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Conserved roles of *Rax/rx3* genes in hypothalamus and pituitary development

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ABSTRACT ***Rax*** **(*****Rx*****) genes encode paired-type homeodomain-containing transcription factors present in virtually all metazoan groups. In vertebrates, studies in fish, amphibian, chick and mouse models have revealed that these genes play important roles in the development of structures located at the anterior portion of the central nervous system, in particular the eyes, the hypothalamus and the pituitary gland. In addition, human patients with eye and brain defects carry mutations in the two human *Rax* paralogues, *RAX* and *RAX2*. Here, we review work done in the last years on *Rax* genes, focusing especially on the function that mouse *Rax* and its zebrafish homologue, *rx3*, play in hypothalamic and pituitary development. Work on both of these model organisms indicate that *Rax* genes are necessary for the patterning, growth and differentiation of the hypothalamus, in particular the ventro-tuberal and dorso-anterior hypothalamus, where they effect their action by controlling expression of the secreted signalling protein, Sonic hedgehog (Shh). In addition, *Rax/rx3* mutations disturb the development of the pituitary gland, mimicking phenotypes observed in human subjects carrying mutations in the *RAX* gene. Thus, along with their crucial role in eye morphogenesis, *Rax* genes play a conserved role in the development of the hypothalamus and adjacent structures in the vertebrate clade.**

KEY WORDS: *rx1*, *rx2*, *diencephalon*, *neurohypophysis*, *adenohypophysis*

Introduction

Rax (Rx) proteins are paired-like homeodomain transcription factors encoded by the *Rax (rx)* genes. *Rax/rx* genes were first described in 1997, when their expression was reported in the anterior neural plate and developing eyes of mice, *Xenopus laevis* and zebrafish embryos (Furukawa *et al*., 1997; Casarosa *et al*., 1997; Mathers *et al*., 1997). Targeted inactivation of the *Rax* gene in mouse was shown to prevent optic cup formation and eye development (Mathers *et al*., 1997), an observation extended by the finding that the classic anophthalmic mouse mutant *eyeless* (Chase and Chase, 1941) carries a hypomorph mutation in the *Rax* gene (Tucker *et al*., 2001). Soon after these findings, loss-of-function studies in *Xenopus* and in teleost fish showed that *Rax* genes have a conserved role in eye development in vertebrates (Winkler *et al*., 2000; Loosli *et al*., 2003; Kennedy *et al*., 2004; Rojas-Muñoz *et al*., 2005; Andreazzoli *et al*., 2003; Bailey *et al*., 2004). Human patients with anophthalmia, microphthalmia and other eye defects carry mutations in *Rax* genes, indicating the relevance of these genes to human pathology (Voronina *et al*., 2004; Wang *et al*., 2004; Van de Sompele *et al*., 2018; Brachet *et al*., 2019).

Rax/rx expression, however, is not limited to the anterior neural plate and eyes. Early studies reported expression of these genes

Abbreviations used in this paper: α-gsu, glycoprotein subunit alpha-chain; ABas, anterobasal nucleus; ACTH, adrenocorticotropic hormone; Arc, arcuate nucleus; Avp, arginine vasopressin; Bmp, bone morphogenetic protein; Chk, chokh; DMH, dorsomedial hypothalamus; E, embryonic day; Fgf, fibroblast growth factor; Gh, growth hormone; hpf, hours post-fertilisation; Lh, luteinising hormone; Oxt, oxytocin; Pomc, proopiomelanocortin; Prl, prolactin; PVN, paraventricular nucleus; Rax, retina and anterior neural fold homeobox; Rx, retinal homeobox; SCN, suprachiasmatic nucleus; Sf1, steroidogenic factor 1; Shh, sonic hedgehog; Sst, somatostatin; Th, tyrosine hydroxylase; Tsh, thyroid stimulating hormone; VMH, ventromedial hypothalamus.

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in the developing hypothalamus (Furukawa *et al*., 1997; Casarosa *et al*., 1997; Mathers *et al*., 1997; Winkler *et al*., 2000; Loosli *et al*., 2003), and indeed, in addition to the obvious eye abnormalities, mouse and zebrafish *Rax* mutants display other forebrain defects, particularly in the hypothalamus and pituitary regions (Stigloher *et al*., 2006; Dickmeis *et al*., 2007; Muthu *et al*., 2016; Zhang *et al*., 2000; Medina-Martínez *et al*., 2009; Orquera *et al*., 2016). Similarly, a *Xenopus tropicalis rax* mutant has altered gene expression in the hypothalamus and telencephalon (Fish *et al*., 2014).

In this review, we focus on the less well-studied, but conserved role of *Rax* genes, in particular mouse *Rax* and zebrafish *rx3*, in the development of the hypothalamus and pituitary. For reviews focusing on the role of *Rax* genes in optic cup formation and differentiation, please see Bailey *et al.* (2004) and Muranishi *et al.* (2012).

Rax/rx paralogue genes in vertebrates

Rax genes are found in all metazoan genomes, with invertebrates having only one *Rax* gene (referred to as *rx*, Mazza *et al*., 2010). In contrast, vertebrate genomes can have one, two or more *Rax* paralogues. Phylogenetic analyses indicate that these can be classified into two groups, *Rax1* and *Rax2*, that originated in polyploidisation events that took place during early vertebrate evolution (Orquera and de Souza, 2017). Zebrafish, medaka and other teleosts possess one member of the *Rax1* subgroup, termed *rx3*, and two members of the *Rax2* subgroup, *rx1* and *rx2*. Reptiles, birds and mammals (including humans) have generally one *Rax1* and one *Rax2* gene. An important exception is the mouse, which possesses only one *Rax1* gene (called simply *Rax*) and lacks a *Rax2* paralogue, a situation common to all rodents and lagomorphs (Table 1).

When comparing different published studies, it is important to take into account the species and paralogues that are being studied, since *Rax1* and *Rax2* genes originated hundreds of millions of years ago and have had time to diverge functionally (Orquera and de Souza, 2017). *Rax2* paralogues are only expressed in the retina and are presumably not involved in hypothalamic development. In zebrafish and medaka, loss-of-function of the *Rax2* paralogues *rx1* and *rx2* affect the differentiation of the retina, with no hypothalamic phenotypes reported (Nelson *et al*., 2009; Reinhardt *et al*., 2015). Recently, Van de Sompele *et al.,* (2019) identified five human patients from unrelated families carrying homozygous mutations in *RAX2*, some of which are predicted to generate proteins with little, if any, activity. The patients present nonsyndromic autosomal recessive retinitis pigmentosa, with an onset age from childhood to late adulthood (Van de Sompele *et al*., 2019). This study indicates that, as in other species, the role of *RAX2* in humans is to maintain the health of the retina throughout life. *Rax1* paralogues, in contrast, are expressed in the hypothalamus and pituitary and play a role in the development of these structures in vertebrates.

