional targets for investigation to more fully understand pituitary development.
RESULTS

Wnt5a Mutant Embryos Exhibit Altered Expression of BMP and FGF in the Ventral Diencephalon

Wnt5a is required for normal pituitary morphology, but the expression pattern of the protein has not been reported and the mechanism of action has been elusive (Cha et al., 2004). WNT5A protein is detectable as early as E10.5 in Rathke’s pouch in the cells lining the presumptive lumen (Fig. 1A). WNT5A is also present in the ventral diencephalon, particularly in the basal cells lining the lumen, with little or no expression in the cells on the apical aspect. At E11.5, WNT5A is expressed in both the rostral domain and caudal domain of the ventral diencephalon, and in Rathke’s pouch (Fig. 1B). The rostral domain is demarcated by the normal expression of

Fig. 1. Loss of Wnt5a may alter pituitary gland patterning in early development. A–F: Immunohistochemical staining for WNT5A protein was performed on wild-type paraffin sections from embryonic day (E) 10.5 to E16.5 and adult pituitary glands. Expression is detected throughout the caudal and rostral domains of the ventral diencephalon from E10.5 to E12.5. WNT5A immunostaining is also present in Rathke’s pouch at E10.5 and E11.5, but the signal is no longer evident in the pouch by E12.5. At E14.5, WNT5A is expressed in the developing posterior lobe, as well as in the ventral anterior lobe, extending into the rostral tip. WNT5A expression is waning by E16.5, but is evident again in the adult pituitary in the anterior and intermediate lobes (A,I), but not in the mature posterior lobe (P). Insets are slides at each time point without primary antibody. G–V: Expression of signaling molecules and transcription factors was performed at E10.5 on Wnt5a wild-type and mutant embryos. G,H: PITX2 antibody staining is detected throughout Rathke’s pouch at E10.5, and in extra oral ectoderm invaginations (G,H, arrows). I–L: TCF4 and β-CATENIN are expressed normally in the ventral diencephalon at this time. M–P: Hexx1 mRNA expression in the oral ectoderm is unaffected (M,N), as is ISL1 protein (O,P). Q,R: LHX3 expression is truncated on the caudal side compared with wild-type (brackets). R: Extra invaginations of oral ectoderm do not express LHX3 (arrow). S,T: Phosphorylated SMAD1 (pSMAD1) is detected by immunohistochemistry. Brackets are used to mark the boundary of pSMAD1 expression from the infundibulum to where expression extends into the ventral diencephalon. U,V: Fgf10 is detected in the developing ventral diencephalon in E10.5 embryos by in situ hybridization, with brackets used to demarcate its domain of expression.
Bmp4 and Fgf10, and the caudal domain includes diencephalon tissue that strongly expresses TCF4 (Brinkmeier et al., 2007; Davis and Camper, 2007). This pattern of protein immunoreactivity is consistent with the reported expression pattern of Wnt5a mRNA (Treier et al., 1998). Protein expression persists in the infundibulum and ventral diencephalon through E14.5, and it is detectable in the ventral region of the anterior lobe at E14.5 (Fig. 1D). The major expression domain of WNT5A in the diencephalon corresponds with the major expression domain of TCF4 (Brinkmeier et al., 2007). Additionally, some protein expression is detected in the anterior and intermediate lobes of the adult pituitary, but no expression is detected in the posterior lobe of the adult (Fig. 1F).

Wnt4 has a mild effect on pituitary cell specification. Immunostaining for the pituitary hormones reveals that each of the major cell types have begun to differentiate properly by embryonic day (E) 16.5 (n = 2) and E18.5 (n = 3). Sagittal sections of embryos were stained with antibodies that recognize the pituitary hormones. A,B: Pro-opiomelanocortin (POMC) and its cleavage product adrenocorticotropic hormone (ACTH) are unchanged in the mutant at E16.5. C,D: Steroidogenic factor 1 (SF1), marking pregonadotropes, is also unchanged at E16.5. E–J: The alpha subunit shared by thyrotropes and gonadotropes, αGSU, appears unchanged both at eE16.5 (E,F) and E18.5 (G,H), as does FOXL2 at E18.5 (I,J). K,L: Growth hormone (GH) is present at E16.5 in the wild-type and mutant. Arrowheads indicate positive growth hormone cells. M, N: Thyroid-stimulating hormone (TSH) is also present by E16.5. Insets show enlarged picture of anterior lobe region. O–R: PIT1 staining identifies preosomatotropes, prelactotropes, and prethyrotropes in the wild-type and mutant at E16.5 (O,P) and E18.5 (Q,R). A slight dysmorphology is seen in anterior lobe tissue surrounding the lumen in mutants at E16.5 (B,D,L,P) and E18.5 (H,R). Immunostaining was developed with fluorescein isothiocyanate or diaminobenzidine (DAB), and sections are counterstained with methyl green or hematoxylin.
share a similar dysmorphic pituitary phenotype with the Wnt5a mutants at E10.5 and E11.5. The dysmorphology in Sox3 mutants has been attributed to the expanded domains of BMP and FGF signaling and Hesx1 expression (Rizzoti et al., 2004). Hesx1 expression is unaltered in Wnt5a mutants at E11.5 (Fig. 1M,N) or at E12.5 (Cha et al., 2004), suggesting that Wnt5a is not upstream of Hesx1. Wnt5a mutants exhibit truncated expression of another homeobox gene, LHX3, at the caudal aspect of the pouch, where multiple invaginations of oral ectoderm tissue occur. Expression of another LIM homeodomain factor, ISL1 is unaltered in the Wnt5a mutants (Fig. 1O–R).

