**Rax** Regulates Hypothalamic Tanyocyte Differentiation and Barrier Function in Mice

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**ABSTRACT**

The wall of the ventral third ventricle is composed of two distinct cell populations: tanyocytes and ependymal cells. Tanyocytes regulate many aspects of hypothalamic physiology, but little is known about the transcriptional network that regulates their development and function. We observed that the retina and anterior neural fold homeobox transcription factor (Rax) is selectively expressed in hypothalamic tanyocytes, and showed a complementary pattern of expression to markers of hypothalamic ependymal cells, such as Rarres2 (retinoic acid receptor responder [tazarotene induced] 2). To determine whether Rax controls tanyocyte differentiation and function, we generated Rax haploinsufficient mice and examined their cellular and molecular phenotype in adulthood. These mice appeared grossly normal, but careful examination revealed a thinning of the third ventricular wall and reduction of both tanyocyte and ependymal markers. These experiments show that Rax is required for hypothalamic tanyocyte and ependymal cell differentiation. Rax haploinsufficiency also resulted in the ectopic presence of ependymal cells in the α2 tanycytic zone, where few ependymal cells are normally found, suggesting that Rax is selectively required for α2 tanyocyte differentiation. These changes in the ventricular wall were associated with reduced diffusion of Evans Blue tracer from the ventricle to the hypothalamic parenchyma, with no apparent repercussion on the gross anatomical or behavioral phenotype of these mice. In conclusion, we have provided evidence that Rax is required for the normal differentiation and patterning of hypothalamic tanyocytes and ependymal cells, as well as for maintenance of the cerebrospinal fluid-hypothalamus barrier. J. Comp. Neurol. 522:876–899, 2014.

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**INDEXING TERMS:** Rax haploinsufficiency; thinning of the third ventricular wall; ectopic ependymal cells; Rarres2 expression; CSF-Brain barrier

The hypothalamus regulates many physiological processes essential for life, including feeding, reproduction, sleep, circadian rhythms, stress response, blood pressure, and core body temperature (Simerly, 1994; Sterenson, 2013). In addition to the functions performed by diverse and distinct hypothalamic neuronal subtypes, a still poorly understood glial cell population called tanyocytes also regulates hypothalamic function. Hypothalamic tanyocytes show elongated radial glial-like morphology, with cell bodies localized in the ventricular layer of the third ventricle and in direct contact with cerebrospinal fluid (CSF). A single long basal process emerges from the tanyocyte cell body. The basal processes of more dorsally located α1 and α2 tanyocytes extend into the hypothalamic parenchyma, where they make contact with endothelial cells, glia, and neurons. In contrast, the basal processes of more ventrally...
located β1 and β2 tanycytes extend to the external layer of the median eminence (ME) or all the way to the pial surface (Millhouse, 1971; Bleier, 1971; Rodríguez et al., 1979). In addition to their morphological differences, these tanycyte subtypes also exhibit distinct gene expression profiles (Rodríguez et al., 2005).

Another cell population present in the wall of the third ventricle is ependymal cells. These cells do not have a long process and are characterized by the presence of multiple cilia projecting to the ventricular lumen where they promote CSF flow and neuroblast migration during development (Doetsch et al., 1997; Sawamoto et al., 2006). Ependymal cells are commingled with tanycytes at the hypothalamus–ventricle interface, and the relative abundance of these two cell types divides the third ventricular wall in the tuberal hypothalamus into three dorsoventral zones: the most ventral tanycytic zone, composed almost exclusively of β1, β2, and α2 tanycytes; the transition zone, which contains α1 tanycytes intercalated with ependymal cells; and the most dorsal ependymal zone, which is composed only of ependymal cells (Mathew, 2008).

Tanycytes have been implicated in regulating multiple processes within the adult hypothalamus, including active transport of growth factors between the CSF and hypothalamic parenchyma (Duenas et al., 1994; Fernandez-Galaz et al., 1996); acting as both a barrier to diffusion and facilitating the diffusion of small molecules between blood, the CSF and hypothalamic parenchyma (Mullier et al., 2010); sensing blood glucose levels (Frayling et al., 2011; Orellana et al., 2012; Langlet et al., 2013a); regulating neuropeptide release (Prevot et al., 1999; Sanchez et al., 2009); and acting as neural progenitor cells (Xu et al., 2005; Li et al., 2012; Lee et al., 2012; Lee and Blackshaw, 2012; Haan et al., 2013; Robins et al., 2013). Tanycytes are able to regulate the activity of hypothalamic neurons, leading to changes in hypothalamic functions such as reproduction (Prevot et al., 1999; Nakao et al., 2008) and energy balance (Coppola et al., 2007; Marsili et al., 2011; Bobborea and Dale, 2013; Langlet et al., 2013a).

In contrast to our rapidly increasing knowledge of tanycyte function, tanycyte development remains poorly understood. Studies in the rat and mouse have shown that tanycytes and ependymal cells of the third ventricle begin to be generated at the end of hypothalamic development apparently from local radial glia (Altman and Bayer, 1978; Rutzel and Schiebler, 1980; Hajas and Basco, 1984). In the rat, ependymogenesis occurs between embryonic day (E)16 and E19 from the caudal to rostral direction along the neural tube, with maturation continuing until the end of the first postnatal week (Altman and Bayer, 1978). Hypothalamic tanycytes start forming at the very end of the rat embryonic development (E19), but some continue to be generated during the first and even the second postnatal week (Altman and Bayer, 1978). Based on cytological, histochemical, and ultrastructural criteria, tanycytes reach their complete morphological and functional maturity during the first month of life (Monroe and Paull, 1974; Altman and Bayer, 1978; Walsh et al., 1978; Rutzel and Schiebler, 1980; Seress, 1980). The stage at which terminal differentiation of tanycytes occurs seems to vary among different subtypes. It has been shown that ventral tanycytes (α2 tanycytes) acquire adult morphology as early as the first postnatal week, and the most dorsal tanycytes (α1 tanycytes) take longer to mature (Walsh et al., 1978).

A genetic fate mapping of Shh-expressing progenitors in the mouse hypothalamus suggested that a subset of tanycytes in the ME arise from Shh-expressing progenitors as early as E8.5 (Alvarez-Bolado et al., 2012). The discrepancy between early birthdating studies and this recent report might arise from the different species and techniques used. Virtually nothing is known about the molecular mechanisms that control tanycyte specification and differentiation, however.

We and others have previously reported that Rax mRNA is prominently and selectively expressed in hypothalamic progenitor cells (Mathers et al., 1997; Shimogori et al., 2010). By E11.5, Rax is expressed along the ventral lateral walls of the hypothalamic primordium and is coexpressed with Shh in a localized ventral region of the midline. From E11.5 to E16.5, Rax is broadly expressed in ventral hypothalamic progenitors (Shimogori et al., 2010; Lu et al., 2013). By E16.5, Rax mRNA is expressed along the entire wall of the hypothalamic third ventricle, and is absent from all other hypothalamic regions (Shimogori et al., 2010). Rax-deficient mice have lethal abnormalities of the anterior neural tube, including complete absence of the hypothalamus, craniofacial defects, and anophthalmia (Voronina et al., 2005). In addition, reduced Rax expression has been associated with suprachiasmatic nucleus (SCN) abnormalities in the eyeless inbred mouse strain ZRDCT (Tucker et al., 2001). A recent mouse study, in which hypothalamic expression of a conditional Rax allele had been disrupted by using Shh:Cre, reported that Rax is required for both proliferation of medio basal hypothalamic progenitors and differentiation of neurons in the ventromedial hypothalamic nucleus (VMH) and the arcuate nucleus (ArcN) (Lu et al., 2013). However, although this study also reported Rax mRNA expression along the ventricular wall of the hypothalamic primordium, the effects of loss of function of Rax on tanycyte differentiation were not characterized.