TABLE 1

***RAX* PARALOGUE GENES IN VERTEBRATES**

Clade	***Rax1* group**	***Rax2* group**
Teleost fish (including zebrafish)	*rx3*	*rx1, rx2*
Reptiles, amphibians, birds, most mammals	*Rax1* (*Rax*)	*Rax2*
Rodents (including mouse), lagomorphs	*Rax*	-

Expression of *Rax/rx3* genes during hypothalamic development

The hypothalamus is an ancient region of the vertebrate brain that regulates basic physiological functions including metabolic rate, reproduction, energy balance, stress, sleep and circadian rhythm. Its neurons, which produce a great variety of neurotransmitters and neuropeptides, are organised in nuclei. Recent years have seen a great advance in the understanding of the cellular and molecular mechanisms involved in the development of this complex structure (reviewed in Pearson and Placzek, 2013; Burbridge *et al*., 2016; Xie and Dorsky, 2017; Álvarez-Bolado, 2019). *Rax1* paralogues are all expressed in the hypothalamus during early vertebrate embryogenesis (Furukawa *et al.,* 1997; Mathers *et al*., 1997; Casarosa *et al*., 1997; Chuang *et al.,* 1999; Ohuchi *et al*., 1999), and work in zebrafish and mouse indicates that their function in hypothalamic development is to a great extent conserved.

In the mouse, *Rax* is detected in a dynamic manner in neural progenitor cells. Expression first appears in a broad domain within the anterior neural plate at embryonic day (E) 7.5 (Furukawa *et al.,* 1997), then is confined to anterior neural fold regions that harbour retinal and hypothalamic progenitors (Furukawa *et al*., 1997; Blaess *et al*., 2015). *Rax* is then maintained in progenitors within the optic vesicles, optic cups and retina into adulthood. However, from E10.5 until at least E15.5, the strongest domain of *Rax* expression is in hypothalamic neuroepithelial progenitor cells located in the future ventricular zone lining the third ventricle (Shimogori *et al*., 2010; Lu *et al*., 2013; Ferrán *et al*., 2015; Orquera *et al*., 2016). Expression is confined to the dorso-anterior and ventro-tuberal hypothalamus (Fig. 1), including the anterobasal nucleus (ABas) and the infundibulum, a region of the tuberal hypothalamus that evaginates/grows to give rise to the posterior lobe of the pituitary gland. In transverse view, *Rax* expression can be seen in three domains from dorso-anterior to ventro-tuberal, named domains I, II and III by Lu *et al.,* (2013). Domain I is located near the dorsomedial hypothalamus (DMH), and is followed by an intermediate domain II (where *Rax* expression is weaker). Domain III is located adjacent to the arcuate (Arc) and ventromedial hypothalamus (VMH) nuclei. In keeping with expression profiling, fate mapping with different Cre recombinase constructs under the control of *Rax* regulatory regions indicates that *Rax*-expressing progenitors contribute to the eyes and hypothalamus, but also to other anterior forebrain structures, including the telencephalon, the pineal gland and the prethalamus (Klimova *et al*., 2013; Lu *et al*., 2013; Pak *et al*., 2014). At present, the lineage-relationship of the different *Rax*-expressing progenitor subsets remains unclear, as do the mechanisms that govern *Rax* expression in distinct progenitor populations. The transcription factor Lhx2 acts upstream of *Rax* expression in retinal progenitor cells (Tétreault *et al*., 2009), but does not appear to be required in all early hypothalamic progenitor cells (Tétreault *et al*., 2009).

In the zebrafish, *rx3* is also expressed in a broad domain in the anterior neural plate at early stages (from 10-30 hours post fertilisation (hpf) (Mathers *et al*., 1997; Chuang *et al*., 1999; Muthu *et al*., 2016), but by 55 hpf, expression becomes confined to the neuroepithelium lining the third ventricle at the level of the hypothalamus (Muthu *et al*., 2016). Here, expression is detected in three neuroepithelial domains (also numbered I, II and III). While the morphology of the developing mouse and zebrafish hypothalamus are different (the tuberal domain of zebrafish appears relatively

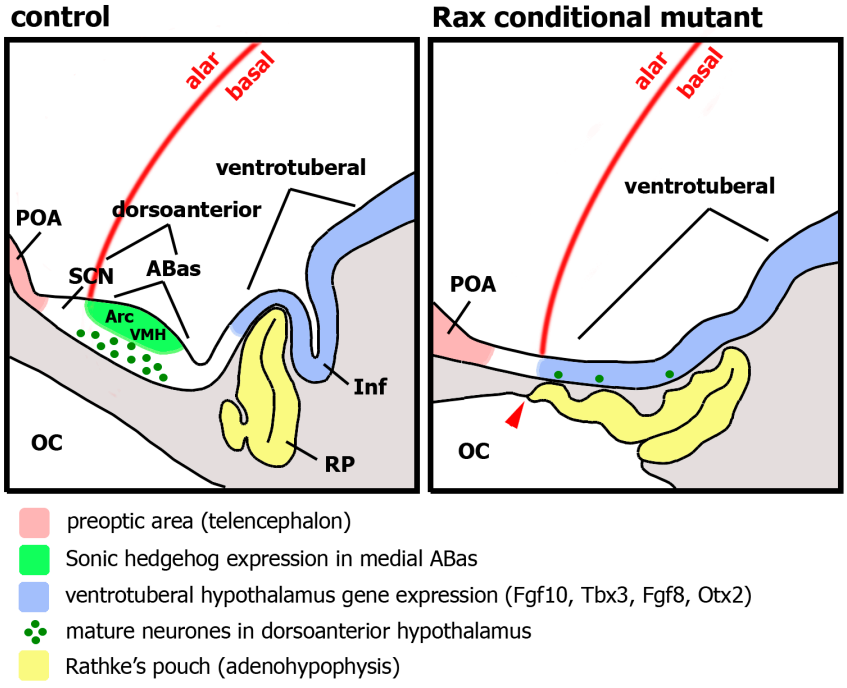

Fig. 1. Hypothalamic and pituitary development in mouse *Rax* mutants. *Schematics of sagittal cuts across the medial hypothalamic region in control (left) and* Rax *conditional mutants (RaxKO@E8.0) at E11.5. In the absence of* Rax, Shh *expression and neuronal differentiation in the dorso-anterior hypothalamus is impaired, and the expression of ventrotuberal gene markers expand towards the dorso-anterior region. In mutants, the infundibulum (Inf) does not form, and Rathke's pouch (RP) detachment from the oral ectoderm (OC) is incomplete, with an expanded hypophyseal placode territory (red arrowhead) and ectopic pouches. POA: preoptic area of the telencephalon; SCN: prospective suprachiasmatic nucleus; ABas: anterobasal nucleus; Arc: prospective arcuate nucleus; VMH: prospective ventromedial hypothalamus. Red line marks the alar/basal limit. Based on Orquera* et al., *(2016). Note that in keeping with the original literature, we use the term dorso-anterior, but this is to denote morphological position, rather than ontogeny (i.e. as explained in the text, evidence in zebrafish and chick suggests that dorso-anterior progenitors/neurons arise from ventro-tuberal progenitors).*

large in comparison to the mouse) a prosaic interpretation is that these are the same as domains I-III in the mouse. Certainly, as in mouse, *rx3*-expressing domain I lies in a Shh-expressing dorso-anterior location, and domain III lies adjacent to regions that express genes homologous to those expressed in the mammalian Arc and VMH (see below). In both mouse and zebrafish the ventro-tuberal domain (domain III) is located immediately dorsal to the developing adenohypophysis (aka anterior pituitary gland), and is likely to include cells of the nascent neurohypophysis (see below) (Muthu *et al.*, 2016; Lu *et al.*, 2013). Thus, the expression pattern of *Rax* and *rx3* in the developing hypothalamus appear to be similar in mouse and zebrafish.