Signals emanating from the ventral diencephalon form distinct boundaries of expression around Rathke's pouch. Early in development, the transcription factor SIX3 and signaling molecules such as BMP4, FGFS, and FGF10 are expressed in the portion of the ventral diencephalon that evacinates to become the infundibulum, and later the posterior lobe of the pituitary gland (Treier et al., 1998; Cha et al., 2004; Davis and Camper, 2007). This domain of expression in the ventral diencephalon forms a distinct rostral–caudal boundary of expression including Sonic hedgehog (Shh) (Davis and Camper, 2007). Immunohistochemistry with an antibody that detects the phosphorylated form of SMAD1 (pSMAD1) was used as an indicator of active BMP signaling. In normal mice, expression of pSMAD1 is detected in the caudal domain of the ventral diencephalon in the same area as Fgf10 transcripts. In the absence of Wnt5a, pSMAD1 expression extends rostrally, beyond the normal border of the caudal domain (Fig. 1S,T). Fgf10 expression is mutually exclusive with TCF4 expression and demarcates the boundaries of the caudal and rostral domains of the ventral diencephalon in wild-type E10.5 embryos. In Wnt5a mutant embryos, however, Fgf10 expression is no longer limited to the caudal domain. The expression domain is expanded along the ventral diencephalon into the area that would normally constitute the rostral domain (Fig. 1U,V). Thus, Wnt5a deficiency causes expansion of both BMP and FGF signaling domains in the ventral diencephalon.

**Wnt4 Has a Mild Effect on Pituitary Cell Specification**

Previous studies report a dramatic decrease in levels of GH, TSHβ, and αGSU immunoreactivity at E17.5 in Wnt4-deficient animals (Treier et al., 1998). To explore the mechanism that underlies the hormone reduction in these animals, we examined the pituitary phenotype of the Wnt4 mutants. To ensure our analysis would be comparable to the previous study, we used the same mutant mice on the same genetic background (Treier et al., 1998). We confirmed that there is no reduction in pro-opiomelanocortin (POMC) immunoreactivity in the anterior or intermediate lobes of mutant embryos, where it marks differentiated corticotropes and melanotropes, respectively (Fig. 2A,B). Equal SF1 expression at E16.5 in wild-type and mutants suggests that the pregonadotrope population is unaffected by loss of Wnt4 (Fig. 2C,D). In contrast to previous reports, we observed no appreciable reduction in choricic gonadotropin alpha (CGA or αGSU) immunoreactivity at E16.5 or E18.5 (Treier et al., 1998). Furthermore, we observed no reduction in levels of FOXL2 (Fig. 2L,J), a protein that is coexpressed with αGSU in pregonadotropes and prethyrotropes, and activates αGSU transcription in cell culture and transgenic mice (Ellsworth et al., 2006). At E16.5 and E18.5, we observed a slight reduction in GH and TSHβ immunoreactivity in Wnt4 mutant pituitaries (Fig. 2K–N). PIT1-positive cells were reduced in the mutant embryos relative to wild-type at E16.5 and E18.5 (Fig. 2O–R), which could underlie the reduced number of somatotropes and thyrotropes. The consistent reduction in PIT1 expression suggests that the differentiated cell populations are reduced and not simply developmentally delayed.

To confirm these qualitative results, we quantified the immunoreactivity of each hormone at E18.5 (Fig. 3). GH immunoreactivity is significantly reduced in the mutants with a 66% reduction in activity relative to wild-type (P = 0.03). TSH immunoreactivity in the anterior lobe of the Wnt4 mutants is reduced 78%, which is significant (P = 0.04). The difference in αGSU immunoreactivity at E18.5 is not obvious, but quantification of αGSU levels in mutants revealed a mild 27% reduction of immunoreactivity compared with wild-type, with a borderline level of significance (P = 0.0495), suggesting that there could be a subtle reduction. As expected, POMC immunoreactivity in the anterior lobe, which did not appear to be grossly altered in Wnt4 mutants, exhibited no statistically significant change when quantified; the 9% difference between wild-type and mutant yields a P value of 0.86.

