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Formation of Rathke's pouch requires dual induction from the diencephalon

Naoyuki Takuma[1,*,‡], Hui Z. Sheng[1,‡], Yasuhide Furuta[3], Jerrold M. Ward[4], Kamal Sharma[5], Brigid L. M. Hogan[3], Samuel L. Pfaff[5], Heiner Westphal[1], Shioko Kimura[2,§,¶] and Kathleen A. Mahon[6,§]

[1]Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development and [2]Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
[3]Howard Hughes Medical Institute, Vanderbilt University Medical Center, Nashville, TN 37232-2175, USA
[4]Veterinary and Tumor Pathology Section, National Cancer Institute, Frederick, MD, 21702-1201, USA
[5]Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA
[6]Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA
*On leave from Department of Obstetrics and Gynecology, Asahikawa Medical College, Nishikagura 4-5, Asahikawa, Hokkaido, 078, Japan
‡These authors contributed equally to the work
§Co-senior authors
¶Author for correspondence (e-mail: shioko@helix.nih.gov)

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SUMMARY

Targeted disruption of the homeobox gene *T/ebp* (*Nkx*2.1, *Ttf*1, *Titf*1) in mice results in ablation of the pituitary. Paradoxically, while *T/ebp* is expressed in the ventral diencephalon during forebrain formation, it is not expressed in Rathke's pouch or in the pituitary gland at any time of embryogenesis. Examination of pituitary development in the *T/ebp* homozygous null mutant embryos revealed that a pouch rudiment is initially formed but is eliminated by programmed cell death before formation of a definitive pouch. In the diencephalon of the mutant, *Bmp*4 expression is maintained, whereas *Fgf*8 expression is not detectable. These data and additional genetic and molecular observations suggest that Rathke's pouch develops in a two-step process that requires at least two sequential inductive signals from the diencephalon. First, BMP4 is required for induction and formation of the pouch rudiment, a role confirmed by analysis of *Bmp*4 homozygous null mutant embryos. Second, FGF8 is necessary for activation of the key regulatory gene *Lhx*3 and subsequent development of the pouch rudiment into a definitive pouch. This study provides firm molecular genetic evidence that morphogenesis of the pituitary primordium is induced in vivo by signals from the adjacent diencephalon.

Key words: Rathke's pouch, Pituitary morphogenesis, Induction, Diencephalon, Homeobox gene, *Bmp*4, *T/ebp*, *Nkx*2.1, *Fgf*8, *Lhx*3, *Isl*1

INTRODUCTION

Rathke's pouch is the primordium of the anterior and intermediate lobes of the pituitary gland. It initially arises from a portion of the midline oral ectoderm that lies in direct contact with the floor of the diencephalon. Subsequently, a portion of the adjacent neuroectoderm (infundibulum) evaginates to form the posterior or neural lobe of the pituitary (Schwind, 1928; Kaufman, 1992). The apposition of Rathke's pouch and the diencephalon is maintained throughout the early stages of pituitary organogenesis, and this close association has long suggested that inductive tissue interactions are involved in the process. Experimental manipulations of amphibian and chick embryos (Etkin, 1967; Ferrand, 1972; Kawamura and Kikuyama, 1995), as well as tissue recombination explants in the rat (Daikoku et al., 1982; Watanabe, 1982a,b) have shown that signals from the diencephalon are essential for the proper differentiation and expansion of certain pituitary cell lineages. However, the molecular nature of extrinsic signals involved in pituitary organogenesis and their influence on the expression of intrinsically expressed factors have only recently been investigated.