Based on the prominent expression of Rax mRNA in late-stage hypothalamic progenitor cells and its known
ROLE IN REGULATING HYPOTHALAMIC CELL FATE SPECIFICATION, we hypothesized that *Rax* might also control the development of hypothalamic ciliary cells, and addressed this question by characterizing the cellular and molecular phenotype of adult mice heterozygous for *Rax*.

**MATERIALS AND METHODS**

**Animals**

All mice used in these studies were maintained and euthanized according to protocols approved by the Institutional Animal Care and Use Committee at the Johns Hopkins School of Medicine. Mice were kept in a 12:12-day cycle in a temperature-controlled room at 23°C. Water and food were available ad libitum.

Six-week-old C56BL/6 female and male wild-type mice were purchased from Charles River (Wilmington, MA) and used for the characterization of wild-type *Rax*, *Rax*<sup>+</sup>/−, and *Rax*<sup>+</sup>/+ littermates, which were then used for all the experiments described in the study.

Male P45 *Rax*<sup>+</sup>/+ and *Rax*<sup>+</sup>/− mice were used for extraction and quantification of RNA from hypothalamus. Female P45 *Rax*<sup>+</sup>/+ and *Rax*<sup>+</sup>/− mice were used for intracerebroventricular injections (i.c.v.) of Evans Blue (EB).

*Rax* and *Rax*<sup>+</sup> FISH for quantification was performed in male and female P45 *Rax*<sup>+</sup>/+ and *Rax*<sup>+</sup>/− mice.

**Tissue preparation**

Brain tissue collection for FISH and IHC was performed as follows. Mice were sacrificed by CO<sub>2</sub> asphyxia, and their brains were immediately dissected. Brains were placed ventral side up, immobilized with needles, and submerged in PBS 1X during dissection. An initial incision was made at the midline, followed by two more lateral cuts made ~2 mm away from the midline, making two rectangular pieces of tissue. Each piece of tissue was placed on its side so that two more cuts could be performed, a rostral cut made just anterior to the optic chiasm (nerve of the optic chiasm) and a caudal cut made posterior to the red nucleus. Hypothalami were placed in Eppendorf tubes containing RNAlater (Life Technologies, Invitrogen, Carlsbad, CA) and stored at −80°C for RNA extraction.

**ANTIBODIES**

Primary antibodies used in this study are described in Table 1. Detyrosinated α tubulin was used as a cilia marker and has been previously characterized (Gundersen et al., 1984). It has been shown to stain primary cilia *in vitro* (Gundersen and Bulinski, 1986) and apical...
null allele was genotyped by using the sense primer 5'-GGACGTGCTTCT-3'. The wild-type and floxed alleles were genotyped by crossing the sense primer AGGAGCTCCAGGAGCTCGAAAGAGC-3'.

Genotyping

DNA for mice genotyping was obtained from tail tips collected carefully with new razor blades to avoid cross-contamination. Tails were incubated at 55°C overnight in lysis solution containing 0.1 μg/μl of Proteinase K (Roche). Rax flox and null alleles were genotyped as previously described (Voronina et al., 2005) The Rax null allele was genotyped by using the sense primer 5'-AGGAGCTCCAGGAGCTCGAAAGAGC-3', and the antisense primer 5'-CGAGTATCCCTACTGCCTGGAAATC-3'. The wild-type and floxed alleles were genotyped by using the sense primer AGGAGCTCCAGGAGCTCGAAAGAGC-3' and the antisense primer 5’-GGACGTGCTTCTCCTTGCTCCTTG-3’. Rax polymerase chain reaction (PCR) genotyping protocol was: 94°C for 5 minutes, 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1.3 minutes, and steps 2–4 were repeated 30 times, 72°C for 5 minutes, 4°C hold.

Probe labeling for in situ hybridization

Clones carrying cDNA for Rax BE951347, Gpr50 B1289437, and Rarres2 AW048638 were amplified with T3/T7 primers by using a Taq PCR Master Mix (Sigma, St. Louis, MO). The amplified PCR product was checked for size and quality by using agarose electrophoresis. PCR products that yielded a single band of the correct size were then used for generating a labeled RNA probe. Probe labeling with DIG or fluorescein was conducted under RNAse-free conditions and using RNA DIG and fluorescein labeling kits (Roche) as recommended by the manufacturer. After RNA amplification the labeling reaction was precipitated by overnight incubation with 4 M lithium chloride (LiCl) and 100% ethanol. Pellets were washed with 70% ethanol and resuspended in 100 μl of 10 mM EDTA, pH 8.0 (Sigma). RNA probe size was then checked by using agarose electrophoresis. Labeled probes were stored at −80°C.

Chromogenic in situ hybridization

In situ hybridizations were performed under RNAse-free conditions, using water treated with diethyl pyrocarbonate (DEPC; Sigma) (DEPC water) for all washes and solutions used prior to probe hybridization. Brain sections were allowed to defrost at room temperature and fixed in 4% PFA prepared in 1X PBS pH 7.5 for 10 minutes. Then they were permeabilized in PBT (1X PBS and 0.1% Tween-20) twice for 5 minutes followed by treatment with 1 μg/ml of Proteinase K solution (PK and 1X PBS) at 37°C. Slides were washed again in PBT twice for 5 minutes and postfixed in 4% PFA for 5 minutes. Background reduction was performed by submerging the slides in acetylation solution (0.1 M triethanolamine, 24.5% acetic anhydride, and DEPC water) for 10 minutes at room temperature (RT). RNA probes were added to the hybridization solution (10 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% sodium...
dodecyl sulfate [SDS], 10% dextran sulfate [American Bioanalytical, Natick, MA; 50% solution], 1X Denhardt’s solution, 200 mg/ml yeast tRNA (Gibco, Grand Island, NY), 50% formamide) and incubated overnight at 68°C in a hybridization chamber humidified with 50% formamide/5X saline-sodium citrate (SSC).

After hybridization, slides were submerged in TNE (Tris, pH 7.5, 10 mM, NaCl 500 mM, EDTA 1 mM) for 10 minutes at 37°C, followed by treatment with RNase solution (0.02 μg/ml of RNase A prepared in TNE) for 25 minutes at 37°C. After the RNase treatment, slides were washed again with TNE for 10 minutes at RT followed by stringency washes in SSC at 65°C as follows; 2X SSC once for 20 minutes, 0.2X SSC twice for 20 minutes each. Then slides were washed in B1T twice (2 M Tris, pH 7.5, 3 M NaCl, and 0.1% Tween-20) at RT for 5 minutes, blocked with 20% blocking solution (20% heat-inactivated sheep serum [HISS] diluted in B1T) for 1 hour, and incubated overnight with an antibody solution (anti-DIG AP 1:2,500 diluted in B1T) at 4°C. The next day, slides were washed in B1T three times for 5 minutes and NTMT for 10 minutes (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2, 1% Tween 20). The riboprobe/mRNA duplexes were detected with a solution containing 0.033 μg/μl of NBT (4-nitro blue tetrazolium chloride) (Roche) and 50 mg/ml of BCIP (5-bromo-4-chloro-3-indolyl-3-phosphate; Sigma) prepared in NTMT. After 1–4 hours of incubation, slides were rinsed with 1X PBS, stained with DAPI (4',6-diamidino-2-phenylindole; Roche) solution 1:5,000 prepared in 1X PBS. When the staining was complete, 100 μl of Vectashield hard-set mounting medium with DAPI (Vector, Burlington, CA) for 20 minutes at RT. The optimal time for the tyramide amplification reaction was determined experimentally: Rax for 16–18 hours and Rarres2 for 1 hour. The reaction was stopped by washing the slides with B1T three times for 5 minutes at RT. One-color fISH was followed by immunohistochemistry.