**Independent Roles of Wnt4 and Wnt5a in Development**

The canonical and noncanonical Wnt signaling pathways may interact and influence one another in development (Topol et al., 2003; Zhou et al., 2007). Wnt4 is frequently associated with the
noncanonical class of WNT molecules, although some WNTs can activate different signaling pathways depending on context (Mikels and Nusse, 2006). To determine whether loss of Wnt4-mediated signaling affects the canonical pathway in the pituitary and ventral diencephalon, we analyzed expression of the activated form of β-catenin in the rostral domain of the ventral diencephalon and found it was undisturbed. We also found no changes in TCF4 immunoreactivity in the ventral diencephalon at E12.5 or Lef1 mRNA levels in Rathke’s pouch at E16.5 in Wnt4 mutants (data not shown).

Wnt5a mutant pituitaries begin to show signs of abnormal development at E10.5. In these mutants, Rathke’s pouch tissue expands rostrally, with extra invaginations of pouch tissue on the caudal side (Fig. 4A, c). This dysmorphology persists through E18.5 (Fig. 4B, c, bracket), although hormone immunoreactivity remains largely unaffected. The dysmorphic region of Wnt5a mutants contains some somatotropes (Fig. 4B, c; Cha et al., 2004), and a portion of differentiated corticotropes and melanotropes as indicated by the
POMC and prohormone convertase 2 (PC2) staining in the region (Fig. 4B, s, w). This expression of PC2 was used to assess the cell specificity of POMC-positive cells in the dysmorphic intermediate lobe of the Wnt5a mutants. Patches of cells in the Wnt5a mutant are positive for PC2 immunoreactivity, suggesting that only part of the dysmorphic tissue is truly intermediate lobe. POMC staining appears normal in the intermediate lobes of all genotypes examined. These hormone-positive cell types cannot account for all of the cells in the dysmorphic region, however, suggesting some of the cells fail to complete a hormone-producing differentiation program.

**Surviving Wnt4, Wnt5a Double Mutants Reveal no Overlapping Function in Pituitary Development**

To assess the potential genetic interaction of Wnt4 and Wnt5a in the pituitary, we produced F1 double heterozygotes and intercrossed them to generate Wnt4, Wnt5a double mutants. The progeny had a genetically heterogeneous background, which arose from the different mixed backgrounds that constituted the stocks for the Wnt4 and Wnt5a mutants. Only two homozygous double mutant embryos were obtained at E10.5, and neither embryo appeared to be viable. Wnt4, Wnt5a double mutants appear to die by an unknown interaction of the two genes early in development. At E10.5, double mutant embryos appear dead or dying, but their pituitaries resemble the Wnt5a single mutant phenotypically at this age (Fig. 4A, c, d). At E12.5, most Wnt4+/−, Wnt5a−/− embryos are necrotic, with development arrested at or before E10.5 (n = 6/8). One double mutant was obtained from 93 embryos at E18.5. Results of a χ² test show that this distribution of genotypes is not attributable to chance at either developmental stage: at E12.5 P < 0.05 and at E18.5 P < 0.005 (Table 1).
The Wnt4$^{-/-}$, Wnt5a$^{-/-}$ pituitary exhibits an additive phenotype. Somatotropes and thyrotropes appear to be slightly reduced in the double mutant relative to wild-type, mimicking the findings for Wnt4 (Fig. 4B, d, h), while αGSU immunoreactivity does not appear drastically reduced. LH and POMC immunoreactivity appears normal at all genotypes examined. The dysmorphology evident around the lumen of the Wnt5a mutants is also apparent in the double mutant (Fig. 4B, d, h, l, p, t, x). Whereas hormone immunoreactivity remains largely unaffected, both Wnt5a single mutants and the Wnt4, Wnt5a double mutant contain a dysmorphic cleft crossing the lumen between the intermediate and anterior lobes. The intermediate lobe of the double mutant expresses PC2 in the same subset of cells as the single Wnt5a mutant (Fig. 4B, w, x), suggesting the cell specification process in the double mutant is not altered beyond that of the single mutant. The combined phenotypes among the rare viable animals observed at either age do not appear more severe than either single mutant, which suggests separate roles for Wnt4 and Wnt5a in pituitary development, despite the interaction in other developing organs that causes reduced viability.

**Wnt6 Does Not Affect Pituitary Gland Development**

A cDNA library generated from dissected Prop$^{+/-}$ Rathke’s pouch tissues at E14.5 contained Wnt6 cDNA (Carninci et al., 2003). Wnt6 transcripts are detected in tissues surrounding the developing pituitary beginning at E10.5. At this stage, Wnt6 is present in the pharyngeal arch. Expression in the oral ectoderm underlying the pituitary is detectable at E12.5 and to a lesser extent at E14.5 (Fig. 5, arrowheads). No expression is detectable in Rathke’s pouch or its derivatives. Expression is extinguished in the oral ectoderm by E18.5. Expression of POMC, GH, TSHβ, LHβ, and αGSU is unchanged in Wnt6$^{-/-}$ embryos at E18.5. This finding suggests that Wnt6 is dispensable for normal differentiation of pituitary cell lineages and morphogenesis.