A great deal is currently known about intrinsic factors expressed in Rathke's pouch or its derivatives that are known or postulated to regulate early pituitary morphogenesis and organogenesis. In particular, several classes of homeodomain-containing transcription factors play important roles in both lineage specification and cell-type-specific gene expression (reviewed by Treier and Rosenfeld, 1996; Watkins-Chow and Camper, 1998). *Prop*1 and *Pit*1, genes defective in the *Ames* and *Snell's* dwarf mutants, respectively, function sequentially in the determination and expansion of thyrotrope, somatotrope and lactotrope cell lineages (Camper et al., 1990; Li et al., 1990; Sornson et al., 1996). The LIM homeobox gene *Lhx*3 is required for proliferation and specification of almost all pituitary cell lineages and for the progression of Rathke's pouch during organogenesis (Sheng et al., 1996). Analyses of mice carrying null mutations of *Lhx*3 (Sheng et al., 1996) and the closely related LIM-homeobox gene *Lhx*4 (*Gsh*4) (Li et al., 1994) has revealed that early pouch development can be

genetically subdivided into at least two stages (Sheng et al., 1997). The pituitary from the *Lhx*3/*Lhx*4 double mutant ceases to develop at the pouch rudiment (placode) stage, whereas *Lhx*3 null embryonic pituitaries arrest at the definitive pouch stage. *Lhx*3 can synergize with *Pit*1 or *Ptx*1 *(P-OTX)*, another homeodomain-containing transcription factor (Lanctot et al., 1997), in the activation of downstream target genes in vitro (Bach et al., 1995, 1997), further underscoring its importance in pituitary ontogeny.

A number of other homeobox genes have been identified that are expressed very early in pituitary ontogeny, before and during pouch formation, suggesting that they play a role during the formation of the pituitary primordium. Rathke's pouch is initially derived from the anterior-most edge of the anterior neural plate, the anterior neural ridge (ANR) (Couly and LeDouarin, 1988). *Rpx*/*Hesx*1 (Hermesz et al., 1996) and *Six*3 (Oliver et al., 1995) are expressed in the anterior neural plate/anterior neural ridge, oral ectoderm and Rathke's pouch. *Ptx*1 (Lamonerie et al., 1996; Bach et al., 1997; Lanctot et al., 1997) and *Ptx*2 (*Rieg, Otlx*2) (Semina et al., 1996; Muccheilli et al., 1996; Gage et al., 1997) transcripts are found in the oral ectoderm and pouch. A particularly informative marker, the LIM-homeobox gene *Isl*1, is transiently expressed in the oral ectoderm and pouch primordium, but is downregulated at about E11.5 (Ericson et al., 1998). These genes are candidates for the regulation of early aspects of pituitary development.

Recently, insight has been obtained into the molecular nature and source of the extrinsic neural cues that influence expression of these intrinsic factors. Targeted mutagenesis of the homeobox gene *T/ebp* (also known as *Ttf*1, *Nkx*2.1, *Titf*1) provided the first clear and convincing evidence that signals from the diencephalon are crucial for pituitary organogenesis (Kimura et al., 1996). *T/ebp* is expressed during early development in the ventral diencephalon, thyroid and lung, but not in the pituitary (Lazzaro et al., 1991). *T/ebp* null mutant mice die at birth with multiple defects in these organs. Interestingly, the entire pituitary gland of the null mutant is missing at birth, suggesting a defect in signaling from *T/ebp*-expressing neuroectoderm (Kimura et al., 1996).

Further insight into the molecular basis of this signaling has come through organ culture or transgene approaches. It has recently been shown that two signaling molecules expressed in the ventral diencephalon, BMP4 (Jones et al., 1991) and FGF8 (Crossley and Martin, 1995), have differential and integrated effects on Rathke's pouch ectoderm (Ericson et al., 1998; Treier et al., 1998). BMP4 can maintain expression in vitro of one of the earliest marker genes expressed in the pituitary primordium, the LIM-homeobox gene *Isl*1 (Ericson et al., 1998). In contrast, FGF8 extinguishes *Isl*1 expression and activates another LIM-homeobox gene, *Lhx*3, which has been shown to be essential for pituitary organogenesis and cell-type-specific gene expression (Bach et al., 1995; Sheng et al., 1996). Antagonizing BMP4 signaling prevents the formation of the pituitary (Treier et al., 1998).