Conserved function of Rax/rx3 in hypothalamic neuronal specification

Loss-of-function models point to a conserved role for *Rax* in vertebrate hypothalamic development. *Rax*-null mice often present extensive craniofacial defects associated with a delay in neural fold closure (Voronina *et al.*, 2004), but some embryos develop relatively normal heads in which the hypothalamus is very thin and the infundibulum does not develop (Zhang *et al.*, 2000). The availability of a *loxP*-flanked (floxed) *Rax* allele (Voronina *et al.*, 2005) has allowed for the study of *Rax* function by temporal and spatial conditional knockout using various Cre recombinase drivers. An extensive analysis of hypothalamic gene expression in conditional *Rax* mutants was done by Orquera *et al.,* (2016) using a floxed *Rax* allele that can be inactivated in a temporal manner using a tamoxifen-inducible Cre recombinase. When *Rax* expression is eliminated at E8.0 (denoted as *Rax*KO@8.0), the dorso-anterior/ventro-tuberal hypothalamus fails to develop normally. The neuroepithelium in the dorso-anterior region becomes very thin, the ABas is not detected, and the infundibulum does not invaginate, similar to the phenotype observed in *Rax*-null embryos (Fig.1). Expression of the proneural genes *Ascl1* and *Ngn3* is abolished, indicating that hypothalamic neurogenesis is interrupted. Transcription factors necessary for the differentiation of hypothalamic neurons/nuclei, in particular, the suprachiasmatic (SCN) markers *Lhx1*, *Six6* and *Nkx2.2*, and the Arc markers *Isl1* and *Orthopedia* (*Otp*), are not detected. Further, molecular markers of mature neurones found in the Arc (the neuropeptide Proopiomelanocortin [Pomc] and the dopaminergic marker gene, tyrosine hydroxylase [Th]) and in other hypothalamic regions (Somatostatin [Sst]) are lost. Interesting, the temporal inactivation of *Rax* at stages later than E8.0 does not cause the loss and thinning of the dorso-anterior/ventro-tuberal hypothalamus (Orquera *et al.*, 2016). This is in agreement with results by Lu *et al.,* (2013), who conditionally eliminated Rax using Cre drivers active at slightly later stages, namely *Six3*-Cre, active after E9.0, and *Shh*-Cre, active after E10.0, and did not observe that the overall development of the dorso-anterior/ventro-tuberal hypothalamus was affected. Instead, after elimination of *Rax* using the *Shh*-Cre driver the authors described a change in the fate of the VMH, evidenced by the loss of expression of the transcription factor *Sf1* - a bona fide VMH marker. The VMH of these mutants also seemed to change its neurotransmitter characteristics, since the expression of the glutamatergic marker VGlut2 was lost and the GABAergic marker Gad67 (along with other molecular markers) was ectopically upregulated, leading Lu *et al.,* to suggest that after E10.0, *Rax* acts as a terminal selector gene for the VMH (Lu *et al.*, 2013). Thus, *Rax* in the mouse seems to have an early function in establishing the dorso-anterior/ventro-tuberal hypothalamus, including domains that will give rise to the SCN, ABas and Arc and a later function in establishing the identity of specific neurons within particular nuclei, notably within the VMH (Orquera *et al.*, 2016; Lu *et al.*, 2013).

In the zebrafish, Muthu *et al.,* (2016) performed a detailed study of the changes in overall architecture and expression of molecular markers in the hypothalamus of *rx3* mutants, more specifically the *chk*^{w29} mutant described by Kennedy *et al.,* (2004), and in *rx3* morphants. As in the mouse, transcription factors that define particular progenitor regions were all but lost in mutant and morphant fish. While these progenitor regions were termed 'anterior/tuberal'

progenitors, it is likely that these are progenitors equivalent to those termed 'dorso-anterior' in the mouse hypothalamus. In particular, in the mutant zebrafish, *otpb* (homologue of mammalian Otp) and *ff1b* (homologue of the mammalian VMH marker Sf1) were all but lost, and *nkx2.1a* (homologue of mammalian Nkx2-1) expression was reduced/lost in specific dorso-anterior domains. Likewise, the mature neuronal markers *pomca* and *th1* (homologues of mammalian Pomc and Th; both found in the Arc) were not detected. This confirms and extends earlier observations by Dickmeis *et al.,* (2007) and Tessmar-Raible *et al.,* (2007) who reported that *rx3* mutants lose hypothalamic expression of *pomca.* Notably, these earlier studies reported, additionally, loss of expression of the fish homologue of arginine vasopressin (*avp*), a neuropeptide that in mammals is expressed in the SCN. (Note that while *avp* is also expressed in the paraventricular nucleus (PVN), other PVN-specific neuropeptides including *corticotropin releasing hormone* (*crh*), *somatostatin 3* (*sst3*) and *isotocin* (the oxytocin homologue in fishes) were not affected in *rx3* mutants (Dickmeis 2007)). Potentially, then, the key role of zebrafish *rx3* is to establish a region equivalent to the 'dorso-anterior' region of the mouse hypothalamus, a region that will give rise to the SCN, Arc and VMH. While further analyses need to be performed, we speculate that in both mouse and zebrafish, Rax/rx3 are required for the formation of hypothalamic territories that will ultimately generate neurons within the SCN, Arc, ABas and VMH.

Similar to the situation in the eyes, where *Rax* persists into adulthood, *Rax* expression is maintained in the adult mouse hypothalamus in the epithelium of the third ventricle adjacent to the Arc, VMH and DMH. Expression is detected in specialised cells called tanycytes (Miranda-Angulo *et al*., 2014; Salvatierra *et al*., 2014), where it is regulated by Lhx2 (Salavatierra *et al*., 2014). Intriguingly, subsets of tanycytes include stem and progenitor populations (Robins *et al*., 2013; Pellegrino *et al*., 2018), suggesting that Rax may continue to play a role in progenitor cell behavior through life. In support of this idea, Miranda-Angulo *et al.,* (2014) found that heterozygote *Rax+/-* adult mice present with a thinner α2-tanycyte area, ectopic ependymal cells and altered cerebrospinal fluid barrier characteristics, indicating that *Rax* is necessary for proper tanycyte differentiation and function in the hypothalamus. No comparable analysis of the neuroepithelium of the third ventricle in adult zebrafish *rx3* mutants has been carried out as yet.