### WNT Pathway Expression in the Pituitary Gland

**Because Wnt4, Wnt5a, and Wnt6 were not critical for pituitary differentiation, we performed a polymerase chain reaction (PCR) screen for the presence of other Wnts in the developing pituitary gland.** Introns spanning primers were used to amplify Wnt and Fzd transcripts from Rathke’s pouch RNA collected at E12.5 and E14.5, and from adult pituitaries. The cDNAs generated from the pituitary RNA, as well as from control tissues, were used as templates. Tissues for positive controls were selected based on previous reports of Wnt or Fzd expression in that tissue (www.informatics.jax.org). Adult testis, kidney, and lung, and E12.5 head, E12.5 body, and E14.5 body RNA were used as positive controls for this assay. From this RT-PCR screening, transcripts from several Wnt and Fzd genes were identified in the pituitary with varying temporal expression patterns (Fig. 6). Identities of the RT-PCR products were confirmed by DNA sequencing.

Wnt11 and Wnt16 were expressed at all time points examined. Other Wnts such as Wnt2b, Wnt3, and Wnt10b were expressed only at one time point (E14.5, E12.5, adult, respectively). Wnt5b, Wnt7a, and Wnt7b expression were not detected in the pituitary gland at any of the times examined (data not shown).

Several receptors of the WNT signaling pathway were also detected in the survey. Fzd1 expression is detected in the embryonic cDNA, and Fzd2, Fzd3, and Fzd4 expression is observed at all three times in pituitary development. Fzd6 and Fzd8 are detected in developing E12.5 and E14.5 pituitary cDNA, but not in the adult tissue. Fzd9 is not detected in the pituitary at any of the times examined (data not shown).

Regulators of the WNT pathway are also present in the pituitary. Wnt inhibitory factor-1 (Wif1) is detected throughout pituitary gland development and in the adult. Wise, the Wnt inhibitor in the surface ectoderm, and its highly related counterpart Sclerostin, Sost, were also detected during embryonic stages.

### Table 1. Wnt4, Wnt5a Double Mutants Are Underrepresented

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*Results of a χ² test show that the distribution of these genotypes is likely not due to chance at e12.5 (P < 0.05) and at e18.5 (P < 0.005). Six severely necrotic embryos were also found at this genotype.*
In Situ Hybridization Analysis for Expression of Wnt Pathway Members

In situ hybridization analysis of Wnts and Frizzleds identified in the initial RT-PCR survey reveal temporally and spatially restricted expression of these genes in the pituitary gland and ventral diencephalon (Fig. 7). Wnt11 and Wnt16 are expressed in Rathke’s pouch beginning at E10.5 and continuing through E16.5. Expression can also be detected in the rostral, lower domain of the ventral diencephalon early in development, from E10.5 to E12.5. Wnt11 and Wnt16 transcripts, however, appear to be excluded from the forming infundibulum at all ages examined. Their expression within Rathke’s pouch is concentrated at all ages examined. Their expression is detectable throughout the ventral diencephalon with no spatially restricted expression of Wnt11 and Wnt16 expression patterns. Fzd3 is detected in the lower domain of the ventral diencephalon with no detectable expression in the infundibulum. This is similar to Wnt11 and Wnt16 expression patterns. Fzd6 expression is detectable throughout the ventral diencephalon and Rathke’s pouch through E16.5. Dvl2 and the negative regulator of WNT signaling, Axin2, are expressed in the ventral diencephalon with Wnt11, Wnt16, and Fzd3. Dvl2 and Axin2 expression are also concentrated in the dorsal aspect of Rathke’s pouch.

DISCUSSION

Wnt5a Affects Patterning of the Ventral Diencephalon

Wnt5a mutant animals exhibit pituitary dysmorphology (Cha et al., 2004). Our data implicate expanded FGF and BMP signaling as the underlying mechanism for the dysmorphology. Bmp4 is required for the invagination of Rathke’s pouch (Takuma et al., 1998), but noggin expression is required to attenuate this dorsal BMP activity (Davis and Camper, 2007). The excess BMP activity that we observed in Wnt5a−/− embryos may induce additional oral ectoderm to invaginate, contributing to the dysmorphology. Sox3- and Tcf4-deficient mice exhibit expanded BMP and FGF signaling and abnormalities in Rathke’s pouch (Rizzoti et al., 2004; Brinkmeier et al., 2007). Taken together, these observations support the idea of cross-talk between the signaling pathways.