In order to determine the molecular events associated with pituitary development in vivo, pituitary ontogeny was examined in *T/ebp* mutant embryos. We found that pituitary organogenesis was arrested very early, just after formation of the rudiment of Rathke's pouch. Combined with analyses of other mutations affecting pituitary development, namely *Bmp*4 and *Isl*1 null embryos, and data obtained with *Lhx*3 and *Lhx*3/*Lhx*4 double null embryos, the molecular mechanisms, both intrinsic and extrinsic, for the initial determination and morphogenesis of the Rathke's pouch primordium were delineated. Here we report that inductive signals from the adjacent diencephalon are essential, not just for pouch-specific gene expression, but for both the induction and morphogenesis of the pituitary primordium.

MATERIALS AND METHODS

Mouse embryos

T/ebp (Kimura et al., 1996), *Bmp*4^{tm1} (Winnier et al., 1995) and *Isl*1 (Pfaff et al., 1996) homozygous null mutants have previously been described. Nominal embryonic age was designated as embryonic day 0.5 (E0.5) on noon of the day in which the copulatory plug was detected. On some occasions, somite number was counted to determine the exact embryonic day.

Histological analyses and immunohistochemistry

A solution of 4% paraformaldehyde in PBS (pH7.2) was used to fix embryos for 2-4 hours. Embryos were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H and E) for histological examinations. Apoptotic cells were identified using the ApopTag Plus *In situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD) by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA.

RNA in situ hybridization

5 μm paraffin sections of mouse embryos were processed for in situ hybridization with ^{33}P-UTP-labeled RNA synthesized from linearized riboprobe vectors as described (Robinson et al., 1991). After exposure and development, slides were stained with bis-benzimide and photographed under simultaneous dark-field and UV illumination.

RESULTS

In order to determine if the absence of the pituitary at birth in the *T/ebp* null mice was due to failure in organogenesis or in failure of pituitary cell survival, homozygous mutant embryos were examined histologically during individual stages of pituitary organogenesis. It was found that a rudimentary Rathke's pouch initially formed during embryogenesis at 10.5 days (Fig. 1C), but that it was histologically abnormal. The wall of the pouch normally is composed of densely packed columnar cells (Fig. 1A,B). The *T/ebp*$^{-/-}$ pouch remained single layered (Fig. 1D), failed to differentiate further and was subsequently eliminated. An immunohistochemical apoptosis assay indicated that the aberrant pouch cells were eliminated through programmed cell death (Fig. 1E).

Genetic dissection of pituitary development through the analysis of mice mutant for *Lhx*3 (Sheng et al., 1996) and the closely related LIM-homeobox gene *Lhx*4 (*Gsh*4) (Li et al., 1994) has demonstrated that Rathke's pouch forms in two steps: a slightly invaginated pouch rudiment is formed initially, followed by further cell proliferation and invagination to generate the definitive pouch (Sheng et al., 1997). While the invaginating pouch rudiment expresses *Isl*1, but not *Lhx*3 or *Lhx*4, the nascent definitive pouch contains transcripts of all three LIM-homeobox genes, and *Isl*1 is subsequently downregulated in most of the pouch by E11.5 (Sheng et al., 1997; Ericson et al., 1998). We examined expression of these

and other pituitary marker genes in the *T/ebp* null mutant to ascertain the time point of developmental arrest in pouch formation. In the *T/ebp*$^{-/-}$ pouch, expression of *Isl*1 was maintained (Fig. 2A,B). *Ptx*1 (*P-OTX*), a homeobox gene that is active in all stomodeal derivatives, including Rathke's pouch (Szeto et al., 1996; Lanctot et al., 1997), was also expressed (Fig. 2C,D). Although both *Isl*1 and *Ptx*1 are expressed in the T/ebp$^{-/-}$ pouch at a decreased level, their transcripts are consistently detected (Fig. 2B,D). Transcripts of *Lhx*3 or *Lhx*4, however, were not detected (Fig. 2E-H). Thus, both morphological and marker gene analysis indicated that, in the absence of *T/ebp* function, pouch development arrests after formation of a rudimentary pouch but before formation of a definitive pouch, similar to the arrest seen in *Lhx*3$^{-/-}$;*Lhx*4$^{-/-}$ double mutants (Sheng et al., 1997).

