Foxg1 deletion impairs the development of the epithalamus

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Abstract

The epithalamus, which is dorsal to the thalamus, consists of the habenula, pineal gland and third ventricle choroid plexus and plays important roles in the stress response and sleep–wake cycle in vertebrates. During development, the epithalamus arises from the most dorsal part of prosomere 2. However, the mechanism underlying epithalamic development remains largely unknown. Foxg1 is critical for the development of the telencephalon, but its role in diencephalic development has been under-investigated. Patients suffering from FOXG1-related disorders exhibit severe anxiety, sleep disturbance and choroid plexus cysts, indicating that Foxg1 likely plays a role in epithalamic development. In this study, we identified the specific expression of Foxg1 in the developing epithalamus. Using a “self-deletion” approach, we found that the habenula significantly expanded and included an increased number of habenular subtype neurons. The innervations, particularly the habenular commissure, were severely impaired. Meanwhile, the Foxg1 mutants exhibited a reduced pineal gland and more branched choroid plexus. After ablation of Foxg1 no obvious changes in Shh and Fgf signalling were observed, suggesting that Foxg1 regulates the development of the epithalamus without the involvement of Shh and Fgfs. Our findings provide new insights into the regulation of the development of the epithalamus.

Keywords: Epithalamus, Habenula, Pineal gland, Choroid plexus, FOXG1-related disorders, Sleep disturbance, Fgf15

Introduction

The epithalamus, which consists of the habenula, pineal gland, and third ventricle choroid plexus (3rdChp), is involved in many functions, including motor control, the sleep–wake cycle and stress responses [1–3]. The habenula is highly conserved in vertebrates and acts as a critical node connecting the forebrain to the midbrain and hindbrain by receiving inputs from the limbic system and the basal ganglia and projecting to the monoaminergic nuclei [4, 5]. The pineal gland is critical for the regulation of circadian rhythms due to its production of melatonin [6], and the choroid plexus synthesizes cerebrospinal fluid (CSF) and many growth factors, including fibroblasts and insulin-like and platelet-derived growth factors, and plays important roles, such as providing a route for nutrients and removing by-products of metabolism [7, 8]. Dysfunction of the epithalamus has been reported to be related to mood disorders, such as major depression, and schizophrenia and sleeping disorders [2, 9–12]. However, knowledge regarding the developmental process of the epithalamus is limited.

During early development, the progenitor domain in the diencephalon is divided into three prosomeres (p), i.e., p1, p2, and p3, along the anterior-posterior axis [13, 14]. P1 and p3 give rise to the pretectum and prethalamus, respectively. The most dorsal region of p2 produces the epithalamus, and the other part generates the thalamus. In the presumptive epithalamic progenitor domain, the most anterior area containing the roof plate develops into the 3rdChp, while the adjacent part generates the habenular commissure, paired habenulas and pineal gland. Previously, a member of the fibroblast growth factor (Fgf) family, Fgf8, has been reported to regulate the development of the habenula and pineal gland in a dose-dependent manner [15]. In zebrafish, Fgf signalling also controls the specification of the pineal complex [16]. However, the molecular and cellular mechanisms underlying the development of the epithalamus still remain largely unknown.
Foxg1 encodes a winged-helix transcriptional repressor and has been reported to play critical roles during telencephalic development [17–20]. Patients with mutations in FOXG1 have been reported to suffer from mental retardation, poor social interactions and severe anxiety [21]. Notably, severe sleep disturbance, deformation of the third ventricle and choroid plexus cysts have also been reported [22, 23]. Thus, Foxg1 may also be involved in the regulation of epithalamic development. In the present study, we found that a disruption of Foxg1 leads to an impaired epithalamus with an expanded habenula, a smaller pineal gland and an extremely complicated cephalic development [17]. FOXG1 has been reported to play critical roles during telencephalic development [22, 23]. Thus, Foxg1 disruption was achieved by an intercross of Foxg1-cre with Foxg1fl/+. B o t h Foxg1cre-cre and Foxg1fl/+ were considered mutants, and their wild-type littermates and Foxg1-cre;Foxg1fl/+ were considered controls. All animals were maintained on an outbred CD1 genetic background and were housed in the animal facility of the Southeast University. All experimental procedures followed the guidelines approved by Southeast University. To stage the embryos, the mice were mated in the afternoon. The day the vaginal plug was found at noon was considered embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0).