Elevated levels or temporal expansion of Hex1 transcription can cause inappropriate induction of Rathke’s pouch tissue from the oral ectoderm (Dattani et al., 1998). We examined Hex1 expression in the oral ectoderm of Wnt5a mutants at E11.5, but we noted no change, suggesting that Hex1 does not contribute to the dysmorphology in Wnt5a mutants. Sox3 is expressed early in development in the ventral diencephalon and presumptive hypothalamus in a similar pattern to that of Wnt5a (Solomon et al., 2004), and mutations in both of these genes result in similar expansion of BMP and FGF signals. Wnt5a and Sox3 may function in a similar pathway or parallel pathways because Sox3 expression is unaltered in Wnt5a mutants (data not shown).

FGF signaling induces proliferation and expansion of pituitary cell types in pituitary explants, and overexpression of FGF in transgenic mice causes excess proliferation and pouch dysmorphology (Ericson et al., 1998; Treier et al., 1998). Based on these observations, we suggest that the expansion of the Fgf10 expression domain in Wnt5a mutants may induce additional oral ectoderm tissue to differentiate into Rathke’s pouch. The excess tissue could result in extra folds along the lumen, causing the characteristic dysmorphology.

FGF overexpression interferes with cell specification and causes striking dysmorphology in transgenic mice, but Wnt5a mutants have normal cell specification and mild dysmorphology (Treier et al., 1998; Cha et al., 2004). This discrepancy could be due to the fact that expansion of FGF expression is transient and within the physiological range in Wnt5a mutants, and the overall level of excess FGF produced in transgenic mice is much higher and potentially nonphysiological. Wnt5a can antagonize the canonical WNT signaling pathway (Topol et al., 2003), and it can stabilize β-catenin under certain circumstances (Mikels and Nusse, 2006). Although the spatial and temporal expression patterns of WNT5A and activated β-catenin overlap in tissues adjacent to the pituitary gland, we observe no difference in levels of activated β-catenin immunoreactivity in the Wnt5a mutant (Fig. 1KL). Thus, Wnt5a is not likely to be the activating signal for β-catenin in this context.

Lhx3 has been shown to be important for pituitary cell survival (Zhao et al., 2006; Ellsworth et al., 2008). For proper LHX3 expression, other pituitary factors such as Lhx4, or the combination of Pitx1 and Pitx2 transcripts are required (Raetzman et al., 2002; Charles et al., 2005). Additionally, inhibition of BMP signaling (Noggin) and Notch activation of transcriptional repressors (Hes1) are required for normal expression of LHX3 (Davis and Camper, 2007; Raetzman et al., 2007). We observe the same exclusion of LHX3 expression from the caudal side of Rathke’s pouch in Wnt5a mutants as was observed in Hes1 mutants. This finding implicates the interplay of several different signaling pathways in the activation of critical pituitary transcription factors.

The dysmorphology characteristic of Wnt5a mutants at E18.5 has a striking similarity to that of the groucho-related Aes mutant mice, which exhibit an abnormal connection of pituitary tissue to the intermediate lobe (Brinkmeier et al., 2003), and some phenotypic similarity to the Prop1 mutant (Ward et al., 2005). The dysmorphic tissue of Wnt5a mutants is comprised of some GH-positive cells, and cells destined to become intermediate lobe, although the dysmorphic region contains some incompletely differentiated cells. These undifferentiated cells may result from an abnormal connection of neural tissue to the intermediate lobe, as seen in the Uncx4.1 mutant mice at birth (Asbreuk et al., 2006).

Interaction between WNT5A, FGF, and BMP signaling pathways has been observed in other organs. Fgf10 is up-regulated in the Wnt5a mutant lung, and overexpression of Wnt5a in developing lung causes increased Fgf10 expression, altered spatial pattern of Bmp4 expression, and reduced epithelial branching (Li et al., 2002, 2005). In addition, expression of Fgfb can reduce Wnt5a expression in the developing mouse cerebral cortex (Shimogori et al., 2004). Together, these findings support our hypothesis that Wnt5a regulates Fgf10 in the ventral diencephalon, which has an
Fig. 7. Spatial expression patterns of Wnt pathway members in the developing pituitary and ventral diencephalon. A–E: Formation of Rathke’s pouch is illustrated from embryonic day (E) 10.5 to E16.5. At E10.5, the oral ectoderm begins to invaginate and pinches off around E11.5 to form Rathke’s pouch. Meanwhile, the ventral diencephalon evaginates to form the infundibulum. From E12.5 to E14.5, the anterior lobe begins to form as cells surrounding the lumen rapidly divide and migrate out of the pouch. By E16.5, the three distinct lobes of the developing pituitary are evident, with the posterior lobe arising from the infundibulum. In situ hybridizations were performed on wild-type sagittal sections from E10.5 to E16.5 to determine temporal and spatial patterns of expression. Slides are oriented with dorsal to the top and rostral to the left. Inset panels (v, aa) show sense slides for negative controls. VD, ventral diencephalon; OE, oral ectoderm; OC, oral cavity; INF, infundibulum; RP, Rathke’s pouch; C, cartilage plate of hard palate; P, developing posterior lobe; I, developing intermediate lobe; A, developing anterior lobe.
indirect effect on Rathke’s pouch. Thus, the balance between each signaling pathway is critical for normal pituitary gland organogenesis.