In order to understand how the ventral forebrain influences development of Rathke's pouch, we analyzed diencephalic defects in the *T/ebp* homozygous null mutant. In the wild-type mouse, the first visible sign of Rathke's pouch formation appears at E8.5 when a portion of the oral epithelium thickens and invaginates (Kusakabe et al., 1984). At the same time, BMP4, a potent signaling molecule of the TGFβ family, is expressed in the ventral diencephalon that lies adjacent to Rathke's pouch. This expression continues beyond E10.5 (Jones et al., 1991; Ericson et al., 1998) (see Fig. 3C). In culture, BMP4 maintains the expression of *Isl*1 (Ericson et al., 1998). In the *T/ebp*$^{-/-}$ mutant, as in the wild-type embryo, the expression domain of *Bmp*4 is maintained at E9.5 (Fig. 3D). This serves to explain the fact that *Isl*1 is induced in the *T/ebp*$^{-/-}$ mutant pouch.

By E9.25, expression of another signaling molecule, FGF8, is initiated in the vicinity of Rathke's pouch (Crossley and Martin, 1995; Ericson et al., 1998). At E10.5, the *Fgf*8 expression domain shifts to the forming infundibulum, the portion of the diencephalon abutting the pouch (Fig. 3E). We detected *Fgf*8 expression in three domains in the developing brain of wild-type E10.5 embryos: the midbrain-hindbrain junction, the commissural plate of the forebrain and the ventral diencephalon/infundibulum (Fig. 3E). Transcripts for the receptor of *Fgf*8 (*Fgfr*2) were found in Rathke's pouch adjacent to the domain of *Fgf*8 expression (Fig. 3G). In the wild-type embryo, the ventral diencephalic domain of *Fgf*8 expression is included in the *T/ebp* expression domain (Fig. 3A,B,E). This domain of *Fgf*8 expression is deleted in the *T/ebp* null mutant (Fig. 3F). Lack of *Fgf*8 expression in a region that directly apposes the developing Rathke's pouch can explain the failure to activate *Lhx*3 and *Lhx*4 expression in the pouch, and thus the failure to form a definitive pouch.

Data derived from both the expression pattern (Ericson et al., 1998) and the *T/ebp*$^{-/-}$ mutant suggest that BMP4 is the signal from the diencephalon responsible for the induction of Rathke's pouch. In order to ascertain the possibility, we examined pituitary development in the *Bmp*4^{tm1} homozygous null mutant (Winnier et al., 1995). At E9.0, formation of a rudimentary pouch is already apparent in the wild-type mouse (Fig. 4A). Most homozygous *Bmp*4$^{-/-}$ embryos die before this stage. However, on some genetic backgrounds (129/SvEv × Black Swiss), a proportion survive to E9.5-10.0, and a few of these develop relatively normal anterior structures (Y. F. and B. L. M. H., unpublished data). Histological examination of pouch development in such mutants revealed no sign of a thickened ectodermal pouch placode, let alone the formation of a pouch (Fig. 4B). This result demonstrates that BMP4 signaling from the diencephalon is absolutely necessary for the induction and formation of a pouch rudiment.

In the wild-type mouse, *Isl*1 is specifically expressed in the pouch rudiment upon BMP4 induction, and its expression precedes that of *Lhx*3 and *Lhx*4 (Ericson et al., 1998). We therefore analyzed *Isl*1 function in pouch formation by histological examination of the *Isl*1$^{-/-}$ targeted mutant (Pfaff et al., 1996) (Fig. 4C,D). *Isl*1 homozygous null mutants die at approximately E10, but analysis at E9.5 revealed that the oral ectoderm of the mutant had invaginated to form a rudimentary pouch. However, the *Isl*1$^{-/-}$ pouch remained small and primitive. Its wall was conspicuously thinner and appeared as a flat, undifferentiated epithelium (Fig. 4D), indicating that, in the absence of *Isl*1, differentiation of the pouch epithelium is blocked at an early stage.