Methods

Animals

Foxg1-Cre (Foxg1 tm1(cre)Skm ) [24] mouse line was purchased from the Jackson laboratory (US, Foxg1-Cre stock: 006084). The Foxg1 β/β line was obtained as previously described [19, 25]. The Fzd10-EGFP transgenic line was generated using standard methods (unpublished data). Foxg1 disruption was achieved by an intercross of Foxg1-cre or crossing Foxg1-cre with Foxg1 β/α. Both Foxg1 cre/cre and Foxg1 cre/ Foxg1 β/α were considered mutants, and their wild-type littermates and Foxg1 β/α were considered controls. All animals were maintained on an outbred CD1 genetic background and were housed in the animal facility of the Southeast University. All experimental procedures followed the guidelines approved by Southeast University. To stage the embryos, the mice were mated in the afternoon. The day the vaginal plug was found at noon was considered embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0).

Tissue processing and Nissl staining

Embryonic brains were directly dissected in cold phosphate buffered saline (PBS) and immediately transferred to 4% paraformaldehyde (PFA, Sigma-Aldrich, 441,244, US) overnight at 4 °C. The brains from P0 were perfused with a mixture of 4% paraformaldehyde (PFA, Sigma-Aldrich, 441,244, US) and 10% sucrose and stored at 4 °C overnight. The brains from P0 were perfused with 4% paraformaldehyde (PFA, Sigma-Aldrich, 441,244, US) and then post-fixed at 4 °C for 12 h. Notably, severe sleep disturbance, deformation of the third ventricle and choroid plexus cysts have also been reported [22, 23]. Thus, Foxg1 may also be involved in the regulation of epithalamic development. In the present study, we found that a disruption of Foxg1 leads to an impaired epithalamus with an expanded habenula, a smaller pineal gland and an extremely complicated cephalic development [17]. FOXG1 has been reported to play critical roles during telencephalic development [22, 23]. Thus, Foxg1 disruption was achieved by an intercross of Foxg1-cre with Foxg1fl/+. Both Foxg1 cre/cre and Foxg1 fl/+ were considered mutants, and their wild-type littermates and Foxg1 fl/+ were considered controls. All animals were maintained on an outbred CD1 genetic background and were housed in the animal facility of the Southeast University. All experimental procedures followed the guidelines approved by Southeast University. To stage the embryos, the mice were mated in the afternoon. The day the vaginal plug was found at noon was considered embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0).

In situ hybridization

E12.5 brains were dissected, immediately transferred to 500 μL of TRI Reagent (Sigma-Aldrich, T9424, US) and processed for total RNA isolation according to the manufacturer’s instructions. After purification using the RNeasyPlus Mini Kit (QIAGEN, 74,106, DE), the RNA concentration was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). In total, 2 μg of purified total RNA was used as the template to synthesize cDNA using the PrimeScript ™ RT Master Mix (Takara, RR036A, CN). The cDNA was then used as the template to amplify DNA fragments by PCR for the probe synthesis. The PCR products were inserted into the pBlueScript vector by T4 ligation polymerase (Takara, 2040A, CN). The probes were synthesized using the Digoxigenin-labelling Mix (Roche, 11,277,073,910, DE) and T3 RNA polymerase (Roche, 11,031,171,001, DE) or T7 RNA polymerase (Roche, 10,881,175,001, DE). In situ hybridization was performed as previously described [26, 27].

Immunofluorescence

Immunofluorescence was performed as previously described [19]. The primary antibodies and dilutions were as follows: anti-Calretinin (Millipore, AB5054, 1:500); anti-Calbindin (Millipore, AB1778, 1:250); anti-Foxg1 (Abcam, ab18259, 1:1000); anti-GFP (Abcam, ab13970, 1:1000); anti-L1 (Millipore, MAB5272, 1:500); anti-Pax6 (BioLegend, 901,301, 1:1000); and anti-Vglut2 (Millipore, MAB5504, 1:500). The secondary antibodies used were Alexa Fluro 488 donkey anti-chicken (Jackson Lab, 703–545-155, 1:500), Alexa Fluor 488 donkey anti-rabbit (Life, A21206, 1:500), Alexa Fluor 546 donkey anti-rabbit (Life, A10040, 1:500), Alexa Fluor 647 donkey anti-rabbit (Life, A31573, 1:500), Alexa Fluor 488 donkey anti-rat (Life, A21208, 1:500), CF 633 donkey anti-rabbit (Sigma-Aldrich, SAB4600133, 1:500) and Alexa Fluor 647 donkey anti-mouse (Invitrogen, A21236, 1:500).