Early hypothalamic patterning depends on *Rax* genes in mice and fish

The same studies that document the loss of dorso-anterior cells in *Rax/rx3* mutants begin to suggest one mechanism through which *Rax/rx3* functions. In murine RaxKO@8.0 mutants, the thinning of the dorso-anterior hypothalamus was accompanied by a shift in the expression of molecular markers of the ventro-tuberal hypothalamus (Orquera *et al*., 2016). In particular, expression of the transcription factors *Tbx3* and *Otx2*, as well as the expression of *Fibroblast growth factor 10* (*Fgf10*), each normally confined to the ventro-tuberal hypothalamus (Zhao *et al*., 2012; Trowe *et al*., 2013; Mortensen *et al*., 2015; Carreno *et al*., 2017), expanded dorso-anteriorly (Orquera *et al*., 2016). Thus, the lack of *Rax* in early development appears to result in a dorso-anterior expansion of ventro-tuberal progenitor cells at the expense of dorso-anterior cells, ie the cells that will normally undergo neurogenesis and

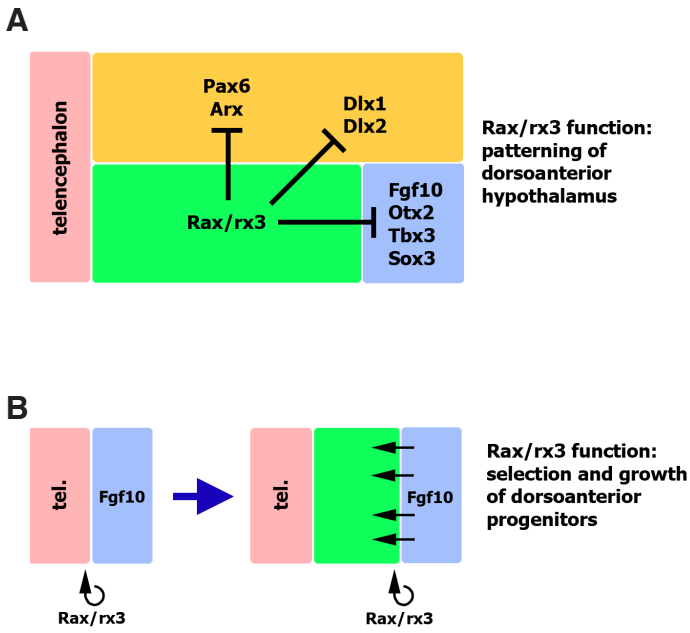

Fig. 2. Rax/rx3 roles in hypothalamic development. (A) *Patterning role of Rax/rx3 in the dorso-anterior hypothalamus. Rax/rx3 activity antagonises the expression of markers genes of the prethalamus (Pax6, Arx), dorsomedial hypothalamus (Dlx1/2) and ventro-tuberal hypothalamus (Fgf10, Otx2, Tbx3, Sox3), as indicated by works in mice,* Xenopus *and zebrafish (Lu* et al.*, 2013; Fish* et al.*, 2014; Muthu* et al.*, 2016; Orquera* et al.*, 2016).* **(B)** *Selection and growth function of Rax/rx3. Rax/rx3 activity selects hypothalamic progenitors of the tuberal hypothalamus (Fgf10+) to proliferate and undergo anisotropic growth, giving rise to dorso-anterior hypothalamic progenitors. This model is supported by work in zebrafish and chick embryos (Muthu* et al.*, 2016; Fu* et al.*, 2017).*

generate neurons of the SCN, Arc, ABas and VMH.

The absence of *rx3* causes a similar change in the early patterning of the zebrafish hypothalamus. During normal development, expression of *rx3* is limited by/overlaps with *fgf3* (a homologue of mammalian *Fgf10*) (Muthu *et al*., 2016), whose expression is normally confined to posterior domains of the ventro-tuberal hypothalamus. In *rx3* mutants, expression of *fgf3* intensifies and extends anteriorly. The transcription factor *sox3*, also expressed in posterior domains of the ventro-tuberal hypothalamus, similarly extends anteriorly in *rx3* morphants (Muthu *et al*., 2016). At the same time, expression of the transcription factor *pax6* expands ventrally from the thalamus and prethalamus into the hypothalamus of *rx3* mutants (Muthu *et al*., 2016). This is reminiscent of *Xenopus tropicalis* embryos carrying a homozygous mutation in *rax*, where *arx*, a transcription factor expressed in the prethalamus, expands ventrally into the hypothalamus (see Fig. 4 in Fish *et al*., 2014). Muthu *et al.,* (2016) also observed a ventral expansion of the transcription factor *dlx1*, a marker of the DMH. Interestingly, Lu *et al.,* (2013) observed similar ectopic expression of *Dlx2* in the VMH territory in *Rax* conditional mutants.

In conclusion, in both mouse and zebrafish (and perhaps in frogs) Rax/rx3 may pattern the early hypothalamus in a cross-talk mechanism(s), specifying the dorso-anterior hypothalamus by repressing transcription factors of the ventro-tuberal hypothalamus (*Otx2, Tbx3, Sox3*), the DMH (*dlx1/Dlx2*) and the prethalamus (*pax6*, *arx*) (Fig. 2A).

Rax genes and anisotropic progenitor growth within the hypothalamus

An emerging concept in hypothalamic development is that of anisotropic growth, i. e. morphogenesis and differentiation caused by the selection and differential proliferation of particular hypothalamic progenitor subsets. This concept has arisen through studies in the embryonic chick hypothalamus, which reveal that 'anterior' hypothalamic progenitors (most likely the equivalent of cells within the region we refer to here as 'dorso-anterior') arise from *Fgf10+* progenitors (most likely the equivalent of the cells that we refer to here as 'ventro-tuberal' progenitors). Thus Fu *et al.* (2017) found that in the chick, *Fgf10*$^{+}$ progenitors initially abut *Foxg1*$^{+}$ telencephalic progenitors; as *Fgf10+* progenitors proliferate, some daughter cells downregulate *Fgf10* and *Fgf* signal pathway components, grow anteriorly, and give rise to anterior progenitors; daughters that maintain *Fgf10* expression are gradually displaced posteriorly from the telencephalon and come to form a constant-size pool of 'ventro-tuberal' progenitors (reviewed in Fu *et al.*, 2019; Fig. 2B).

Along with a patterning role in the hypothalamus, *rx3* may direct the selection and growth of cells equivalent to 'dorso-anterior' progenitor subsets. Indeed, the anisotropic growth of such progenitors may contribute to/account for the phenotype of zebrafish *rx3* mutant embryos. Muthu *et al.,* (2016) noticed that the 'dorso-anterior' hypothalamic region of the zebrafish greatly increases in size between 30 hpf and 55 hpf, with growth apparently driven from proliferating *rx3*-expressing cells; over this period the posterior hypothalamus does not show a similar proportional increase in size. In *rx3* mutants, hypothalamic proliferation is maintained but cells accumulate in an aberrant manner around the recesses of the third ventricle in an expanded *fgf3*-expressing posterior ventro-tuberal hypothalamus, failing to differentiate and often undergoing apoptosis (Muthu *et al.*, 2016). The third ventricle does not extend anteriorly in these mutants, and, as discussed earlier, the 'dorso-anterior' hypothalamus fails to form. Thus, *rx3* is needed to select 'dorso-anterior' progenitor cells that normally differentially expand to generate/contribute to this region (Muthu *et al.*, 2016). Future fate-mapping studies are needed to confirm that, as in chick, dorso-anterior progenitors arise from *Fgf*-expressing ventro-tuberal progenitors that proliferate but fail to differentiate in the absence of *rx3*.