Two-color fluorescence in situ hybridization

Two-color fISH was performed as in the single color method except the overnight hybridization was done by using two riboprobes added simultaneously; one riboprobe was DIG labeled (Rarres2 or Gpr50) and the other riboprobe was fluorescence labeled (Rax). The DIG-labeled riboprobe was developed first (Rarres2, 1 hour or Gpr50, 1 hour) by using Cy3 diluted 1:50 in the amplification buffer provided in the TSA kit. The reaction was stopped with three washes of B1T for 5 minutes at RT and it was followed by quenching the peroxidase activity in 1% H2O2 for 15 minutes. Then the fluorescein-labeled riboprobe was developed (Rax, 16 hours) by using fluorescein diluted 1:50 in the amplification buffer provided in the TSA kit. The second color reaction was stopped with B1T. The two-color in situ hybridization was followed by immunohistochemistry.

Immunohistochemistry

Permeabilization/blocking was performed by using three washes with PBS plus solution for 5 minutes at RT (0.3% Triton X-100 and 5% normal horse serum, 0.1 M 1X PBS), followed by blocking in Superblock (ScyTek, North Hollywood, CA) for 5 minutes. When the anti-tubulin dehydrogenase antibody was used, the blocking was preceded by antigen retrieval with L.A.B. Solution (Liberate Antibody Binding Solution) (Polysciences, Warrington, PA) for 20 minutes at RT. After blocking, slides were incubated overnight at 4°C with primary antibodies prepared in PBS Plus. On the next day, slides were incubated with Alexa secondary antibodies diluted in PBS Plus (1:500) for 2 hours at RT. DAPI staining was performed by submerging the slides in a DAPI 1:5,000 solution prepared in 1X PBS. When the staining was complete, 100 μl of Vectashield hard-set mounting medium with DAPI (Vector, Burlington, CA) was applied to the slides and covered with a coverslip. Vectashield was allowed to dry in the dark for 1 hour, and the slides were stored at 4°C until image acquisition.

Cannulation and intracerebroventricular injections of Evans Blue dye

EB powder dye content 75% (Sigma) was dissolved in sterile 0.9% saline solution to a 1% final concentration. The EB solution was prepared fresh and kept in the dark at RT.
P45 female $Rax^{+/+}$ and $Rax^{+/-}$ mice were anesthetized with an intraperitoneal injection (i.p) of a Dormitor (medetomidine, selective $\alpha_2$-receptor adrenergic agonist; 1 mg/kg; Novartis Animal Health, Greensboro, NC) and ketamine (N-methyl-D-aspartate [NMDA] antagonist; 80 mg/kg) cocktail in a 5:2 ratio (Dormitor:ketamine). The scalp incision area of the anesthetized mice was prepared for surgery by shaving off their hair, followed by swabbing their skin with a povidone–iodine swab stick, an antiseptic germicide, and 70% ethanol. The mice were then placed in a stereotaxic instrument (Kopf, Tujunga, CA) by adjusting the incisor and ear bars and elevating the mice to a skull level position. An incision was made into the skin along the midline of the scalp starting at a point slightly posterior from the eyes to a point approximately 1 mm caudal to lambda reference point. Then a hole was drilled into the skull 0.6 mm caudal to Bregma and 1.2 mm lateral to midline. To let the cannula adhere into place, the surface of the skull was etched with a sterile scalpel, coated with superglue, and etched again with a scalpel when the glue was dry. An extra–thin-walled 24-gauge stainless steel cannula was inserted 2.2 mm below the skull surface, and secured in place by using dental cement. A 30-gauge obturator was inserted 2.2 mm below the skull surface, and secured in place by using dental cement. A 30-gauge obturator was inserted into the cannula to maintain patency. The wound was closed using silk sutures. To prevent hypothermia and death, the mice around the base of the dental cement was closed using Sof-inserted into the cannula to maintain patency. The wound in place by using dental cement. A 30-gauge obturator was secured dry. An extra–thin-walled 24-gauge stainless steel cannula.

The landmarks used for the identification of the different tanycytes along the ventrodorsal axis of the medial hypothalamus were the following: medial zone of the 

| Gene          | Forward primer                                    | Reverse primer                               |
|---------------|---------------------------------------------------|----------------------------------------------|
| $Rax$         | 5'-TGGGCTTTACCACAAGGAAGCC-3'                      | 5'-GGTAGAGGGCCTAGTAGCCTT-3'                  |
| Gpr50         | 5'-AGACAGCAGTGCAACACCG-3'                         | 5'-GCGAAATTTGCGCTCCTGTT-3'                  |
| Hes1          | 5'-CCGCGATAGTGAACACGA-3'                          | 5'-AAGCCCCGGGAGCTATCTTCTT-3'                |
| Hes5          | 5'-GAAAAAACCGACTGGGAAGCC-3'                       | 5'-CGCCGCGGAGGTTTCT-3'                      |
| Fox1          | 5'-GCGCCCCACTGCTATGCA-3'                          | 5'-GGCCAGGAGGTGATCTGTG-3'                   |
| Raves2        | 5'-GGTGTATCCCTAGGCTATG-3'                         | 5'-CCAATCAGCAACACTACACT-3'                  |
| Vimentin      | 5'-CGTGTCATCGGCTAGTACAG-3'                        | 5'-GGGGATAGGAATAGAGGCT-3'                   |
| GFAP          | 5'-CCCTGGGTGCTGTTGATT-3'                          | 5'-GGACGGATACCTCCTCCTC-3'                   |

RNA extraction and qRT-PCR

mRNA was extracted from dissected hypothalami by using RNeasy Mini kits (Qiagen, Chatsworth, CA). RNA was quantified by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was used to create cDNA from 1,000 ng of RNA. Levels of mRNA for specific genes were quantified with reverse transcription quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) by using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA). The qRT-PCR output generated a Ct value that was transformed to $\Delta$Ct by normalizing each sample to 18S gene (sense primer 5'-GACCGATACCTGCTT-3' and antisense primer 5'-GGGATAGGAATAGAGGCT-3'). The $\Delta$Ct value was then calculated relative to the average control $\Delta$Ct value. The $\Delta$Ct values were analyzed for statistical significance using unpaired t-tests with $P < 0.05$ as significant.

Primers were obtained from the validated pools of primers in the PrimerBank website http://pga.mgh.harvard.edu/primerbank/ (Spandidos et al., 2008, 2010) except for Hes5 primers, which were donated by Dr. Nicholas Gaiano, National Institutes of Health, Bethesda, Maryland. Primer sequences used for qRT-PCR are described in Table 2.