Statistical analysis and cell counting

The measurements for the volumetric analyses were performed using every tenth 8 μm coronal section stained with anti-Calretinin. The regions of the habenula were measured using ImageJ software as previously described [28, 29]. The volumes (V) were calculated as $V = \Sigma A \times i \times d$, according to Cavalieri’s principle, where $A$ represents the sum of the areas in the habenula, $I$ represents the intervals between the sections, and $d$ represents the thickness of the sections. The measurements for analysis of the thickness of habenular commissure were performed using every third 10 μm coronal section by the immunofluorescence of L1. The thickness at the midline area were measured by ImageJ software and calculated by the average. Cells of each distinct cell type in the sub-nuclei of the habenula were counted, and the numbers of CR+, CB+, Tac1+ and Pax6+ cells were counted in every tenth 8 μm coronal section from each side of the habenula and summed to obtain the
total number. We considered every strong Tac1+ staining dot as a single cell under high magnification views. Very weak staining was not taken into account. Both controls and mutants were counted under the same criterion. The area of Brn3a+ cells in the medial habenula was measured by ImageJ software in every third 10 μm coronal section from one side of the habenula and averaged to obtain the mean area per section. All experiments were performed using at least three different litters, and the data were statistically analysed using GraphPad Prism software. Two-tailed Student’s t-test was performed to analyse the statistical significance at \( p < 0.05 \) (*), \( p < 0.01 \) (**), \( p < 0.001 \) (***)

Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR was carried out according to the protocols as previously described [19]. The dorsal part of E12.5 diencephalon at least from three different litters were used. The specific primers for Fgf15 is: 5’-GAGGAAGC CAGAAGGTATGAAG-3’ and 5’-GGCAAGCTAAGA TCCCCATGA-3’.

Results

**Foxg1 is specifically expressed in the developing dorsal diencephalon, and ablation of Foxg1 leads to an impaired epithalamus**

The forkhead box transcription factor Foxg1 has been reported to be critical for telencephalic development [17]. However, the function of Foxg1 in the diencephalon has been under-investigated. Considering the symptoms, including poor sleep patterns, emotional disorders and choroid plexus cysts, observed in patients suffering from FOXG1 syndrome [21–23], we suspect that Foxg1 likely plays an important role in the developing diencephalon. Previously, the forced overexpression of Foxg1 in chicks has been shown to downregulate Otx2 in the alar plate of the diencephalon, indicating that Foxg1 likely plays a role in telencephalic development [30]. In this study, we first analysed the expression of Foxg1 in detail using in situ hybridization. As shown in Fig. 1a, at E12.5, although extremely strong staining was detected in the developing telencephalon, Foxg1 was also found to be weakly expressed in the progenitors in the third ventricular zone (3rdVZ) and their postmitotic derivatives. This expression pattern was confirmed by immunostaining with anti-Foxg1 (Fig. 1b, arrow). Strong staining was particularly detected at the dorsal-most region of the diencephalon, which was presumably the epithalamus area from which the habenula, pineal gland and 3rdChp arise (Fig. 1a, b). As development proceeded, the expression level of Foxg1 gradually increased with stronger expression in the medial habenula (MHb) and weaker expression in the lateral habenula (LHb) at E18.5 (Fig. 1d, e). Previously, a new Foxg1-IRES-Cre line that faithfully recapitulates the endogenous Foxg1 expression also exhibited Cre-mediated recombination in the developing epithalamus [31], which is consistent with our observations. Collectively, Foxg1 is specifically expressed in the developing diencephalon, particularly in the dorsal part of the 3rdVZ, strongly supporting that Foxg1 plays a role in the development of the epithalamus.