DISCUSSION

Both BMP4 and FGF8 have been shown to play critical roles in the development of pituitary-specific cell lineages (Ericson et al., 1998; Treier et al., 1998). However, transcripts of these two factors already present in the diencephalon at earlier stages of embryonic development, at a time when Rathke's pouch forms. The present study examines this phase of pituitary development. Our results allow us to conclude that BMP4 and FGF8 convey consecutive signals from the diencephalon that play critical roles in the induction and formation of the pituitary primordium.

At the time when Rathke's pouch is induced, *Bmp*4 is the only member of the *Bmp* family that is expressed in the presumptive ventral diencephalon directly apposing the oral ectoderm (Ericson et al., 1998; Treier et al., 1998). We find that embryos that lack *Bmp*4 gene function fail to develop an ectodermal pouch placode, indicating that this factor is essential for the induction of Rathke's pouch.

The role of the *Fgf*8 gene in pituitary induction cannot be examined by a lack-of-function approach since the *Fgf*8$^{-/-}$ mutant dies during gastrulation (Meyers et al., 1998). An *Fgf*8 mutant with a specific deletion of the diencephalic expression domain is not currently available. Fortunately, however, analysis of the *T/ebp*$^{-/-}$ mutant has provided us with critical information regarding *Fgf*8 function during the formation of Rathke's pouch. Expression of *T/ebp* and *Fgf*8 in the diencephalon is spatially related. *T/ebp* is expressed in a broad area of the ventral diencephalon around E8.0 to E8.5 (Shimamura et al., 1995). *Fgf*8, and perhaps other members of the family as well, is expressed at E9.25 in the posterior portion of the diencephalon (Crossley and Martin, 1995; Ericson et al., 1998). The *Fgf*8 expression domain is included in the *T/ebp*-positive region. In the *T/ebp*$^{-/-}$ mutant, the *Fgf*8 expression domain is deleted. Since null mutation of *T/ebp* causes an ablation of the posterior ventral diencephalon, it prevents compensation of the *Fgf*8 function, if any, by other members of the *Fgf* family expressed in the same area. Defects in the diencephalon development cause corresponding changes in the nascent pouch. The oral ectoderm in the *T/ebp*$^{-/-}$ mutant involutes to form a rudimentary pouch. This pouch, however, does not develop further. Rather, it is subsequently eliminated through apoptosis. This result indicates that *Fgf*8 constitutes a

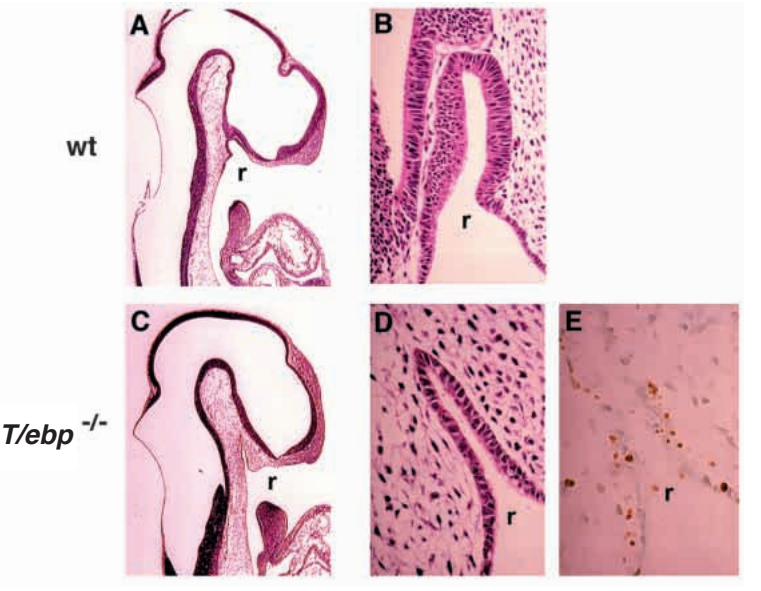

Fig. 1. Formation of Rathke's pouch in the wild-type embryo (A,B) and in the *T/ebp*$^{-/-}$ mouse (C-E). At E10.5, a definitive Rathke's pouch is developing in the wild-type embryo (A,B). Only a thin, rudimentary pouch is formed in the *T/ebp* null mutant (C,D), that contains many apoptotic cells (brown spots in E). The wild-type pouch contains few apoptotic cells (not shown).

signal derived from the diencephalon that is essential for the formation of a definitive pouch.