**Wnt4 Causes Reduction in the PIT1 Lineage**

Wnt4 is expressed early in development, from E9.5 in Rathke’s pouch, with limited expression in the dorsal aspect of the pouch through E14.5 (Treier et al., 1998; Olson et al., 2006). Wnt4 is also expressed in the oral ectoderm throughout development. Wnt4 was implicated as a necessary element in anterior pituitary precursor cell expansion and αGSU expression (Treier et al., 1998).

Our analysis of Wnt4 mutants at E16.5 and E18.5 reveals a mild reduction of anterior lobe size, although, in contrast to the previous report, we see no drastic reduction in αGSU cell number at either E16.5 or E18.5, and quantification of αGSU immunostaining results in a barely significant change in expression (P = 0.0495).

The pituitary transcription factor PIT1 is responsible for differentiation and expansion of somatotropes, thyrotropes, and lactotropes (Camper et al., 1990; Li et al., 1990). We have found Wnt4 mice have reduced PIT1, which is likely the cause for the reduction in numbers of somatotropes and thyrotropes. Pit1 deficiency does not alter the size of the pituitary gland until several days after birth, so the delay in Pit1 expression does not account for the pituitary hypoplasia in Wnt4 mutants during gestation (Ward et al., 2006).

**Wnt4 and Wnt5α Function Independently in the Pituitary Gland**

Currently, 19 WNT-related genes have been identified in the mouse (http://www.stanford.edu/~russwe/wntwindow.html). Due to the large number of mammalian WNT family members, WNTs are likely to compensate for one another in development. Wnt4 and Wnt5α may both function in the noncanonical WNT/Ca²⁺ pathway (reviewed in Kuhl et al., 2000). Because Wnt4 and Wnt5α are expressed in complementary regions in and surrounding the pituitary gland, and have overlapping temporal expression patterns, we conducted a classic double mutant analysis to test for interaction between the two genes.

Conditional mutants for Wnt4 and Wnt5α would be ideal for studying genetic interaction in the pituitary gland because double mutants were underrepresented at E10.5 and E18.5, suggesting that Wnt4 and Wnt5α are interacting early in development, resulting in lethality of some embryos before E10.5. Because pituitary hormones are not necessary for fetal growth or survival to term, the lethality must arise from a requirement for either Wnt4 or Wnt5α in other organs. We do not observe a genetic interaction between Wnt4 and Wnt5α in the pituitary glands of the surviving double mutants. Wnt4 mutants do not have an observable morphological phenotype at E10.5, and a double mutant at this age exhibits the characteristic Wnt5α phenotype. At E18.5, an additive phenotype is observed in the double mutant. This finding suggests that the roles of Wnt4 and Wnt5α in the pituitary gland are functionally distinct and not synergistic or overlapping.

**Wnt6 Is Not Required for Pituitary Gland Development**

Using in situ hybridization, expression of Wnt6 was localized to the oral ectoderm, but Wnt6 was not detected in Rathke’s pouch tissue at any time examined throughout development. Expression of Wnt6 was detected by RT-PCR in E12.5 laser-captured Rathke’s pouch cDNA (Olson et al., 2006). This finding may indicate that there are low levels of Wnt6 expression in Rathke’s pouch that precluded detection by in situ hybridization, or the laser capture may have included oral ectoderm outside the pituitary anlage. Despite this discrepancy in expression patterns, it is clear that Wnt6 mutants exhibit no obvious pituitary morphological abnormalities and undergo cell differentiation appropriately. Thus, the expression of Wnt6 is not required for pituitary gland development.

**Multiple Opportunities for Wnt Signaling to Regulate Pituitary Growth and Development**

TCF4 was detected in Rathke’s pouch, and expression was localized by immunohistochemistry to the rostral domain of the ventral diencephalon (Douglas et al., 2001; Brinkmeier et al., 2007). In the absence of Tcf4, the pituitary gland exhibits a profound increase in anterior lobe size, demonstrating an important role for TCP4 in repressing pituitary gland growth (Brinkmeier et al., 2003).

It is not known which WNT, if any, regulates Tcf4 expression. An RT-PCR survey performed on laser-captured E12.5 Rathke’s pouch cDNA also reports expression of Wnts 3, 11, and 16 at E12.5. Additionally, it was reported that Wnts 5b, 7a, and 7b were present at E12.5 (Olson et al., 2006), although these transcripts were not detected in our survey. Our analysis suggests that Wnt11 and Wnt16 are expressed in a pattern that might permit them to activate Tcf4 expression (Fig. 8). Such a role has been suggested for Wnt16 in synovial joint formation (Guo et al., 2004) where activated β-catenin is detected in the rostral domain concurrent with Wnt11 and Wnt16 expression. Furthermore, LEF/TCF reporter expression is activated in the caudal domain of the ventral diencephalon in an overlapping pattern with Fgf10 and Bmp4, suggesting that canonical WNT signaling may modulate FGF and BMP signaling in that region (Maretto et al., 2003).