Subsequently, we adopted the “self-deletion” approach by crossing Foxg1-Cre with Foxg1cre/cre to obtain compound homozygous Foxg1cre/cre mice [32]. Both Foxg1cre/cre and Foxg1cre/+ were considered Foxg1-phenotypic null mutants and in this study, all results were obtained in comparable levels from serial sections of the habenula. As shown in Fig. 1c and f, the expression of Foxg1 in the dorsal diencephalon were effectively eliminated in the mutants at E12.5 and E18.5. We first analysed the changes of the epithalamus during early development. Previously, Brn3a (also called Pou4f1) has been reported to be strongly expressed in postmitotic neurons in the MHb and weakly expressed in the LHB and critical for the development of the habenula [33]. At the stages of E12.5 and E14.5, in situ staining of Brn3a showed the habenula was slightly enlarged and expanded dorsal-laterally after Foxg1 deletion (Fig. 1g, h; i, h’). This is confirmed by Nissl staining as well (Fig. 1i, i’). At E18.5, the habenula visualized by Nissl staining expanded much more than that of observed in E12.5 and E14.5. The third ventricle was found to be significantly enlarged which could be a result of the changes caused in the lateral ventricle due to the decrease in the size of the cortex as previously reported [34, 35] (Fig. 1j, j’). However, the pineal gland was smaller (Fig. 1k, k’). Interestingly, the 3rdChp were more branched in Foxg1 mutants compared to the controls (Fig. 1k, k’, asterisk). To further confirm these abnormalities, we performed in situ hybridization for Frizzled10 (Fzd10), one of the Wnt receptors, specifically expressed in the MHb progenitors and postmitotic neurons [36]. As shown in Fig. 1l–l’, Fzd10+ region was enlarged. We also generated a Fzd10-EGFP transgenic mouse line in which EGFP faithfully reflected the endogenous Fzd10 expression (unpublished data). As shown in Fig. 1m–m’, the MHb appeared to expand to the lateral side, resulting in an irregular shape, and the habenular commissure was lengthened and became thinner. A smaller pineal gland was also observed, which is consistent with the observations using Nissl staining. Finally, we evaluated the volume of the habenula and found that it was relative increased by approximately 12% in mutants (Fig. 1n). Collectively, the disruption of Foxg1 caused an impaired epithalamus.

**Increased numbers of epithalamic progenitors and habenular subtype neurons**

To further investigate the cause of the enlarged habenula following the Foxg1 deletion, we examined the progenitor
pool at E18.5. In the controls, Pax6 was expressed in the dorsal 3rdVZ, and its expression was particularly intense in the epithalamic progenitors. A portion of the Pax6+ progenitor cells was also dispersed within the dorsal MHb (dMHb) (Fig. 2a, dotted line) and the lateral division of the LHb (LHbL) (Fig. 2a, yellow arrowhead). In the mutants, the epithalamic VZ seemed thicker than that in the controls, and more Pax6+ cells were scattered in the dMHb and LHbL; the total number of Pax6+ cells was significantly increased by approximately 37% (Fig. 2a, a’, i), demonstrating that disruption of Foxg1 results in an increased number of progenitors in the developing epithalamus. Then, we examined the alterations in several subtypes of habenular neurons. Calretinin (CR) and Calbindin (CB) are two members of the EF-hand family of calcium-binding proteins that are required for the differentiation of early generated thalamic neurons [37]. Here, we found that CR+ neurons were mainly populated in the dMHb and LHbL, which are the similar regions in which the Pax6+ progenitors were located; additionally, the CR+ neurons were distributed in the central part of the medial division of the LHb (LHbMC). The total number of CR+ cells in the habenula was remarkably increased by approximately 33%, a 42% increase in the dMHb and a 22% increase in the LHbL were observed (Fig. 2b, b’; j). The CB+ neurons were mainly detected in the boundary between the MHb and the LHb. In the mutants, the number of CB+ neurons was also significantly increased by approximately 78%, and more CB+ neurons were scattered in the LHb (Fig. 2c, c’; k). In situ staining showed Brn3a+ areas in both the mutant MHb and LHb were expanded (Fig. 2d, d’). We measured the Brn3a+ area at the central level of the MHb and found there was approximate 51% increase in mutants (Fig. 2i).
We further analysed the changes in several other neuronal subtypes in the MHB and LHb. The dMHb has been previously shown to contain a group of neurons that release the neuropeptide substance P, and the ventral part of the MHB (vMHB) contains glutamatergic neurons [5, 38]. According to the in situ staining of Tachykinin1 (Tac1), which acts as a precursor of substance P, and the stained areas were expanded with irregular morphologies in the Foxg1 mutants. Substance P-ergic neurons in the dMHb showing Tac1 in situ hybridization and Etv1+ glutamatergic neurons in the vMHB. 