As shown previously, morphogenesis of Rathke's pouch occurs in steps. The pouch is induced around E8.5 when a portion of the oral ectoderm invaginates to form a pouch rudiment. This rudimentary pouch grows subsequently to a definitive pouch, a step that requires the function of *Lhx*3 or *Lhx*4 (Sheng et al., 1997). The analysis of the *T/ebp*$^{-/-}$ mutant shows that formation of a pouch rudiment does not by default lead to a definitive pouch. Activation of *Lhx*3/4, which is critical for formation of the definitive pouch, can not be achieved through upstream transcription regulators intrinsic to the pouch rudiment. Rather, a second signal derived from the diencephalon, FGF8, is essential for activation of *Lhx*3 and *Lhx*4 in the pouch. In the absence of this factor, *Lhx*3 and *Lhx*4 are not activated, and Rathke's pouch does not develop beyond the rudimentary stage. Loss of FGF activity in the diencephalon or of *Lhx*3 in the pouch rudiment both abort further pituitary development, implying that FGF8 and *Lhx*3 are on the same regulatory pathway.

Our finding suggests the following dual induction model. Rathke's pouch develops in a stepwise process requiring at least two sequential inductive signals from the diencephalon. BMP4 is needed for the induction and formation of the pouch rudiment, whereas FGF8 subsequently controls activation of *Lhx*3 and *Lhx*4, a prerequisite for development of the pouch rudiment into a definitive pouch.

In an independent study, Treier et al. (1998) have also examined the role of BMP molecules in pituitary formation, albeit using a transgenic approach. These authors linked regulatory sequences of *Ptx*1 (*P-OTX*), a transcription factor expressed in the oral ectoderm at very early stage, to the coding sequences of *noggin*, a factor known to antagonize BMP function (Zimmerman et al., 1996). In mouse mutants expressing the *Ptx-noggin* transgene, pituitary development is arrested after formation of a definitive pouch, and most of the pituitary-specific cell lineages are missing, with the exception of a few corticotropes (Treier et al., 1998). These results raise two questions concerning the function of BMPs in pituitary