### Table 2.

| Gene          | Forward primer                                    | Reverse primer                               |
|---------------|---------------------------------------------------|----------------------------------------------|
| $Rax$         | 5'-TGGGCTTTACCACAAGGAAGCC-3'                      | 5'-GGTAGAGGGCCTAGTAGCCTT-3'                  |
| Gpr50         | 5'-AGACAGCAGTGCAACACCG-3'                         | 5'-GCGAAATTTGCGCTCCTGTT-3'                  |
| Hes1          | 5'-CCGCGATAGTGAACACGA-3'                          | 5'-AAGCCCCGGGAGCTATCTTCTT-3'                |
| Hes5          | 5'-GAAAAAACCGACTGGGAAGCC-3'                       | 5'-CGCCGCGGAGGTTTCT-3'                      |
| Fox1          | 5'-GCGCCCCACTGCTATGCA-3'                          | 5'-GGCCAGGAGGTGATCTGTG-3'                   |
| Raves2        | 5'-GGTGTATCCCTAGGCTATG-3'                         | 5'-CCAATCAGCAACACTACACT-3'                  |
| Vimentin      | 5'-CGTGTCATCGGCTAGTACAG-3'                        | 5'-GGGGATAGGAATAGAGGCT-3'                   |
| GFAP          | 5'-CCCTGGGTGCTGTTGATT-3'                          | 5'-GGACGGATACCTCCTCCTC-3'                   |

### Image acquisition

Low-resolution images of chromogenic in situ hybridization and fISH were obtained in an Axioskop-2 microscope (Carl Zeiss, Thornwood, NY) equipped with AxioVision software (Carl Zeiss).

z-stack images were taken in a Zeiss LSM510 Meta confocal microscope by using a 63× or 100× objective and digital zoom 0.7 or 1, equipped with Zen 2009 software. To ensure that the z-stack images to be used for quantification in the two genotype groups ($Rax^{+/+}$ and $Rax^{+/-}$) were taken in identical conditions, we collected them together during the same confocal session, using the same zoom, pinhole, gain, contrast, and optical slice interval.

The landmarks used for the identification of the different tanycytes along the ventrodorsal axis of the medial hypothalamus were the following: medial zone of
the ME (β2 tanycytes), lateral evagination of the infundibular recess (β1 tanycytes). To differentiate α1 tanycytes from α2 tanycytes we used as a reference the point where the infundibular recess starts opening which we named the deflection point. Then, α2 tanycytes were located immediately ventral to the deflection point and α1 tanycyte were located immediately dorsal to the deflection point. Ependymal cells were located at the roof of the third ventricle.

Identification of Bregma points along the anteroposterior axis was done by comparing our images with the Allen Mouse Brain Atlas (Sunkin et al., 2013) and using the shape of the ME, the lateral region of the pars tuberalis, and the shape of the different hypothalamic nuclei stained with DAPI as landmarks.

Low-resolution images of brain sections containing EB were obtained with an Axioskop-2 Mot (Carl Zeiss) equipped with AxiosVision software and using 5× and 10× objectives. Two sets of pictures were taken in these sections. The first set was taken right after the slides were dried and used to examine the general distribution of EB in the third ventricle. The second set was taken 4 months after the initial set of pictures and it was used to obtain images for pixel quantification. Slides were frozen at −80°C during the period between the two sets of pictures and defrosted at room temperature in the dark for 15 minutes prior to image acquisition.

Image analysis and statistics

Quantification of z-stack images was done by one blind evaluator and using Imaris software version 7.1.1 (Bitplane, South Windsor, CT).\(\text{Rax}^+/+\) and \(\text{Rax}^+/-\) IISH signal was quantified by using the automatic three-dimensional quantification of spots followed by editing for excess or lack of signal detection. Spot quantifications were performed in three mice per genotype group, and we used a two-tailed unpaired \(t\)-test to analyze statistical significance considering \(P < 0.05\) as significant.

Quantification of DAPI volume was done semiautomatically by using the cell tool of the Imaris software and manually adjusting the threshold to correspond to the area of interest. Visualization of volume reconstruction was done with the surface tool of Imaris, and visualization of the two-dimensional images of the z stacks were obtained by grouping the z stack in ImageJ software. Volume quantifications were performed in three mice per genotype group, and we used a two-tailed unpaired \(t\)-test to analyze statistical significance considering \(P < 0.05\) as significant.

Cilia quantification was performed in brain sections from \(\text{Rax}^+/+\) and \(\text{Rax}^+/-\) mice stained with detyrosinated α tubulin (G-TUB), which labels cilia, and Vim, which labels ependymal cells and tanycytes. Confocal z-stack images of the α2 tanycytic zone were analyzed for the presence of multiple cilia as follows: each digital slide of the confocal z-stack was visualized in the slice gridded viewer of the Imaris software. One blind observer manually counted the number of cilia longer than 2 μm present in each square of the grid for each digital slide. Cilia shorter than 2 μm were excluded because these most likely corresponded to primary cilia. When a cillum was clearly part of a cilia cluster, it was counted only once. Cilia quantifications were performed in three \(\text{Rax}^+/+\) mice and four \(\text{Rax}^+/-\) mice, and we used a two-tailed unpaired \(t\)-test to analyze statistical significance considering \(P < 0.05\) as significant.

Quantification of EB signal was performed as follows. All 10× images from the entire medial hypothalamus of each mouse were used for pixel quantification using ImageJ software. In ImageJ, all images had the same background subtraction and brightness adjustment before they were converted into binary images (0 or 255 pixels). The threshold used for all images was the same and was set automatically after choosing the max-entropy thresholding method. Then two different regions of interest (ROIs) were drawn in each image for quantification: ROI(b) was a fixed rectangular box that contained all pixels in the third ventricle and the adjacent hypothalamic parenchyma where EB diffused. ROI(a) was an irregularly shaped ROI unique to each image and contained only the pixels in the parenchyma. When there were no pixels in the parenchyma we assigned a default number of pixels that was the lowest number of pixels in the entire sample (359) to be able to calculate a ratio. The total amount of pixels for each ROI was the “area measurement” obtained from the analyze measure tool of the software. Finally, the distal diffusion of EB was calculated as a ratio \(R\) of the EB in the parenchyma (ROIa) divided by the total amount of EB in the medial hypothalamus (ROIb), where \(R = (\text{ROIa}/\text{ROIb})\). The R value for each image obtained from the \(\text{Rax}^+/+\) mice \((n=3, \text{total images} = 44)\) was compared with the ratio for each image obtained from \(\text{Rax}^+/-\) mice \((n=3, \text{total images} = 41)\) and analyzed for statistical significant difference using an unpaired nonparametric two-tailed Mann–Whitney test with \(P < 0.05\) as significant.

All z-stack images were stored in the Zen 2009 software as LSM images. To generate figures for publication, z-stack images were processed with ImageJ by using the following tools: autocontrast, split color, and group z-projector, which generated flattened images of individual channels. Then the merge channel tool was used to generate merged images. Images of individual channels and merged channels were converted to tiff and processed in Photoshop version 10.0.1 (Adobe Systems, San Jose, CA) to adjust image size. These images were then moved to Illustrator version
13.0.2 (Adobe Systems) to build the figures. When necessary, the figure was converted from an Illustrator file to a tiff file and processed in Photoshop to adjust the input level of each channel in all panels of the figure equally and simultaneously. Sometimes shadows, highlights, and contrast were also modified in order to improve the quality of the figure.