**Impaired habenular innervations after Foxg1 deletion**

Due to the remarkable structural alteration in the mutant epithalamus, we investigated the changes in the innervations. Immunostaining of anti-L1, which is a neural cell adhesion molecule, was performed. As shown in Fig. 3a-a', the stria medullaris (SM), which project forebrain inputs to the habenula [39], were dramatically impaired, which may also be a consequence of the severely impaired telencephalon in the mutants. The habenular commissure, which conveys information between the paired habenulas, was much thinner than that in the controls, although it could cross the midline (Fig. 3b-b'). The control processes were well fasciculated and projected dorsally across the midline. However, the mutant processes were poorly fasciculated and significantly decreased. The decrease was consistent throughout the rostro-caudal axis when observed in serial
sections. We then analysed the thickness of habenular commissure in the midline area and found it was reduced by approximate 28% in Foxg1 mutants (Fig. 3c).

The dramatically decreased habenular commissure was also detected by immunostaining with anti-CR and Vglut2 at E18.5 as shown in Fig. 2b, b'; h, and h'. Despite the increased numbers of neuron subtypes, the severely decreased habenular commissure indicates that neuronal differentiation may be affected by the Foxg1 deletion as well.

Interestingly, the fasciculus retroflexus (FR), through which the habenula projects to the interpeduncular nucleus (IPN) [39, 40], appeared to project correctly, although it was less fasciculated and its projecting angle was slightly changed, which may be due to the irregular morphology of the habenula (Fig. 3d, d'). Thus, Foxg1 is essential for the development of habenu lar innervations, particularly the habenular commissure.

Reduced pineal gland and extremely complicated Chp after Foxg1 deletion

During development, both the pineal gland and the 3rdChp, along with the habenula, arise from the dorsal region of p2, which is the presumptive epithalamic domain. In addition to the habenula, the pineal gland and 3rdChp were also impaired in Foxg1 null mutants. According to the in situ hybridization of Ttr, the 3rdChp and lateral ventricle Chp displayed extremely complicated morphologies compared to those in the controls (Fig. 3e, e') and, to a certain extent, reflected the Chp cyst in FOXG1 patients. Meanwhile, the size of the pineal gland was reduced viewed by immunostaining of Pax6 (Fig. 3f, f'). Previous studies have shown that misregulation of Foxg1 in chick prosencephalon causes the downregulation of Otx2, which is required for the development of the pineal gland and Chp [30, 41, 42]. However, here, we found that in the absence of Foxg1, the
expression level of Otx2 in the pineal gland appears normal (Fig. 3g, g’), indicating that Otx2 is not involved in the Foxg1-regulated development of the pineal gland in mice. We observed strong expression of Follistatin-like 1 (Fstl1), which is a secreted glycoprotein that functions as an antagonist of BMP signalling in the developing pineal gland [43, 44]. As shown in Fig. 3h-i’, at E18.5 in the controls, Fstl1 was densely expressed in a distinct cell population in the pineal gland, pineal stalk, and habenula commissure. However, in the mutants, the Fstl1+ cells were not well organized, and the Fstl1+ pineal stalk was remarkably shortened with a lengthened habenula commissure. In summary, Foxg1 may be essential for the regional identities of the dorsal part of p2.