In mouse *Rax* mutants, no obvious deficit in proliferation have been observed in the dorso-anterior hypothalamus (see for instance Fig. S2 in Orquera *et al.*, 2016), but a detailed proliferation analysis at early time-points has not yet been performed. In both chick and zebrafish, dorso-anterior progenitors are generated in an early narrow time-window, that would equate to an E8.0-E8.25 mouse. Further studies are needed to establish, therefore, whether the expanded *Fgf10+* territory in the Rax mouse occurs solely through the cross-repressive mechanism (described in Fig. 2A), or whether *Fgf10*-expressing cells proliferate abnormally, forming an expanded ventro-tuberal domain. It therefore remains possible that in these mutants, the highly proliferative *Fgf10+* ventro-tuberal hypothalamus invades the dorso-anterior territory.

Rax genes control hypothalamic *Sonic hedgehog* expression

Sonic hedgehog (Shh) is a secreted protein that acts as a key morphogen in the induction and patterning of the ventral neural tube in vertebrates, including the hypothalamus (reviewed in Blaess *et al.*, 2015; Placzek and Briscoe, 2018). In the mouse, immunolabelling studies show that Rax protein colocalises with Shh in the anterior midline of the neural fold of the E8.5 embryo (Orquera *et al.*, 2016), i.e the region fated to give rise to the hypothalamus (Blaess *et al.*, 2015). By E10.5, Shh expression has been downregulated from the ventro-tuberal hypothalamus, including the developing infundibulum, but is expressed in a torus that includes a set of dorso-anterior hypothalamic progenitors (Fig. 1; Álvarez-Bolado *et al.*, 2012), where it colocalises with *Rax* (Orquera *et al.*, 2016). Together with the thinning of the neuroepithelium, impaired neurogenesis and neuronal differentiation discussed earlier, the deletion of *Rax* at E8.0 causes a complete loss of *Shh* expression from dorso-anterior hypothalamic progenitors. Importantly, the conditional deletion of *Rax* at later stages does not affect *Shh* expression, suggesting that the drastic developmental defects caused by the early loss of *Rax* in the hypothalamus could be mediated, at least in part, by the loss of *Shh* expression (Orquera *et al.*, 2016). In support of this idea, conditional inactivation of *Shh* specifically from the early hypothalamic neuroepithelium leads to an anatomical phenotype that bears a striking resemblance to that of eliminating *Rax*, notably a thinning of the neuroepithelium and absence of the infundibulum (Szabó *et al.*, 2009; Shimogori *et al.*, 2010; Zhao *et al.*, 2012). In addition, the elimination of *Shh* also prevents hypothalamic neurogenesis, evidenced by the lack of the proneural factor Ascl1, and a general failure of the differentiation of Arc, VMH and DMH neurones, as shown by the reduced or absent expression of Pomc, Th, Sst, Sf1 and Hmx3 (Corman *et al.*, 2018), the same nuclei and molecular markers affected in *Rax* mutants (Lu *et al.*, 2013; Orquera *et al.*, 2016). Remarkably, the loss of Shh also causes the expression of *Fgf10* and *Tbx3* to expand anteriorly (Zhao *et al.*, 2012; Corman *et al.*, 2018), similar to their expansion when *Rax* is eliminated (Orquera *et al.*, 2016). Nkx2.1, an early marker of the hypothalamus, is still expressed in both *Rax* and in neuroepithelial *Shh* mutants, showing that these genes are not important for conferring hypothalamic character per se but for the patterning/development of this region (Zhao *et al.*, 2012; Orquera *et al.*, 2016; Corman *et al.*, 2018).

In the zebrafish, *Shh* is also expressed in the hypothalamus at 30 hpf, largely colocalising with *rx3* in progenitor cells at the ventricular zone in domains I, II and III (Muthu *et al.*, 2016). As outlined above, by 55 hpf, the third ventricle has grown dorso-anteriorly and in this territory, a set of *Shh+/rx3+* progenitors is maintained; just rostral to these, *Shh+*/*rx3-* ventricular zone cells are detected (Muthu *et al.*, 2016). In *rx3* mutants, the *Shh+* dorso-anterior ventricular zone fails to form and, as discussed earlier, hypothalamic neuronal types typical for this region fail to differentiate. Thus, there is a correlation between the lack of *Shh* expression and reduced neuronal differentiation in the dorso-anterior hypothalamus in *rx3* mutants, reminiscent of what is observed in mice. Interestingly, Muthu *et al.,* (2016) showed that exposure of embryos to the Shh antagonist cyclopamine between 10 and 28 hpf blocks the induction of *rx3* in the hypothalamus and prevents the development of neurones of the dorso-anterior hypothalamus. Remarkably, however, a late exposure (28-55 hpf) of embryos to cyclopamine caused *rx3* to be upregulated, while still preventing neuronal differentiation, suggesting that a late Shh-mediated downregulation of *rx3* is needed for *rx3+* progenitors to commit to a dorso-anterior hypothalamic identity. Support for this idea was provided by a late rescue experiment, in which embryos injected with *rx3*-morpholinos (*rx3*-morphants) were exposed to

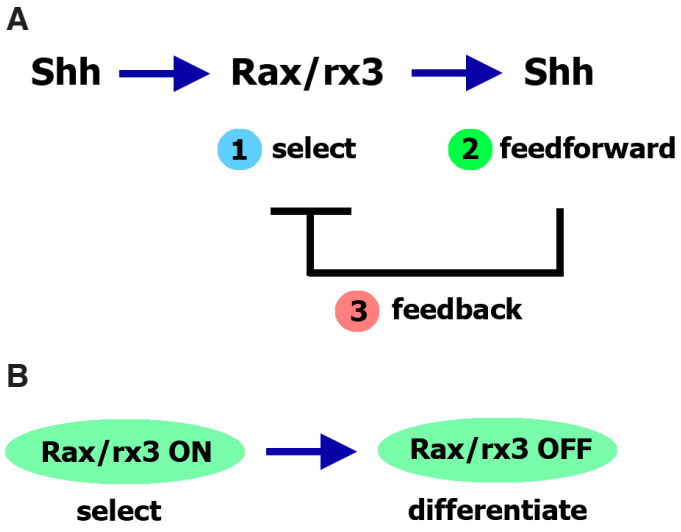

Fig. 3. Shh and Rax/rx3 regulatory circuitry in the hypothalamus. (A) *Early Shh signalling induces* Rax/rx3 *expression in hypothalamic progenitors (step 1). Then, Rax/rx3 turns on* Shh *in dorso-anterior hypothalamic progenitors (step 2). Finally, Shh signalling represses* Rax/rx3 *expression in the dorso-anterior hypothalamus (step 3), which is necessary for terminal differentiation. This regulatory circuitry has been described in zebrafish (Muthu* et al.*, 2016), with step 2 having been attested in the mouse (Orquera* et al.*, 2016).* **(B)** *Dual role Rax/rx3 in the hypothalamus. Early on, Rax/rx3 activity is needed to select progenitors that grow and expand into the dorso-anterior portion of the hypothalamus (ON); later, Shh-mediated inhibition of Rax/rx3 (OFF) is needed for neuronal differentiation to proceed (Muthu* et al.*, 2016).*