EB images were stored in the AxioVision software and converted to tiff images to prepare figures for publication. These images were processed in Photoshop version 10.0.1 to adjust size and to pseudocolor them in black and white. Figures were built in Illustrator and exported to tiff again to allow modification of input levels in the entire figure equally and simultaneously using Photoshop.

RESULTS

Rax mRNA is expressed in terminally differentiated hypothalamic tanycytes

By using in situ hybridization, we characterized Rax expression in mature hypothalamus and found that in postnatal day (P) 40 mice, Rax mRNA expression was restricted to cells lining the ventral hypothalamic third ventricle, closely matching the distribution of hypothalamic tanycytes (Fig. 1). In the anterior hypothalamus, Rax was expressed in a small region of the ventral midline (Fig. 1B). In the medial hypothalamus, the zone of Rax expression expanded dorsally, reaching the walls of the third ventricle adjacent to the VMH and dorsomedial hypothalamic nucleus (DMH) (Fig.1C). In the
posterior hypothalamus, *Rax* was strongly expressed in the ventral and lateral walls of the third ventricle (Fig 1D). *Rax* expression was absent from the dorsal, purely ependymal portion of the third ventricular wall (Fig. 1). *Rax* expression appears to be specific to tanyocytes of the mediobasal hypothalamus, as expression was not detected in any other circumventricular organ where cells with tanyocyte-like morphology have been identified (PMID:23649873) Langlet et al., 2013b.

To confirm that *Rax* mRNA was selectively expressed in hypothalamic tanyocytes, we performed fISH for *Rax* followed by IHC for the intermediate filament protein vimentin. Vimentin is enriched in tanyocyte cell bodies and basal processes, as well as ependymal cell bodies (Rodriguez et al., 2005). We observed that *Rax* was coexpressed with vimentin in cell bodies lining the wall of the hypothalamic third ventricle. *Rax* mRNA was selectively expressed in tanyocytes, which were readily identifiable by the presence of both a Vim+ cell body and a vimentin-positive basal process that extends into the hypothalamic parenchyma. In contrast, *Rax* was completely absent from the ependymal cells, identified by a Vim+ cell body lacking a basal process (Fig. 2).

As an additional test for *Rax* mRNA enrichment in tanyocytes, we performed double-fluorescence in situ hybridization for *Rax* and the G protein–coupled receptor 50 (*Gpr50*), a known tanyocyte marker (Batailler et al., 2012) in combination with immunohistochemistry for vimentin. We found that *Rax* mRNA expression colocalized with *Gpr50* mRNA in hypothalamic ventricular cells (Fig. 3). We also observed that *Gpr50* mRNA was prominently expressed in neurons of the DMH, as previously reported (Batailler et al., 2012), but that *Rax* mRNA was not detectable in these cells (Fig. 1).

In addition to ventricular tanyocytes, we detected *Rax* expression in the ME of the tuberal hypothalamus (Figs. 1B, 3, 4). By using confocal imaging, we observed that *Rax* mRNA was not only localized in cell bodies of β2 tanyocytes in the ependymal layer of the ME, but was also expressed in cells whose nuclei were localized in deeper layers of the ME (Fig. 4). In addition, some *Rax* mRNA was not associated with any nuclei, but was associated with tanyocyte processes (Fig. 4). Interestingly, *Gpr50* showed a similar pattern of expression in the ME, suggesting that *Rax*, *Gpr50*+ cells may correspond to astrocytic tanyocytes previously identified using Golgi staining, which lack identified molecular markers (Millhouse, 1971).

**Rarres2** mRNA is selectively expressed in ependymal cells

To differentiate tanyocytes from ependymal cells, we used the Allen Brain Atlas (Sunkin et al., 2013) to identify genes expressed exclusively in ependymal cells. By using this approach, we identified *Rarres2* as a candidate ependymal marker. *Rarres2* encodes chemerin, a secreted protein that functions as an adipokine and is involved in immune response and inflammation (Ernst and Sinal, 2010). By using in situ hybridization, we found that *Rarres2* mRNA expression in the hypothalamus was restricted to the dorsal ventricular wall of the anterior, medial, and posterior hypothalamus (Fig. 5A). *Rarres2* expression was absent from the ventral portion of the hypothalamic ventricular zone, where tanyocytes are located (Fig. 5B). Because ependymal cells, unlike tanyocytes, have multiple apical cilia, we used a cilia marker, detyrosinated α tubulin (G-TUB) to label ependymal cells (Mullier et al., 2010). To confirm that *Rarres2* was expressed in ependymal cells, we examined its presence in ciliated cells. We found that *Rarres2* expression matched G-TUB expression in the anterior, medial, and posterior hypothalamus (Fig. 6A). Moreover, *Rarres2* mRNA was located in cell nuclei right beneath the multiple cilia of ependymal cells of the dorsal third ventricle (Fig. 6B).

**Reduced expression of both tanyctic and ependymal markers is seen in *Rax*+/− mice**

Having shown that *Rax* is selectively expressed in tanyocytes and *Rarres2* is selectively expressed in ependymal cells, we next investigated whether loss of function of *Rax* disrupted the development of cells of the hypothalamic ventricular zone. To avoid the early lethality seen in *Rax* null animals (Mathers et al., 1997), we examined *Rax* heterozygous mice (*Rax*+/−). As expected, adult *Rax*+/− mice showed an approximately twofold reduction in *Rax* mRNA expression compared with wild-type controls, as assessed by both quantification of *Rax* fISH signal in α2 tanyocytes (Fig. 7) and qRT-PCR analysis of hypothalamic mRNA (Fig. 8). In addition, mRNA levels of other genes expressed in developing and terminally differentiated tanyocytes such as *Hes1*, *Hes5*, and *Gpr50* (Lee et al., 2012) were significantly reduced in *Rax*+/− hypothalamus as determined by qRT-PCR (Fig. 8). Interestingly, mRNA levels of genes specific to ependymal cells such as the forkhead transcription factor J1 (*Foxj1*) (Yu et al., 2008) were also reduced in *Rax*+/− hypothalamus. In contrast, levels of *Vim* and *Gfap* mRNA did not show any significant difference in expression between *Rax*+/− and *Rax*+/− mice (Fig. 8).