Early sub-regionalization of the presumptive epithalamic domain was disrupted after Foxg1 deletion
To further examine whether the regionalization of the epithalamic domain was affected by the loss of Foxg1, in situ hybridization was performed during the early developmental stage at E12.5. As shown in Fig. 4a-a”, the high-level expression of the homeodomain gene Dbx1 normally demarcates the habenular progenitor region [45]. In the absence of Foxg1, the Dbx1+ domain was obviously expanded and shifted dorsolaterally (Fig. 4b-b”). The whole-mount in situ hybridization further confirmed the expansion of Dbx1 in Foxg1 mutants (Fig. 4a” and b”). Ngn2, which is a member of the proneural bHLH transcription factor family, has been shown to be widely expressed in the habenular VZ, caudal progenitor domain of the thalamus (pTH-C), and key diencephalic organizer zona limitans intrathalamica (ZLI) which is located at the interval between p2 and p3, but specifically excluded from the rostral progenitor domain of the thalamus (pTH-R) in controls [46] (Fig. 4c-c’). In the mutants, the Ngn2+ habenular VZ was expanded, while ZLI, pTH-C and pTH-R appeared comparable to those in the controls (Fig. 4d-d”). The similar results were obtained by the whole-mount hybridization (Fig. 4c’ and d’”). Next, we examined whether the primordium of the pineal gland was affected by the in situ staining of Fzd10 and found that the mutant pineal recess was

![Fig. 4](image)

*Fig. 4* Foxg1 is required for early epithalamic sub-regionalization. (a b’): In situ hybridization of Dbx1 showing that the habenular progenitor region (bracket) was obviously expanded and shifted dorsolaterally after Foxg1 deletion. (a’ b’): Whole-mount in situ hybridization for Dbx1 at E12.5. The bracket indicates the epithalamus. (c–d’): Expanded Ngn2+ habenular VZ (black bracket) but normal pTH-C (red bracket), pTH-R (yellow bracket) and ZLI (arrow) in the mutants. (c’ d’): Whole-mount in situ hybridization for Ngn2 at E12.5. The bracket indicates the epithalamus. (e f’): Smaller pineal recess (black bracket) shown by in situ staining of Fzd10. The red bracket marked the Fzd10+ stripe between the pineal recess and the habenular ventricle zone. (g–j’): In situ hybridization of Mash1 showing normal pTH-R (arrow) and ZLI (h j’): No obvious changes in the patterning of p1 were revealed by the in situ staining with Pax3, which labels the pretectal VZ (h h’); Lhx1, which labels the mantle zone of the rostral pretectum (j j’); and BHLHB4, which labels the mantle zone of the rostral pretectum (i i’). The black dashed line in h j’ outlined the diencephalon. Epi, epithalamus; Hb, habenula; pTH-C, caudal progenitor domain of the thalamus; pTH-R, rostral progenitor domain of the thalamus; Pr, pineal recess; p1, prosomere 1; cPT, caudal pre-rectum; rPT, rostral pre-rectum; ZLI, zona limitans intrathalamica. Scale bars: 100 μm (scale bar in a”, b”, c” and d”: 2 mm; g and g’: 200 μm)
obviously smaller than that in the control (Fig. 4e-f). Thus, sub-regionalization in the developing epithalamus was severely impaired.

To further investigate whether Foxg1 affects pTH-C, pTH-R and ZLI, in situ hybridization of Mash1 was also performed. As shown in Fig. 4g-g`, Mash1 was strongly expressed in pTH-R and p3 but specifically excluded from the ZLI [46]. There were no differences to be detected between the control and the mutant, consistent with that viewed by in situ hybridization of Ngn2 (Fig. 4c-d`). We further analysed the patterning of p1 by Pax3, which labels the pretectal VZ [47]; LIM-homeodomain transcription factor 1 (Lhx1), which is a commonly used marker of the mantle zone of the caudal prepectum [48]. BHLHB4, which is a member of the basic helix-loop-helix (bHLH) family; and a specific marker for the mantle zone of the rostral prepectum [49]. No obvious alterations were observed (Fig. 4h-j`). Collectively, Foxg1 is required for the sub-regionalization of the presumptive epithalamic domain but has no effects on the other parts of p2 and p1 during early diencephalic development.

