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

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

RUNNING TITLE: Rax in hypothalamic and pituitary development

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

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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 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.
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 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.

**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 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 RaxKO@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\textsuperscript{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, i.e. the cells that will normally undergo neurogenesis and 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 lack 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 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. 3A,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 bloodstream, 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 coincide 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-null cells, Medina-Martínez et al. (2009) observed that Rax-null 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-null 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-null 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 inter-regulation 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<sup>125327</sup> (Rojas-Muñoz et al., 2005), a strain that causes a less severe reduction in rx3 than the chk<sup>p29</sup> 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 adenohypophysial 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-tuberan 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., 2025; 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 phylogenetically 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, adenohypophyseal 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, \textit{rx3} and \textit{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 \textit{Shh} mutant mice available. In addition, zebrafish \textit{rx3} controls the differential growth of dorso-anterior progenitors but thorough proliferation analyses in mouse \textit{Rax} mutants are still to be performed. In future, comparative transcriptomic analysis focused on the hypothalamus of \textit{Rax/rx3} mutants might further unveil the conserved and divergent molecular functions of these genes. Work on \textit{Rax} function in the hypothalamus of other models including \textit{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 \textit{Rax/rx3} in model organisms, as well as case studies of human patients carrying \textit{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.

\textit{Acknowledgements}

Work in FSJS’s laboratory is supported by CONICET and ANPCyT (PICT 2015-3566), Argentina and in MP’s laboratory is supported by the Wellcome Trust (212247/Z/18/Z).

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### 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*        | -            |
**FIGURE S**

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 ventro-tuberal 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 arise from ventro-tuberal progenitors).
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).
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).