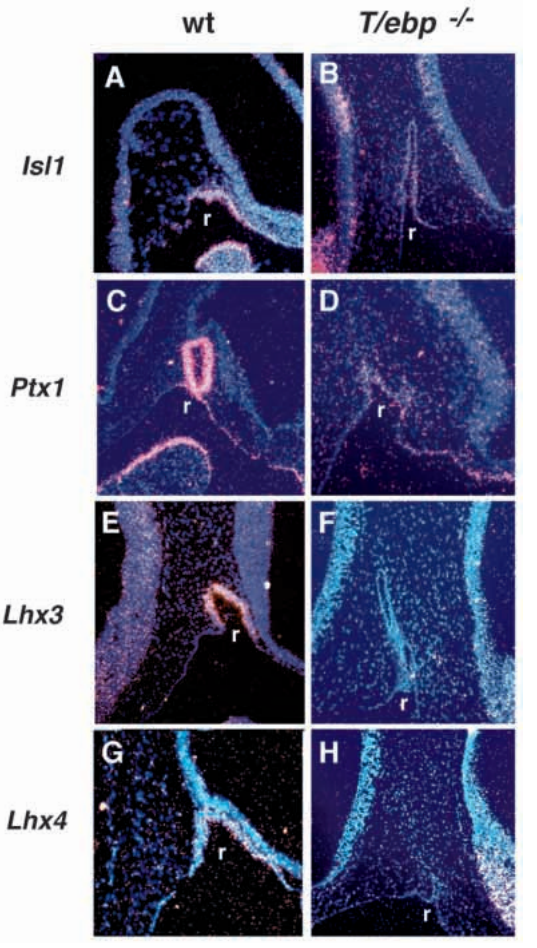

Fig. 2. The wild-type pouch expresses *Isl*1(A), *Ptx*1(C), *Lhx*3 (E) and *Lhx*4 (G), whereas the *T/ebp*$^{-/-}$ pouch only expresses early pouch markers *Isl*1 (B) and *Ptx*1 (D), but not definitive pouch markers *Lhx*3 (F) and *Lhx*4 (H). r, Rathke's pouch.

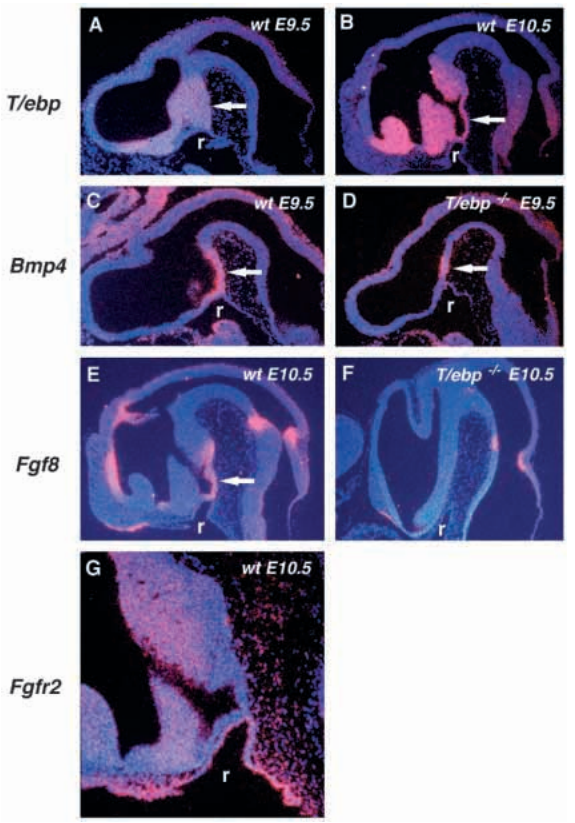

Fig. 3. Development of the diencephalon in the *T/ebp* null mutant. Expression domains of *T/ebp* (A,B), *Bmp*4 (C,D), *Fgf*8 (E,F) and *Fgfr*2 (G) in sagittal sections of E9.5 and E10.5 wild-type (A,B,C,E,G) and *T/ebp*$^{-/-}$ mutant (D,F) mouse brains as shown by in situ hybridization. The diencephalic expression domains of *T/ebp*, *Bmp*4 and *Fgf*8 are immediately above the nascent Rathke's pouch. A high level of *Fgfr*2 expression is detected in Rathke's pouch (G) suggesting that the pouch is competent to respond to FGF8 signaling. The *Bmp*4 expression in the diencephalon remains in the *T/ebp* null mutant (D), whereas the *Fgf*8 expression domain is eliminated, although the domain in the hindbrain is maintained (F). r, Rathke's pouch. Arrows indicate *T/ebp*, *Bmp4* or *Fgf8* expression domains in the diencephalon.