a Shh agonist (SAG, Smoothened agonist) at 28-55 hpf. In these embryos, rx3 expression in domains I and II was restored, as was the differentiation of *pomc+* and *ff1b/sf1+* neurons (Muthu *et al.*, 2016). Thus, the regulation of *Shh* and *rx3* are linked in a reciprocal feedback loop of the Shh-rx3 ON and Shh-rx3 OFF type: at first, *Shh* is needed for the induction of *rx3* in the hypothalamus; *rx3* is then needed for Shh expression in dorso-anterior progenitors, but a Shh-mediated downregulation of *rx3* is later necessary for these progenitors to give rise to terminally differentiated dorso-anterior hypothalamic neurones (Muthu *et al.*, 2016; Fig. 3 A,B).

In the mouse, it remains to be established whether *Shh* and *Rax* interact in a similar feedback loop as in the zebrafish. The induction of *Rax* has not as yet been analysed in *Shh* loss-of-function models that target the hypothalamus (Szabó *et al.*, 2009; Zhao *et al.*, 2012; Haddad-Tóvolli *et al.*, 2015; Corman *et al.*, 2018). Nevertheless, it seems clear that the role of Rax and rx3 in the patterning, growth and neuronal differentiation in the hypothalamus is, at least partially, carried out by its capacity to induce Shh expression in the neuroepithelium.

Function of *Rax* in pituitary development

The pituitary is a “master” gland that works in concert with the hypothalamus to govern the physiology of all vertebrates. It is located ventral to the hypothalamus and has two parts - the adenohypophysis and the neurohypophysis. These have distinct developmental origins: the adenohypophysis is derived from a hypophyseal placode of the oral ectoderm, which invaginates to form Rathke’s pouch, while the neurohypophysis is derived from the infundibulum (reviewed in Davis *et al.*, 2013; Pearson and Placzek, 2013; Rizzoti, 2015). The adenohypophysis is highly vascularised and contains cells that secrete key hormones that control growth, reproduction, stress and metabolic rate in response to neuropeptides/neurohormones that reach the gland from the hypothalamus. The neurohypophysis receives axonal endfeet from hypothalamic nuclei that reach the gland through the pituitary stalk and liberate the hormones oxytocin and vasopressin into the blood stream, where they regulate water balance and reproductive functions (Davis *et al.*, 2013; Rizzoti, 2015).

Rax genes are not expressed in the hypophyseal placode or adenohypophysis in any known vertebrate, but, as detailed above, *Rax1* group paralogues are expressed in the overlying hypothalamus and developing neurohypophysis (infundibulum) in *Xenopus*, chicken and mouse (Mathers *et al.*, 1997; Casarosa *et al.*, 1997; Ohuchi *et al.*, 1999; Chen and Cepko, 2002; Orquera *et al.*, 2016). Likewise, expression of *rx3* in domain III of zebrafish (Muthu *et al.*, 2016) appears to co-incide with expression of *crabp1a* (Liu *et al.*, 2013), a marker of the zebrafish neurohypophysis (Löhr and Hammerschmidt, 2011), meaning that it is highly likely that *rx3* is expressed in the neurohypophyseal anlage of zebrafish embryos, as in tetrapods.

As mentioned before, mouse *Rax* mutants do not display the evagination/growth of the infundibulum that characterises the developing neurohypophysis in tetrapods (Medina-Martínez *et al.*, 2009; Orquera *et al.*, 2016). Using chimaeric embryos consisting of wild-type and *Rax-/-* cells, Medina-Martínez *et al.*, (2009) observed that *Rax-/-* cells cannot contribute to the developing infundibulum, showing that *Rax* is required for the morphogenesis of the neurohypophysis in a cell-autonomous manner. Interestingly, *Rax-/-* cells are unable to contribute to the evaginating optic cup in mouse, suggesting a general function for Rax in establishing fields of cells with specific morphogenetic properties (i.e. cup-like evaginations) (Medina-Martínez *et al.*, 2009). Transplantation experiments in medaka (Winkler *et al.*, 2000) and zebrafish (Stigloher *et al.*, 2006) embryos indicate that *rx3-/-* cells similarly cannot populate the evaginating optic cup in teleosts, which suggests that this morphogenetic function is conserved in vertebrates (Medina-Martínez *et al.*, 2009). In the zebrafish eye field, a coherent morphogenetic field is achieved, at least in part, by the control of adhesion proteins of the Eph/Ephrin family by *rx3* (Cavodeassi *et al.*, 2013). Whether *Rax* is required for the induction and/or maintenance of neurohypophyseal-expressed genes, including adhesion molecules, remains to be determined. As mentioned earlier, *Otx2*, *Fgf8*, *Fgf10* and *Tbx3* are maintained/expanded, rather than abolished in RaxKO@8.0 mutants (Orquera *et al.*, 2016), but this could reflect the expansion of ventro-posterior hypothalamic progenitors, rather than a reflection of the function of Rax in the control of neurohypophyseal genes.

Adenohypophysis development depends on hypothalamic *Rax* expression

Importantly, the lack of *Rax* does not only affect the developing neurohypophysis. Even though the gene is not expressed in the forming or mature adenohypophysis, *Rax*-null and RaxKO@8.0 mice display an abnormally developed Rathke’s pouch at early developmental stages (E10.5-E12.5). *Rax* mutants show an expansion of the pre-pouch territory, evidenced by the appearance of multiple evaginations and the expanded expression of typical pre-pouch transcription factor genes including *Lhx3*, *Six6* and *Pit1* (Orquera