Shh and Fgf signalling were not affected during the development of the epithalamus

Multiple signals, including fibroblast growth factor (Fgfs) are expressed in the dorsal midline of the diencephalon [15] and coordinate with Shh, which is secreted from the ZLI and basal plate, to establish regional identity in the developing diencephalon [50, 51]. Fgf8 has been reported to be involved in the patterning of the p2 region. In Fgf8 hypomorphic mice, the pineal gland and habenula are lost or reduced, exhibiting dose-dependent changes [15]. Fgf15, which is another member of the Fgf family, has been shown to act as a downstream target of Shh that regulates the development of the diencephalon [52]. Using in situ hybridization, we explored whether Fgf signalling is involved in the regulation of Foxg1 in the sub-regionalization of the epithalamic domain. As shown in Fig. 5a, at E12.5, Fgf15 was expressed in the future habenula and thalamic VZ with no detectable expression in the developing pineal gland and 3rdChp in the controls; the transcription level of Fgf15 in the mutant presumptive habenula was comparable to that of controls, no significant changes were observed in p2 (Fig. 5a, a`, arrowhead). The same result was obtained using whole-mount hybridization and qRT-PCR (Fig. 5b, b`; c). No detectable changes were observed in Fgf8 either along the AP axis (Fig. 5d-d`; e-e`). Therefore, Fgf15, as well as Fgf8, were not involved in the Foxg1-mediated regulation of the development of the epithalamus. Previously Wnt3a has been reported to be expressed in the dorsal p2 and Wnt8b is expressed in the prethalamus [15].

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**Fig. 5** Shh and Fgf signalling were not affected in the epithalamic development. (a-a'): Transcription level of Fgf15 in the presumptive habenula (bracket) was not obviously affected in the mutants, no changes were observed in the region of pTH-R (arrowhead) in p2. (b-b'): The whole mount in situ from E12.5 embryos also showing a comparable transcription level in the epithalamus (bracket); c: Relative mRNA levels of Fgf15 (n = 4, p = 0.898). (d-e'): No obvious differences in Fgf8 were observed in the Foxg1 mutants. The white dashed line outlined the diencephalon. (f-h'): Staining of Wnt3a in dorsal P2 and Wnt8b in TE and P3 in mutants were comparable to that of controls. Arrows in f and f' indicate the cortical hem, arrowheads in g and h indicate the thalamic eminence. (i-i'): The activity of the Shh signalling pathway appeared normal in the mutants. The white arrow in i and i' indicates the ZLI. CH, cortical hem; Ep, epithalamus; Hb, habenula; IPN, interpeduncular nucleus; TE, thalamic eminence; ZLI, zona limitans intrathalamica. Scale bars: 100 μm (scale bar in b and b`: 1 mm, g-h`: 200 μm)
Foxg1 has been shown to suppress Wnt function in the developing telencephalon [15, 53]. To explore whether Foxg1 regulate the development of epithalamus through Wnt signalling, we then examined Wnt3a and Wnt8b; however, no obvious alteration were detected either (Fig. 5f-h). Shh, which is secreted by the ZLI, is critical for the development of the diencephalon [50, 54–56]. The ventral high-dorsal low gradient of Shh specifies the diencephalic regional identity [57]. Therefore, we examined whether Shh signalling contributes to the epithalamic defects. At E12.5, in the Foxg1 mutants, the expression of Shh in the ZLI was comparable to that in the controls (Fig. 5i, i’). We then examined the activity of the Shh signalling pathway as reflected by Ptch1 [58]. No obvious differences were detected (Fig. 5j, j’). Meanwhile, the level of Gli3, which is a member of the Glioma-associated oncogene (Gli) family that has been reported to inhibit Shh signalling [59], also appeared normal in the epithalamic VZ (Fig. 5k-k”; l-l”). Collectively, Foxg1 may regulate the development of the epithalamus independently of Shh signalling.

Discussion
Foxg1 has been reported to be critical for telencephalic development [17, 19]. However, its role in the development of the diencephalon remains unclear. Individuals with FOXG1 syndrome exhibit a disturbed sleep pattern, Chp cysts and emotional disorders [21–23], suggesting that Foxg1 likely plays a role in epithalamic development. In this study, we demonstrate that Foxg1 is essential for the development of the epithalamus. The disruption of Foxg1 leads to an extremely complicated Chp, a reduced pineal gland and an enlarged habenula. Moreover, we demonstrate that Foxg1 may be required for the regional specification of the epithalamic progenitor domain independently of Shh and Fgf signalling. Our findings shed light on the molecular mechanism underlying the subdivision of the epithalamic domain.