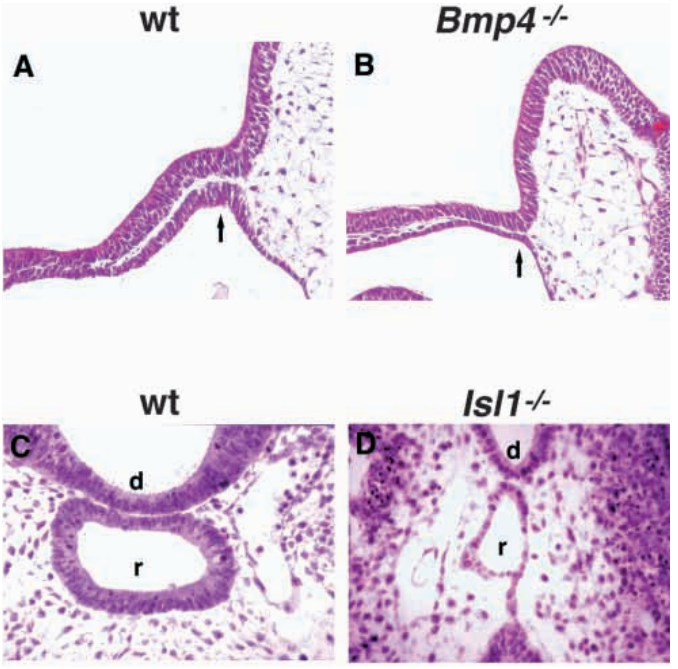

Fig. 4. (A,B) Histological examination of Rathke's pouch formation in wild-type and *Bmp*4$^{-/-}$ mutant embryos. An invaginating pouch is seen in 20 somite (E9.0) wild-type embryo (A). There is no sign of Rathke's pouch formation (either ectodermal thickening or invagination) in E9.5-9.75 *Bmp*4$^{-/-}$ mutant embryos (B). Arrows point to the oral ectoderm in mid-sagittal sections. (C,D) Comparison of Rathke's pouch formation in a stage matched wild-type (C) and an E9.5 *Isl*1$^{-/-}$ mutant (D) embryo. Transverse sections demonstrate that Rathke's pouch is formed in the *Isl*1$^{-/-}$ mutant. However, the pouch cells are aberrant and fail to differentiate (D). r, Rathke's pouch; d, diencephalon. All sections are stained by Hematoxylin and Eosin.

organogenesis. First, the experiment shows that a pouch rudiment is formed, indicating that induction by BMP4 has occurred in an animal in which *noggin* is targeted to block BMP function. By contrast, our loss-of-function experiment clearly shows that BMP4 is required for the induction. It is possible that at least some BMP4 function has escaped *noggin* blockage in the *Ptx-noggin* transgenic mouse. Second, the *Ptx-noggin* transgenic mouse displays a pituitary phenotype almost identical to that of the *Lhx*3$^{-/-}$ mutant (Sheng et al., 1996; Treier et al., 1998). This result suggests that *Lhx*3 does not function in the transgenic mouse, implying that BMP affects the function of *Lhx*3. Our results have implied FGF8 function in the induction of *Lhx*3 activity, and Ericson et al. (1998) have shown that FGF8 alone is sufficient for re-initiation of *Lhx*3 expression in the E11.5 pouch after prolonged culture. Experiments remain to be designed to test whether activation of *Lhx*3 in E9.5 Rathke's pouch requires both FGF8 and BMP4.

A rudimentary pouch is initially formed and subsequently developmentally arrested in both the *T/ebp*$^{-/-}$ and *Isl*1$^{-/-}$ mutants. Despite this similarity in pituitary phenotypes, the basis for the developmental arrest is different in each scenario. *Isl*1 encodes a transcription factor that is intrinsically expressed in the pouch rudiment. Null mutation of *Isl*1 could conceivably cause developmental arrest of pouch precursor cells, as is the case for motor neurons (Pfaff et al., 1996). Inactivation of *T/ebp*, in contrast, causes a deletion of the *Fgf*8 expression domain in the diencephalon. This, in turn, leads to developmental arrest of the pouch, presumably through failed induction of *Lhx*3 and *Lhx*4 expression.

This study demonstrates that the ectodermal primordium of Rathke's pouch is induced by signals emanating from the juxtaposed neural tissue. Previous studies on manipulated embryos and explant cultures have focused primarily on the role of the brain in eliciting expression of cell-type-specific genes or pituitary hormones. We provide direct in vivo evidence that Rathke's pouch is induced from an ectodermal placode by neural contact, and have delineated the molecular mechanisms underlying initial inductive events in pituitary morphogenesis.

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