The spatial and temporal pattern of Wnt11 expression mimics the expression of Wnt16 (Fig. 8). While Wnt16 is thought to activate the canonical Wnt/β-catenin pathway, Wnt11 is often classified with Wnt5α in the noncanonical WNT pathway. It is possible that both canonical and noncanonical signaling pathways are actively participating in patterning of the ventral diencephalon and Rathke’s pouch. Wnt11 is proposed to have overlapping functions with FGF ligands and receptors and members of the BMP family in regulation of ureteric branching (Majumdar et al., 2003). Wnt11 and Wnt5α regulate convergent extension movements in the zebrafish, and their activities are negatively regulated by a gradient of BMP signaling (Myers et al., 2002). This finding suggests that Wnt11 and Wnt5α may similarly pattern the pituitary gland, in part by influencing BMP signaling, but may not be in-
volved in specification of the hormone-producing cell types.

The expression patterns of Axin2 and Dvl2 are also similar to the expression patterns of Wnt11 and Wnt16 (Fig. 8). AXIN2, known to be an inhibitor of canonical WNT signaling, was reportedly expressed in the ventral aspect of Rathke’s pouch from E11.5 to E14.5 (Olson et al., 2006). Our Axin2 probe was generated from a full-length cDNA that was completely sequenced. This probe revealed an expression pattern beginning at E10.5 in Rathke’s pouch, and continuing through E16.5. The specific pattern of expression, including concentrated mRNA signal in the dorsal aspect of the pouch, as well as in the rostral domain of the ventral diencephalon, supports our hypothesis that Wnt signaling is active in the pituitary and adjacent ventral diencephalon. Whereas Olsen et al. (2006) report no change in expression of Axin2 in E14.5 Prop1 null embryos, Axin2 expression has been shown to be downregulated in Prop1 (null) P1 cDNA compared with wild-type by means of gene expression microarray analysis, suggesting more than just a circumstantial connection between Prop1 and WNT signaling (Mortensen and Camper, unpublished observations).

Wnt10b is unique among the WNTs we surveyed in that it is expressed only in the adult pituitary gland. Wnt10b expression is detected in a high fraction (11/14) of human pituitary adenomas (Howg et al., 2002). WNT expression in adult pituitary might affect BMP signaling in the same way that we observed in pituitary development. This finding could be significant because BMP4 promotes cell proliferation in the prolactinomas, the most common type of pituitary adenoma (Puez-Pereda et al., 2003).

In conclusion, we have clearly defined the roles of Wnt5a, Wnt4, and Wnt6 in development of the pituitary gland, and implicate additional WNT family members that may play functional roles. The ability of WNTs to influence BMP and FGF signaling pathways emerges as a common theme in the pituitary gland (Camper, 2004; Rizzoti et al., 2004; Brinkmeier et al., 2007; Davis and Camper, 2007). Disruption of this balance in two WNT mutants results in patterning defects, dysmorphology, and a developmental delay in cell specification. The discovery of numerous other WNT family members expressed in the pituitary gland and ventral diencephalon suggests that there may be multiple roles for WNT genes in pituitary development and a great deal of overlapping function among WNT family members.

EXPERIMENTAL PROCEDURES
Mouse Care and Embryo Preparation
Wnt5a mutant mice on a mixed 129S1/Hsd and C57BL/6 background were obtained from Stephen Jones (Yamaguchi et al., 1999) and maintained at the University of Michigan by heterozygote matings. The 129-Wnt4<sup>tm1Amc</sup>/J mutant mice were obtained from The Jackson Laboratory and maintained at the University of Michigan by heterozygote matings and matings with wild-type C57BL/6J females, also from The Jackson Laboratory. We will refer to these mice as Wnt4<sup>−/−</sup>, Wnt4<sup>+</sup>, Wnt4<sup>++/−</sup> double mutants were obtained by intercrossing double heterozygous animals obtained from a Wnt4<sup>−/−</sup> × Wnt5a<sup>−/−</sup> parental cross. A Wnt6 null allele was generated by Andreas Kispert at the Institute for Molecular Biology at Hannover Medical School, Germany, where E18.5 embryos were generated by heterozygous matings. Exon 3 and exon 4 were deleted from the Wnt6 locus to ensure generation of a null allele. Integrity of the null allele was ascertained by restriction fragment...
length polymorphism analysis. The generation of the Wnt6 allele will be fully reported elsewhere. Mice were housed under the supervision of the Unit for Laboratory Animal Medicine and the University Committee for Usage and Care of Animals, and all procedures were in compliance with the principles outlined in the NIH Guidelines for the Care and Use of Experimental Animals. Genotyping was performed as previously described for Wnt4 (Stark et al., 1994) and Wnt5a (Cha et al., 2004). Noon of the day of the vaginal plug is designated as embryonic day 0.5. Embryos were dissected and fixed 30 min overnight in 3.7% formaldehyde in phosphate buffered saline at 4°C. Embryos were dehydrated to 100% ethanol, embedded in a Citadel 1000 (Thermo Electric, Cheshire, England) paraffin-embedding machine and sectioned sagittally or coronally at 6μm thickness for immunohistochemistry and in situ hybridization.