**Rax*+/− mice show reduced cell volume of the third ventricular wall**

Reduced mRNA levels for multiple tanyocyte and ependymal markers in *Rax*+/− hypothalamus suggested that these mice might have fewer cells in the ventricular zone of the third ventricle. Quantification of total cell
Figure 2. Rax is coexpressed with vimentin in the ventral portion of the third ventricle wall. Confocal z-stack reconstruction of Rax fluorescent in situ hybridization (fISH; magenta) and vimentin (Vim) immunohistochemistry (green) counterstained with DAPI in brain sections of adult C57BL/6 wild-type mice (P45). A–T: Rax is expressed along the anteroposterior axis of the ventral hypothalamus and colocalizes with vimentin in the ventral portion of the wall of the third ventricle (white arrowheads). Numbers in left bottom corner correspond to different Bregma points. Bregma −1.555 mm (A–D) represents the anterior hypothalamus, Bregmas −1.655 mm to −1.955 mm (E–P) represent the medial hypothalamus, and Bregma −2.055 mm (Q–T) represents the posterior hypothalamus. 3V, third ventricle. Scale bar = 100 μm in A (applies to A–T).
number in the walls of the third ventricle using confocal images was not possible due to the tight packing of tanycytes and ependymal cells. However, the cell nuclei of the ventricular layer, which are comprised exclusively of tanycytes and/or ependymal cells, can be easily separated from the subventricular zone and hypothalamic parenchyma, and the volume can be measured using digital analysis. To do this, we measured the volume of the wall of the third ventricle by using digital quantification of confocal z-stack reconstruction images of DAPI staining in the ventricular layer. We measured the volume of the cell nuclei present in the ventricular wall of the tuberal hypothalamus located at the lateral evaginations of the infundibular recess (LEIR) containing β1 tanycytes, the region ventral to the deflection point where the infundibular recess starts opening (α2 tanycytes), the region dorsal to the deflection point (α1 tanycytes), and the roof of the ventral third ventricle (ependymal cells). We found that Rax heterozygous mice showed a significant reduction in third ventricle wall volume in the LEIR, the α2 tanycytic

Figure 3. Rax and Gpr50 are expressed in tanycytes. A–Y: Confocal z-stack reconstruction of double fISH for Rax (green), Gpr50 (magenta) mRNA, and vimentin (Vim) immunohistochemistry (white) counterstained with DAPI (blue) in brain sections of adult C57BL/6 wild-type mice (P45). Rax and Gpr50 are expressed by α1 (F–J), α2 (K–O), β1(P–T), and β2 (U–Y) tanycytes (Vim+ cells with process, white arrowheads), and they are absent from ependymal cells (A–E) (Vim− cells without process, yellow arrowheads). Rax and Gpr50 are also expressed in the median eminence (U–W). 3V, third ventricle; ME, median eminence. Scale bar = 20 µm in A (applies to A–Y).
zone, and the ependymal zone. Interestingly, the transition zone where α1 tanycytes are located did not show any significant difference in volume (Fig. 9). β2 tanycytes were not examined, because most sections lost the ME during sectioning or staining.

*Rax*+/− mice show a ventralization of Rarres2 expression and ectopic ependymal cells in the α2 tanycytic zone

After quantifying Rarres2 mRNA in fISH confocal images, we found that *Rax*+/− mice selectively showed significantly higher levels of Rarres2 mRNA in the ventricular wall of the α2 tanycytic zone at Bregma −1.655 mm (Fig. 10). This difference was not statistically significant in the α2 tanycytic zone at other anteroposterior points examined inside the tuberal hypothalamus (Bregma −1.755 mm and −1.855 mm), although a significant increase of Rarres2 mRNA expression in the overall *Rax*+/− α2 tanycytic zone was detected when data from all Bregma points were combined (Fig. 10 I).

Having previously found that Rarres2 is expressed in ependymal cells of the third ventricle, we used immunohistochemical analysis to visualize the presence of

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**Figure 4.** Rax and Gpr50 are expressed within the median eminence. Orthogonal view of Rax (green) and Gpr50 (magenta) double fISH combined with vimentin (Vim) immunohistochemistry (white) counterstained with DAPI (blue) in the median eminence of adult C57BL/6 wild-type mice (P45). 

A: Rax and Gpr50 mRNA is expressed not only in β tanycytes located in the ependymal layer (EL) of the ME (white arrowheads), but also in other layers of the ME in close association with tanycyte processes (white arrows). This localization suggests that Rax and Gpr50 are translated in tanycyte processes. B–K: Orthogonal view of the dashed square area showing a Rax+, Gpr50+ nucleus in close association with Vim staining. These Rax+, Gpr50+ cells might correspond to “astrocytic tanycytes.” SE, subependymal layer; FL, fiber layer; RL, reticular layer; PL, palisade layer; 3V, third ventricle. Scale bar = 20 μm in A (applies to A–K).
Figure 5. Rax and Rarres2 show complementary expression patterns in hypothalamic ependymal cells. mRNA expression of Rax and Rarres2 along the hypothalamic anteroposterior axis (A–F) and ventrodorsal axis (G–Z). A–F: Rarres2 (A–C) and Rax (D–F) chromogenic in situ hybridization in the anterior (A,D), medial (B,E), and posterior (C,F) hypothalamus of adult C57BL/6 wild-type mice (P45). Rarres2 and Rax mRNA is not expressed in the same zones of the third ventricle wall along the anteroposterior axis of the hypothalamus. Rarres2 mRNA is absent at the ventral portion of the wall of the third ventricle where tanycytes are located (white arrowheads). Rax mRNA expression is absent at the dorsal portion of the wall of the third ventricle where ependymal cells are located (black arrowheads). G–Z: Confocal z-stack reconstruction of double FISH for Rarres2 (magenta) and Rax (green) mRNA and vimentin (Vim) immunohistochemistry (white). Panels correspond to the ependymal zone (G–J), transition zone (K–N), dorsal α2 tanycytic zone (O–R), β1 tanycytic zone (S–V), and β2 tanycytic zone (W–Z). Rarres2 and Rax mRNA is not expressed in the same zones along the ventrodorsal axis of the wall of the third ventricle. Rarres2 mRNA is predominantly expressed in the most dorsal region, where ependymal cells are the exclusive cell population (G–J), and in the transition zone, where tanycytes are intermingled with ependymal cells (G–N) (yellow arrowheads). Rarres2 mRNA expression decreases ventrally, where tanycytes are the predominant cell population (O–Z; white arrowheads). There is some expression of Rarres2 in the dorsal α2 tanycytic zone (O–R) as ependymal cells start intercalating with tanycytes to form the transition zone more dorsally (K–N). Rax mRNA is predominantly expressed in the ventral region of the wall of the third ventricle where tanycytes are abundant (O–Z) (white arrowheads) and it progressively decreases dorsally (K–N) until it completely disappears in the ependymal zone (G–J). 3V, third ventricle. Scale bar = 200 μm in D (applies to A–F); 20 μm in G (applies to G–Z).
multiple cilia in the α2 tanycytic zone to confirm the presence of ependymal cells (Mullier et al., 2010). Confocal z-stack visualization of G-TUB in the α2 tanycytic zone showed an increase in cilia projecting to the ventricular lumen (Fig. 11). However, we noticed that G-TUB was present not only in the motile cilia of the ependymal cells (multiple cilia), but also in the nonmotile cilia (primary cilia), which are detectable in some α2 tanycytes, but are more abundant in β1 and β2 tanycytes (data not shown).

Figure 6. Rarres2 is expressed in hypothalamic ependymal cells. Confocal z-stack reconstruction of Rarres2 FISH (magenta) and immunohistochemistry for detyrosinated α tubulin (G-TUB; green) counterstained with DAPI (blue) in the anterior (A), medial (B), and posterior (C) hypothalamus of adult C57BL/6 wild-type mice (P45). A–C: G-TUB is expressed in the dorsal region of the wall of the third ventricle along the anteroposterior axis similar to Rarres2 expression (white arrowheads). D–K: Orthogonal view of the box in A where G-TUB (D,E) and Rarres2 (F,G) signal is observed in ependymal cells identified by the expression of G-TUB (green) in their multiple cilia projecting to the third ventricle. Rarres2 signal is underneath G-TUB multiple cilia (J–L). G-TUB signal in hypothalamic parenchyma corresponds to primary cilia. 3V, third ventricle. Scale bar = 100 μm in A (applies to A–C); 20 μm in D (applies to D–K).
Primary cilia in tanycytes and motile cilia of the ependymal cells can be differentiated by their length and arrangement. Primary cilia are shorter than motile cilia, <1 μm for primary cilia and >2–8 μm for motile cilia (O’Callaghan et al., 1999) and, more importantly for discrimination between the two, motile cilia are found in clusters, whereas primary cilia are found as solitary cilia arising from the cell body (Satir and Christensen, 2007). Taking these differences in account, we quantified the clusters of cilia longer than 2 μm in the tanycytic zone of three Bregma points in each slide of our confocal z-stacks. We found that there was a significant increase in the number of multiple cilia clusters in the tanycytic zone of Rax<sup>+/−</sup> mice at Bregma −1.655 mm, where we found ventralization of Rarres2, as well as at Bregma −1.755 mm (Fig. 11).