et al., 2016; Fig.1); subsequently, the lumen of Rathke's pouch remains connected to the oral cavity, instead of detaching from it (Zhang *et al*., 2000; Medina-Martínez *et al*., 2009; Orquera *et al*., 2016). Brachet *et al.,* (2019) studied *Rax*-null late embryonic (E16.5) and newborn (P0) mice. A neurohypophysis could not be identified in these mutants, and, consistent with the early defects in pouch formation and invagination, a properly-structured adenohypophysis with anterior and intermediate lobes separated by a lumen was not observed. Adenohypophyseal hormones (including adrenocorticotropic hormone (ACTH), growth hormone (GH), thyroid stimulating hormone (TSH), luteinising hormone (LH) and glycoprotein subunit alpha-chain (α-GSU)) were still detected, but were spread over a large surface of the oral epithelium, or concentrated in clusters in a tissue that did not resemble an adenohypophysis. Thus, the absence of *Rax* in the overlying hypothalamus leads to profound morphological defects in the adenohypophysis of mice. It is known that the induction and initial development of the hypophyseal placode and Rathke's pouch depends on Bmp4 (Bone morphogenetic protein 4), Fgf and Shh signals provided by the ventral hypothalamus (Ohuchi *et al*., 2000; Davis *et al*., 2013; Rizzoti, 2015; Carreno *et al*., 2017). Numerous lines of evidence indicate that the precise balance and finely-regulated expression of these signals (all of which show interregulation in the ventral hypothalamus: see Burbridge *et al.,* 2016) is critical for the proper induction and development of Rathke's pouch and the adenohypophysis. In RaxKO8.0 embryos, the defects in Rathke's pouch development are associated with an expansion of *Fgf10* expression towards the dorso-anterior hypothalamus (Orquera *et al*., 2016; Fig. 1). Likewise, the conditional elimination of *Shh* expression from the hypothalamus causes an expansion of *Fgf10* expression and the formation of extra Rathke's pouches (Zhao *et al*., 2012), while mice lacking the transcription factor *Vax1* develop an extra site of *Fgf10* expression in the neuroepithelium and an ectopic pouch adjacent to it (Bharti *et al*., 2011). Thus, it is possible that the expanded hypophyseal placode territory and the extra Rathke's pouches in *Rax* mutant mice are the result of an expansion of *Fgf10* expression in the hypothalamic neuroepithelium (Orquera *et al*., 2016). The absence of an evaginating infundibulum in *Rax* embryos might also contribute to the hypoplastic Rathke's pouch, as the infundibulum is a source of Bmp4 and Fgf signals necessary for continued proper adenohypophysis development (Davis *et al*., 2013; Rizzoti, 2015).

Shh signalling from the developing hypothalamus is likewise necessary for Rathke's pouch induction, as revealed by the conditional elimination of *Shh* expression from the anterior forebrain of mouse with a *Hesx1*-cre driver: in these embryos, a rudimentary pouch forms, but it does not express Lhx3 (the master transcriptional regulator of Rathke's pouch development) and is separated from the overlying hypothalamus by loose mesenchymal cells (Carreno *et al*., 2017). Intriguingly, the early treatment of chick embryos with the Shh antagonist cyclopamine eliminates/reduces Shh-responsive dorso-anterior progenitors, and exacts an identical pouch phenotype (Fu *et al*., 2017). Since, in mouse and zebrafish, dorso-anterior progenitors require *Rax/rx3* (see above) this raises the possibility that *Rax* indirectly governs Rathke's pouch induction via its ability to direct dorso-anterior hypothalamic progenitor development. However, although *Rax* mutant embryos lack *Shh* expression in the dorso-anterior hypothalamus, the fact that Rathke's pouch is still present and develops pituitary cell types in these mutants (Orquera *et al*., 2016; Brachet *et al*., 2019) indicates that this reduction in *Shh* expression happens after the temporal window of adenohypophysis induction by Shh. Indeed, RaxKO@E8.0 embryos are still expressing *Shh* in the developing forebrain at E9.0 (Orquera *et al*., 2016), when Rathke's pouch induction has already begun.

In the zebrafish, the adenohypophysis has a similar organisation and function as in tetrapods (reviewed in Löhr and Hammerschmidt, 2011). In addition, the processes of induction and differentiation of the zebrafish adenohypophysis also depend on Shh and Fgf (in this case, Fgf3) signals from the developing forebrain/hypothalamus (Liu *et al*., 2013; reviewed in Pogoda and Hammerschmidt, 2009). The zebrafish *rx3* mutant *chk*^{t25327} (Rojas-Muñoz *et al*., 2005), a strain that causes a less severe reduction in *rx3* than the *chk*^{w29} strain used by Muthu *et al.,* (2016), develop an adenohypophysis that expresses *pit1*, *gh*, *prolactin* (*prl*) and α*-gsu* in an appropriate way (Dickmeis *et al*., 2007). Detailed anatomical studies have not been performed, but the general organisation and development of cell populations within the adenohypophysis do not seem to be greatly disturbed in these *rx3* mutants, except for the pomc lineage (Dickmeis *et al*., 2007). The pomc prohormone is produced by two adenohypophysial cell types, namely corticotropes, where it is processed to release the peptide ACTH, and melanotropes, where it is processed as melanocyte-stimulating hormone, MSH. While the pomc-expressing melanotropes are unaltered in *rx3* mutants, corticotropes are completely absent, causing glucocorticoid deficiency in mutant fish (Dickmeis *et al*., 2007). Thus, as in mouse *Rax* mutants, a reduction in *rx3* does not affect most adenohypophyseal hormone-secreting cells. As for the pomc lineage, mice also possess corticotropes and melanotropes, located in the anterior and intermediate lobes of the adenohypophysis, respectively. No attempt has been made to differentiate corticotrope from melanotrope cell types in *Rax* mutants, but this could be done with gene markers for each lineage, for instance *Pax7*, which is specifically expressed in melanotropes (Budry *et al*., 2012).

The neurohypophysis of the zebrafish does not form from a recognisable infundibulum and no anatomical defect of this region has yet been observed in *rx3* mutants. In fish and chick embryos, Fgf3/Fgf10 signals from the developing neurohypophysis are needed for the proper vascularisation and innervation of the pituitary by hypothalamic neurones (Liu *et al*., 2013). Importantly, Fgf signals seem to act in a dose-dependent way, with attractant and repellent axon guidance effects being exerted at low and high concentrations, respectively, indicating that disturbances in Fgf expression could alter axon guidance in the neurohypophysis (Liu *et al*., 2013). It remains to be evaluated whether the innervation of the neurohypophysis by oxytocin and vasopressin neurones is altered in *Rax* and *rx3* mutants, since in these animals the distribution and intensity of Fgf3 and Fgf10 expression domains in the ventro-tuberal hypothalamus are changed.

RAX in human hypothalamic and pituitary development

Human patients carrying homozygous or compound heterozygous mutations in *RAX* (a *Rax1* paralogue, Table 1) can develop anophthalmia or microphthalmia (Voronina *et al*., 2004; Lequeux *et al*., 2008; Abouzeid *et al*., 2012; Chassaing *et al*., 2014; Brachet *et al*., 2019). In addition, homozygous point mutations in *RAX* have been found in a patient with coloboma and retinoschisis (Huang *et al*., 2017), while heterozygous mutations in *RAX* have been found in two patients with unilateral microphthalmia (González-Rodríguez *et*

al., 2010) and one patient with unilateral coloboma (London *et al*., 2009). In addition to these obvious phenotypes, a few patients with *RAX* mutations show brain abnormalities in magnetic resonance imaging (MRI) analyses, including cortical atrophy (Abouzeid *et al*., 2012) or cognitive impairments, but these have been difficult to relate to the phenotypes of *Rax1* genes in model organisms.