Previously, the function of Foxg1 was under-investigated in the developing diencephalon. In this study, we identified specific expression of Foxg1 in the dorsal part of p2 from which the epithalamus derives and further elucidated its function during epithalamic development. The habenula has been reported to be closely related to emotional disorders and has recently attracted increasing attention [2, 9, 10]. By receiving inputs from the limbic system and basal ganglia and projecting to monoaminergic nuclei, the habenula acts as a node that connects the forebrain to the brainstem [39]. Here, we found that the disruption of Foxg1 results in an enlarged habenula with an increased number of subtype habenular neurons. The loss of Foxg1 also caused differentiation defects in habenular neurons, which led to impaired habenular innervations and ultimately resulted in abnormal information conveyance among the forebrain, brainstem and paired habenula, which may account for the emotional disorders observed in patients with FOXG1 mutations. To the best of our knowledge, this is the first report illustrating that Foxg1 regulates the development of the epithalamus.

During development, the most dorsal domain of p2 gives rise to the epithalamus, which consists of 3rd Chp, pineal gland, habenular commissure and habenula. However, the mechanism by which the sub-regional identities are established is unknown. Multiple signals, including Fgfs have been found to be specifically expressed in the dorsal region of p2 and involved in the development of the epithalamus [15, 60, 61]. Previous studies have shown that the pineal gland and habenula are lost or reduced in Fgf8 hypomorphic mice, which exhibit dosage-dependent changes in the epithalamus [15]. Previously, we have reported a strong expression of Fstl1 in the pineal gland [43]. In this study, we have detected Fstl1 is also expressed in the habenular commissure. The remarkably short pineal stalk with the lengthened habenular commissure observed in the Foxg1 mutants indicate Fstl1 may be required for the development of the pineal gland and the habenular commissure. Further study is needed to elucidate its function. Shh is secreted from the ZLI and basal plate, and by coordinating with signals from the dorsal region, Shh is critical for the regionalization of the diencephalon [45, 50, 51]. Fgf15 has been identified as a downstream target of Shh that suppresses cell proliferation and promotes differentiation in the developing telencephalon [62]. In this study, we did not detect obvious changes in Shh and Fgf signalling in our mutants. The mutant ZLI was comparable to that in the controls, and the activity of the Shh signalling pathway, as shown by Ptch1 and Gli3, appeared normal, suggesting that the regulation of Foxg1 during epithalamic development is independent of Shh and Fgf signalings. Further studies are required to determine the downstream targets of Foxg1 during the development of the epithalamus.

Conclusions
In the present study, we have identified a specific expression of Foxg1 in the developing epithalamus and further found that disruption of Foxg1 resulted in an impaired epithalamus with an expanded habenula, a reduced pineal gland and more branched choroid plexus. No obvious changes in Shh and Fgf signaling were detected in Foxg1 mutants, indicating that Foxg1 may regulates the development of the epithalamus independent of Shh and Fgfs. Our findings provide new insights into the regulation of the development of the epithalamus. Further study is required to elucidate the molecular mechanism.
Abbreviations
3rdChp: Third ventricle choroid plexus; 3rdV: Third ventricle; 3rdVZ: Ventricular zone of the third ventricle; CH: Cortical hem; CPT: Caudal pretectum; dHb: Dorsal medial habenula; FR: Fasciculus retroflexus; Hb: Habenula; Hbc: Habenular commissure; IPH: Interpeduncular nucleus; LHb: Lateral habenula; LHbC: Lateral division of the lateral habenula; LHbMC: Central part of the medial division of the lateral habenula; Mhb: Medial habenula; P: Prosomere; Pg: Pineal gland; Pp: Pineal recess; Ps: Pineal stalk; pTH-C: Caudal part of the habenular complex in monoamine transmission and cognition. Neurosci Lett. 2004;355(1):255–60.

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Availability of data and materials
The generated data are included in this published article. The mouse lines and antiprobes are available from the corresponding author upon request.

Authors' contributions
Designed the study: BL and CZ. Conducted the experiments: BL. Analysed and interpreted the data: BL, CZ, XW and KZ. Prepared the manuscript: BL, CZ. Designed the study: BL and CZ. Analysis and antiprobes are available from the corresponding author upon request. The generated data are included in this published article. The mouse lines and antiprobes are available from the corresponding author upon request.

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The authors declare that they have no competing interests.

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