**Rax<sup>+/−</sup>** mice display reduced distal diffusion of Evans Blue

Because tanycyte and ependymal cells form the CSF-brain barrier in the hypothalamus, and because
we observed both fewer tanycytes and ependymal cells in the third ventricle and ectopic ependymal cells in the α2 tanycytic zone, we hypothesized that \( Rax^{+/−} \) mice would exhibit altered tracer diffusion from the CSF to the hypothalamic parenchyma. To test this hypothesis, we injected EB dye in the lateral ventricle of \( Rax^{+/+} \) and \( Rax^{+/−} \) mice. Because EB is a low-molecular-mass tracer with high albumin affinity that readily diffuses across paracellular space (Fry et al., 1977), we quantified EB signal in the hypothalamic parenchyma 1 minute after i.c.v. injection (Fig. 12).

In \( Rax^{+/+} \) mice, we found that EB distal diffusion was higher in the hypothalamus compared with other brain regions adjacent to ventricular compartments (Fig. 12 B–G). Also, within the medial hypothalamus, EB diffusion varied along the anteroposterior axis in both genotypes, with no distal diffusion in the anterior hypothalamus, and more distal diffusion in the medial and posterior hypothalamus (Fig. 12 B–G). When we compared the EB signal in \( Rax^{+/−} \) and \( Rax^{+/+} \) animals by simple visualization, we found a consistent difference only in the medial hypothalamus. Based on this result, and also owing to a desire to compare our results with

**Figure 8.** \( Rax \) heterozygotes show a twofold reduction in tanycyte and ependymal markers. qRT-PCR quantification of relative mRNA obtained from hypothalami dissected from adult mice (P45). Twofold reduction of relative \( Rax \) mRNA in heterozygous mice (\( Rax^{+/−} \)), \( P = 0.05 \). Relative mRNA of other genes expressed by terminally differentiated tanycytes is also reduced (Gpr50, \( P = 0.01 \); Hes1, \( P = 0.01 \); Hes5, \( P = 0.04 \)). Ependymal genes Foxj1 (\( P = 0.03 \)) and Rarres2 (\( P = 0.03 \)) are significantly reduced in \( Rax^{+/−} \) mice. There is no significant reduction of Gfap and vimentin relative mRNA. Unpaired t-test \( Rax^{+/+} (n = 5), Rax^{+/−} (n = 6) \).
Figure 9. Rax heterozygotes show a reduction in volume in the third ventricle wall. A–H: Confocal z-stack reconstruction of DAPI staining along the third ventricle wall of the medial hypothalamus (dotted area) in adult mice (P45). I–P: Digital three-dimensional reconstruction of the dotted areas in A–H. Q: Digital volume quantification of the wall of the third ventricle using the cell tool of the Imaris software. Rax heterozygotes have reduced volume of the ventricular wall at the ependymal zone (B,J,Q) as well as at the α2 (F,N,Q) and β1 (H,P,Q) tanycytic zones. Unpaired t-test (n = 3), β1 tanycytes, P = 0.01; α2 tanycytes, P = 0.03; α1 tanycytes, P = 0.08; ependymal cells, P = 0.02. 3V, third ventricle. Scale bar = 50 μm in A (applies to A–P).
the previous report of EB diffusion in wild-type mice (Mullier et al. 2010), we quantified the total amount of EB in all brain sections of the medial hypothalamus, as well as EB in the parenchyma, by using the pixel quantification ImageJ tool. Distal diffusion was calculated as a ratio between the levels of EB in the parenchyma of each brain section divided by the total amount of EB in the same brain section. In Rax\(^{+/−}\) mice, distal diffusion into the medial hypothalamic parenchyma was significantly lower than in Rax\(^{+/+}\) mice (Fig. 12 H–N).

### DISCUSSION

Previous studies have shown that Rax mRNA is expressed in progenitors located along the ventricular wall of the third ventricle. Our data showed that Rax expression persisted through adulthood, when its expression became restricted to hypothalamic tanyocytes and the ME (Figs. 1–5). Characterization of Rax\(^{+/−}\) mice showed that Rax haploinsufficiency during development leads to a reduction in both tanyocyte...
and ependymal cell numbers in the hypothalamic third ventricle in adult animals, as demonstrated by reduced marker expression for both cell types and reduced volume of the ventricular wall (Figs. 8 and 9). Along with the overall reduction of ependymal cells in Rax$^{+/−}$ mice, we observed that some ependymal cells were ectopically located in the α2 tanyctic zone, where few of these cells are usually found (Figs. 10 and 11). Furthermore, we found that Rax$^{+/−}$ mice displayed changes in the hypothalamic CSF-brain barrier that resulted in reduced distal diffusion of EB from the CSF to the parenchyma of the medial hypothalamus (Fig. 12). Our main findings are summarized in Fig. 13.

**Rax haploinsufficiency leads to reduced volume of the third ventricle wall**

Rax$^{+/−}$ mice showed reduced ventricular wall volume due to a reduction in both tanyocyte and ependymal cell numbers (Figs. 8 and 9). This likely arises at least in part from Rax haploinsufficiency defects in late-stage hypothalamic progenitor cells, which give rise to both cell types (Mathers et al., 1997). The mechanism by which Rax controls hypothalamic progenitor proliferation is unknown, although recent studies of Xrx1, the *Xenopus* ortholog of Rax, have shown that it causes smaller eye and brain size due to reduced proliferation (Andreazzoli et al., 2003; Casarosa et al., 2003). Terada and Furukawa (2010) have shown that Xrx1 binds to the chromatin modulator Xhmgb3 (*Xenopus* high mobility group 3) and to the Six family transcription factor XOptx2 (Six6) to promote cell proliferation in the eye and brain. Xhmgb3 in turn binds to the SUMO E2 ligase UBC9 and inactivates transcription of the kinase inhibitor p27Xic1, leading to increased proliferation of retinal progenitor cells (Terada and Furukawa, 2010). Both Six6 and Hmgb2, a close homologue of Hmgb3, are prominently and selectively expressed in hypothalamic progenitor cells (Shimogori et al., 2010), suggesting that Rax might be regulating the proliferation of hypothalamic ventricular radial glia through a similar mechanism, with reduction of Rax expression leading to increased p27 levels and suppression of tanyocyte progenitor proliferation.