Recently, however, Brachet *et al.,* (2019) reported the case of a child carrying homozygous mutations in *RAX* who displays both anophthalmia and severe pituitary defects. In this patient, MRI revealed the presence of a pituitary stalk but neither the adenohypophysis nor the neurohypophysis could be visualised. Other midline defects were present, including bilateral cleft palate and absence of the sella turcica, the sphenoid bone depression where the pituitary is normally located. Consistent with the MRI observations, the patient presented several clinical signs of deficiency in the hypothalamus-pituitary axis a few days after birth, including greatly reduced levels of growth hormone, thyroxin (T4), ACTH, cortisol and testosterone, while prolactin and TSH were detected within normal levels. The patient also displayed diabetes insipidus, a possible sign of arginine vasopressin deficiency. Thus, the absence of a visible pituitary and panhypopituitarism with greatly reduced, albeit still detectable, pituitary hormones in the patient are compatible with the phenotypes observed in the mouse (Zhang *et al*., 2000; Medina-Martínez *et al*., 2009; Orquera *et al.,* 2016; Brachet *et al*., 2019). *Rax*-null mice analysed at birth lack the basosphenoid bone and palate, a phenotype that is also related to the midline defects in the patient (Brachet *et al*., 2019) and might be the result of the lack of colonisation and accumulation of mesenchyme cells between the ventral hypothalamus and the oral cavity that is observed in *Rax* mutants at early embryonic stages (Zhang *et al*., 2000; Orquera *et al*., 2016).

Patients with homozygous or compound heterozygous mutations in *RAX* usually have at least one allele with a mutation that is predicted to be less severe for protein function, such as missense mutations or truncations that preserve part of, or the whole, homeodomain (see Table 2 in Brachet *et al*., 2019). Surprisingly, the patient described by Brachet *et al.,* (2019) is homozygous for a truncating mutation in exon 1 at proline 89 (p.Pro89Argfs*114), predicted to give rise to a protein that lacks the homeodomain and thus be a null or severely hypomorphic mutation. *Rax*-null mice are non-viable and show complete penetrance of eye and hypothalamic phenotypes (Zhang *et al*., 2000), suggesting that *RAX*-null human patients should also be largely non-viable. Studies of mouse *Rax* mutants begin to resolve this puzzle, revealing a variability in certain phenotypes. For instance, *Rax*-null mice show partial penetrance of general forebrain defects, with some embryos having relatively normal heads while others have severe forebrain and craniofacial midline defects (Zhang *et al*., 2000; Voronina *et al*., 2005). Likewise, the *eyeless* mouse mutant, a *Rax* hypomorph, displays variable anophthalmia penetrance in different strains (Chase, 1942) and variable hypothalamic penetrance: 30% of homozygous mutants show defects in the SCN, while 70% have normal SCN anatomy (Silver, 1977). Thus, the phenotypic penetrance of *Rax* homozygous mutations is likely to depend on genetic background and, potentially, to stochastic developmental effects.

Clearly, the study of Rax in the human hypothalamus is an important goal, and may be aided through techniques for the directed differentiation of pluripotent cells (Sasai *et al*., 2012). Human and mouse pluripotent stem cells can be induced to differentiate into *RAX/Rax*-expressing hypothalamic progenitors (Wataya *et al*., 2008; Merkle *et al*., 2015; Wang *et al*., 2015; Ogawa *et al*., 2018), that can induce adenohypophysial tissue in culture (Ozone *et al*., 2016). This indicates that hypothalamic organoids might eventually be used to study the function of *RAX* during the development of the hypothalamus and the pituitary in humans.

Rax genes in pineal gland development

Briefly, it is worth noting that the conserved expression and function of *Rax* in the eye, hypothalamus and pituitary gland does not imply a conservation and key role in all regions of the brain. The pineal gland, for instance, a dorsal placodal/diencephalic-derived structure that secretes melatonin to adjust bodily functions to circadian rhythm, shows a variable expression, and only subtle requirement for *Rax* in different species. In *Xenopus*, mouse and rat, *Rax1* paralogues are expressed in the developing pineal gland (Casarosa *et al*., 1997; Bailey *et al*., 2004; Rhode *et al*., 2011; Rhode *et al*., 2017). Using a floxed *Rax* allele and a *Crx-cre* driver to knock-out the gene in the developing retina and pineal gland, Rhode *et al.,* (2017) showed that the mutant mice were anophthalmic, as expected, but showed only a subtle pineal phenotype: morphogenesis of the pineal was unaltered and detailed analysis of marker genes revealed only a reduced expression of the enzyme *Aanat* (aralkylamine N-acetyltransferase), which is necessary for melatonin synthesis. Expression of *Rax* genes has not been reported in the pineal of medaka, zebrafish or chick embryos (Chen and Cepko, 2002; Chuang *et al*., 1999; Deschet *et al*., 1999), but Kennedy *et al.,* (2004) and Dickmeis *et al.,* (2007) analysed the expression of *aanat2* in the pineal of different *rx3* mutant strains and found no changes in the circadian rhythm of the enzyme. Thus, the function of *Rax* in pineal gene expression is relatively subtle and seems not to be phylogeneticaly conserved.

Conclusions

Mouse *Rax* and zebrafish *rx3* play conserved roles in hypothalamic progenitors. In both model organisms, the genes are not necessary for hypothalamic induction, but are essential for the development and growth of the dorso-anterior hypothalamus and the differentiation of key neurons and nuclei that form from this region. Studies reveal that *Rax/rx3* are necessary for the expression of *Shh* in developing dorso-anterior progenitor cells at a critical temporal window, and it is very likely that the control of *Shh* expression is an essential, conserved function of *Rax* genes in dorso-anterior hypothalamic specification. The pituitary, which develops intimately with the hypothalamus, likewise requires *Rax/rx3* genes for its proper development in both species, although here, the mechanisms of *Rax/rx3* function remain unknown. Similar to dorso-anterior hypothalamic progenitors, neurohypophyseal cells appear to require Rax cell-autonomously; by contrast, adenohypophyeal development depends on an unknown non cell-autonomous function for Rax. The phylogenetic conservation of important cellular and mechanistic details for Rax/rx3 function remains to be tested, even in the specification of dorso-anterior hypothalamic progenitors. For instance, in the zebrafish, *rx3* and *Shh* are involved in an intricate regulatory loop that has not been described in the mouse, but which could be explored using the many conditional *Shh* mutant mice available. In addition, zebrafish *rx3* controls the differential growth of dorso-anterior progenitors

but thorough proliferation analyses in mouse *Rax* mutants are still to be performed. In future, comparative transcriptomic analysis focused on the hypothalamus of *Rax/rx3* mutants might further unveil the conserved and divergent molecular functions of these genes. Work on *Rax* function in the hypothalamus of other models including *Xenopus* and chicken embryos, as well as human hypothalamic organoids, will help to elucidate a fuller picture of the role and mechanism of action of these genes. Importantly, the work on *Rax/rx3* in model organisms, as well as case studies of human patients carrying *RAX* mutations, illustrates how different and complementary approaches in different organisms can synergise to shed light on the development of this ancient structure of the vertebrate brain.

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