**Immunohistochemistry, In Situ Hybridizations, and Histology**

Immunohistochemistry for the pituitary hormones was performed on paraffin sections as previously described (Kendall et al., 1994), and visualized with diaminobenzidine (DAB) chromogen. Antibodies used in fluorescent immunohistochemistry were incubated at 4°C overnight. Rabbit anti-phosphorylated SMAD1 (pSMAD1; Cell Signaling Technology, Inc., Danvers, MA) was used at 1:200 dilution overnight, and goat anti-WNT5A (R&D Systems Inc., Minneapolis, MN) at a 1:100 dilution overnight. Mouse anti-Lim3 (LHX3) monoclonal antibody (Developmental Studies Hybridoma Bank, U of Iowa) was used at a 1:200 dilution and mouse anti-TCF4 (Upstate Cell Signaling, Charlestown, MA) was used at a 1:100 dilution overnight. Rabbit anti-LIM3, biotin-conjugated anti-mouse secondary antibody with the TSA Fluorescein System (Perkin-Elmer, Wellesley, MA) according to the manufacturer’s directions.

Quantification of immunostained pituitary sections was performed using the ImagePro Plus 6.2 software program (Media Cybernetics, Inc., Bethesda, MD; Meynen et al., 2007; Mitchell et al., 2007). Three sagittal slides were taken from each of three Wnt4 mutant and three wild-type or heterozygous embryos at E18.5. The anterior lobes of these sections were selected as the area of interest, and immunoreactive sites exclusively in the anterior lobes were identified using the program’s histogram based color selection, which was set to recognize the DAB-stained cells. For GH, TSHβ, αGSU, and adrenocorticotropic hormone, the ImagePro Plus integrated optical density (IOD) was chosen to quantify the amount of DAB staining for each slide, and the sums of optical density for each section were recorded, resulting in a calculation in arbitrary units for the amount of each hormone present. Each section was analyzed three separate times to ensure the software program was reading the samples consistently, and therefore the optical density for each slide was averaged. The averaged IOD values for the three slides for each of the six embryos examined were analyzed for significance using a repeated measures analysis of variance test in StatView 5.0.1 from SAS Institute, Inc. (Cary, NC), and P-values were generated using P < 0.05 as significant.

In situ hybridizations were performed on paraffin sections using digoxigenin-labeled antisense riboprobes (Roche, Indianapolis, IN) as previously described (Douglas et al., 2001). Probes for Wnt11, Wnt16, and Fzd3 were generated from RT-PCR products as described below, cloned into pGEM-T Easy cloning vector (Promega, Madison, WI), and verified by DNA sequencing. Wnt11 was subcloned into pBluescript (Stratagene, La Jolla, CA), and the antisense probe was linearized with Kpn1 and labeled with T3 polymerase. Wnt16 was linearized with NcoI and labeled with SP6 polymerase, and Fzd3 was linearized with Sph1 and labeled with SP6 polymerase. Probes were diluted 1:50 and hybridized at 50°C overnight. Probes for Wnt6, Fzd6, Axin2, and Dvl2 were obtained from embryonic pituitary cDNA libraries (Carninci et al., 2003). Wnt6 was linearized with NsiI and labeled with T3 polymerase and hybridized at 55°C. Fzd6, Axin2, and Dvl2 were linearized with Sall, labeled with T3, and hybridized at 53°C. A plasmid containing Fgf10 was a gift from Brigid Hogan (Duke University Medical Center), and a probe was generated using BamHI to linearize and T3 polymerase to label. A plasmid containing Hes1 was a gift from Paul Q. Thomas (University of Adelaide, Australia). An antisense probe was generated by BamHI linearization and labeling with T3 polymerase, and sense probe generated with EcoRI and T7. For negative controls, sense probes were generated or no probe was added for hybridization. Hematoxylin and eosin staining were performed as previously described (Cha et al., 2004).

**RT-PCR**

RNA was isolated from Rathke’s pouches dissected from wild-type embryos at E12.5 and E14.5, and from wild-type adult pituitaries using the Trizol method per manufacturer directions (Invitrogen, Carlsbad, CA). Five micrograms total RNA was treated for 30 min with DNaseI (Promega) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as suggested by the manufacturer. One microliter of resulting cDNA was used as template in a 25-μl PCR reaction. Intronspanning primers were used to am-
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