**Rax haploinsufficiency is associated with an increased fraction of cells with multiple cilia in the tanyctic zone**

Under normal conditions, there are few ependymal cells in the ventral third ventricle zone where α2 tanyocytes are located. However, we found that in Rax$^{+/−}$...
mice there was both a substantial increase in the number of cells with multiple cilia, characteristic of ependymal cells, and a ventral extension of the ependymal cell marker Rarres2 expression in this region (Fig. 10). Persistent and selective expression of Rax mRNA in mature hypothalamic tanycytes but not ependymal cells suggests that, in addition to regulating late-stage hypothalamic progenitor proliferation, Rax may actively promote tanycyte differentiation at the expense of ependymal identity.

Alternatively, it has been shown that motile cilia can develop from single ciliated cells during development of airway epithelial cells (Jain et al., 2010). This opens the possibility that cells in the ventricular zone with single apical cilia, such as tanycytes, could develop motile cilia in response to cell-autonomous and non-cell-autonomous
cues similar to what happens in the airway. Two key cell-autonomous regulators of multiciliogenesis, multicilin and miR449, promote cilia formation in skin, kidney, and airway by blocking Notch signaling (Marcet et al., 2011; Stubbs et al., 2012). Interestingly, Notch signaling components are expressed in developing and mature tanycytes, but not ependymal cells (Shimogori et al, 2010), and showed reduced expression in \textit{Rax}^{1/2} mice (Fig. 8), in line with previous reports that \textit{Rax} probably participates in tanycyte and ependymal cell progenitor proliferation and differentiation. Hypocellularity of the ventricular wall leads to reduced distal diffusion of the Evans Blue tracer. 3V, third ventricle.

![Figure 13](image-url) Schematic representation of the composition and distribution of cells in the ventral third ventricle wall of \textit{Rax} haploinsufficient mice. Reduction of \textit{Rax} gene dose during the development of \textit{Rax}^{+/−} mice leads to a thinner wall of the ventral third ventricle and ectopic presence of ependymal cells in the α2 tanycytic zone, suggesting that \textit{Rax} probably participates in tanycyte and ependymal cell progenitor proliferation and differentiation. Hypocellularity of the ventricular wall leads to reduced distal diffusion of the Evans Blue tracer.

The cellular composition of the third ventricle wall influences the distal diffusion of CSF-derived EB in the hypothalamus of \textit{Rax}^{+/−} mice

We investigated permeability of the third ventricle wall in \textit{Rax}^{+/−} mice, which have a reduced overall number of ependymal cells and tanycytes along the hypothalamic third ventricle wall and ectopic ependymal cells in the α2 tanycytic zone. We found that in \textit{Rax}^{+/−} mice there was reduced distal diffusion of EB from the CSF to the medial hypothalamic parenchyma (Fig. 12). We observed proximal EB diffusion all along the anterior to posterior hypothalamic axis, but distal EB diffusion was only present in the medial and posterior hypothalamic wall of \textit{Rax}^{+/−} mice (Fig. 12). This might be related to the variable composition of the hypothalamic ventricular wall along this axis. The ventricular zone of the ventral anterior hypothalamus is composed exclusively of ependymal cells, whereas tanycytes are only present in a small area at the floor of the third ventricle. In contrast, the ventral medial and posterior hypothalamus features more tanycytes than ependymal cells (Rodriguez et al., 2005).

Our observations suggest that distal diffusion of EB is higher in hypothalamic areas enriched with tanycytes. Both tanycytes and ependymal cells can form gap junctions that permit proximal diffusion of a tracer, but only tanycytes have long processes that project distally from the ventricle. Tanycytes may facilitate distal diffusion of EB by endocytosis of the EB followed by transcellular transport. In agreement with a previous publication, we observed lateral diffusion of ventricular EB in the ArcN (α2 tanycytic zone) and in the ependymal zone of the medial hypothalamic (Mullier et al., 2010). However, we also observed lateral EB diffusion in \textit{Rax}^{+/−} mice in the transition zone at the level of the VMH and DMH, were both where α1 tanycytes and ependymal cells are intermingled. Diffusion at this level was not reported by the authors of this study seems to correspond to tanycytes. The authors suggested that when Six3 is absent, the transition from “radial glia” to ependymal cells is defective, leading to a hybrid cellular phenotype. Using vimentin staining alone, we are unable to discriminate between a hybrid, multiciliated tanycyte and an ependymal cell located directly on top of a tanycyte cell body, giving the appearance of a multiciliated tanycyte. Analysis of individual tanycyte morphology using mice expressing fluorescent reporter genes, in combination with colabeling with cilia markers, would ultimately allow us to determine whether such hybrid tanycyte-ependymal cells exist in \textit{Rax}^{+/−} mice.
Mullier et al. 2010. We believe that this discrepancy might be explained because the referenced study focused on EB diffusion at a single bregma point. Due to we observed that EB diffusion varies along the anteroposterior axis, it is possible that his study missed spatially restricted domains of lateral EB diffusion in the α1 tanycytic zone.

Finally, we found reduced distal diffusion of EB in the medial hypothalamus of Rax$^{+/−}$ mice. If distal EB diffusion was facilitated by the presence of tanycytes, the reduction of tanycytes in Rax$^{+/−}$ mice would explain a reduction in distal EB diffusion. However, reduced EB diffusion was observed not only ventrally in the tanycytic zone but also dorsally in the ependymal region of the hypothalamus. This finding suggests that there might be other factors that affect hypothalamic ventricular wall permeability beyond simply the number of tanycytes, such as altered expression of tight and gap junction proteins. There are abundant tight and gap junctions between ependymal cells and tanycytes of the hypothalamic third ventricle wall (Peruzzo et al., 2000; Rodriguez et al., 2005; Mullier et al., 2010; Langlet et al. 2013a). Because paracellular permeability depends on the type of intercellular junctions, with gap junctions facilitating diffusion whereas tight junctions restrict diffusion, reduced distal diffusion of EB in Rax$^{+/−}$ mice suggests that a possible compensatory increase of tight junctions resulting from a reduced number of cells along the wall of the third ventricle may have occurred. However, we were unable to successfully perform staining with antibodies against tight or gap junction proteins in conjunction with EB visualization.

The disrupted EB diffusion between the CSF and hypothalamic parenchyma seen in Rax$^{+/−}$ mice may have physiological consequences. Protein-mediated signaling between the CSF and adjacent neurons is important for normal brain development and function (Dziegielewsk et al., 2000; Sharma and Johanson, 2007). Signaling may be receptor-mediated or may occur by paracellular transport (Agnati et al., 1995). It has been suggested that based on the expression of cell–cell junctions and the pattern of EB diffusion in the ArcN, there is an open communication between the CSF and this nucleus (Mullier et al., 2010). Molecules such as T3 and leptin have been proposed to diffuse through the CSF to regulate the activity of neurons of the ArcN (Rodriguez et al., 2010). As a result, it would be expected that changes in protein permeability might affect hypothalamic function. Although the Rax$^{+/−}$ mice used in this study did not display any gross anatomical or behavioral abnormalities, this does not exclude the presence of more subtle changes in tanycyte-mediated hypothalamic functions probably minimized by compensatory mechanisms induced by Rax haploinsufficiency.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: ALMA, SB. Acquisition of data: ALMA, SB, MB, JM, HW. Analysis and interpretation of data: ALMA, SB, MB. Drafting of the manuscript: ALMA, SB. Critical revision of the manuscript for important intellectual content: ALMA, SB. Statistical analysis: ALMA, SB, MB. Obtained funding: SB. Administrative, technical, and material support: ALMA, SB, MB, JM, HW. Study supervision: SB

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