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Homeobox genes in the rodent pineal gland: roles in development and phenotype maintenance

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Abstract

The pineal gland is a neuroendocrine gland responsible for nocturnal synthesis of melatonin. During early development of the rodent pineal gland from the roof of the diencephalon, homeobox genes of the orthodenticle homeobox (Otx)- and paired box (Pax)-families are expressed and are essential for normal pineal development consistent with the well-established role that homeobox genes play in developmental processes. However, the pineal gland appears to be unusual because strong homeobox gene expression persists in the pineal gland of the adult brain. Accordingly, in addition to developmental functions, homeobox genes appear to be key regulators in postnatal phenotype maintenance in this tissue.

In this paper, we review ontogenetic and phylogenetic aspects of pineal development and recent progress in understanding the involvement of homeobox genes in rodent pineal development and adult function. A working model is proposed for understanding the sequential action of homeobox genes in controlling development and mature circadian function of the mammalian pinealocyte based on knowledge from detailed developmental and daily gene expression analyses in rats, the pineal phenotypes of homeobox gene-deficient mice and studies on development of the retinal photoreceptor; the pinealocyte and retinal photoreceptor share features not seen in other tissues and are likely to have evolved from the same ancestral photodetector cell.

Keywords

pineal gland; homeobox gene; rodent; brain development; circadian rhythm

1. Introduction: homeobox genes as developmental regulators

Homeobox genes encode a large family of conserved transcription factors; these genes are molecularly defined by the presence of the homeobox, a well-defined 180 bp sequence encoding a definitive DNA-binding homeodomain [1,2]. Homeobox genes are involved in regulation of developmental processes, cellular differentiation and morphogenesis in all metazoans [3]. The initially identified homeobox genes, referred to as Hox genes, are arranged in genomic clusters, the organization of which closely resembles the nested expression patterns along the cranio-caudal axis of the animal [4]. This relation is now the text book example of colinearity; however, no Hox code exists for the most rostral part of

the central nervous system, where development is controlled by so-called dispersed homeobox genes including members of the Otx (orthodenticle homeobox), Rax (retina and anterior neural fold homeobox) and Pax (paired-box) families.

Although the general developmental role of homeobox genes is well-established, a growing body of evidence suggests a second function in healthy adult tissues [5,6] primarily including cell types undergoing frequent renewal such as blood cells, endometrium and epithelia of the gastrointestinal tract; in these tissues, homeobox genes appear to be involved in adult cell proliferation and differentiation. However, within recent years persistent and restricted expression of homeobox genes in the postnatal central nervous system especially in the pineal gland has been reported [7–11]. In the following sections, we highlight ontogenetic and phylogenetic aspects of pineal development and recent progress in understanding the roles homeobox genes play in the rodent pineal gland throughout life.

2. Development and circadian function of the mammalian pineal gland

The mammalian pineal gland is a neuroendocrine organ, which converts photoperiodic information into the nocturnal hormonal signal of melatonin [12]. In most mammals, the pineal gland is located directly on the dorsal part of the brain stem at the mesencephalic-diencephalic border, whereas in rodents it is subdivided into a superficial part located on top of the brain just caudal to the hemispheres and a deep part consisting of a collection of pinealocytes located between the habenular and posterior commissures. The superficial and deep parts are connected by a thin pineal stalk (Fig. 1). The parenchyma of the pineal gland consists of the predominant melatonin-producing pinealocytes (about 95%), astrocyte-like interstitial cells (about 4%) and macrophages (< 1%) [13].

2.1 Cellular development of the rat pineal gland

The pineal gland develops as a tubular evagination, the pineal recess, from the most caudal part of the diencephalic roof between the habenular and posterior commissures (Fig. 1). The pineal evagination appears around embryonic day 16 (E); in the following days of development, the pineal recess elongates (E18), before the lumen gradually disappears. Around birth, the pineal gland appears as a solid organ with a small proximal cleft communicating with the third ventricle (E21), but postnatally, the superficial pineal gland is only connected to the third ventricle via the pineal stalk and the deep pineal gland (P30) [14–16]. Pineal cell proliferation is high in the prenatal gland and ceases rapidly during the two first postnatal weeks [17]. These observations are in line with ultrastructural studies showing that pre- and neonatal pineal cells are undifferentiated pinealoblasts and that differentiation into pinealocytes takes place also during the first two weeks of postnatal life [18,19], thus suggesting that the pinealocytes should be regarded as mature cells afterwards.

2.2 Development of pineal circadian function

The physiological function of the pineal gland is to entrain circadian rhythms to the daily photoperiod via nocturnal secretion of melatonin; melatonin is synthesized from serotonin by sequential action of the pineal enzymes arylalkylamine *N*-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT). The activity of AANAT exhibits large daily changes driving the daily rhythm in pineal melatonin synthesis [20]. The pineal gland is innervated by peripheral ganglia and directly from the central nervous system; the most prominent input to the gland is the dense innervation with sympathetic fibers originating from perikarya in the superior cervical ganglia [21]. The sympathetic nerve terminals in the pineal are located in the perivascular spaces but also penetrate into the pineal parenchyma; however, the sympathetic terminals do not make synapse-like contacts with pinealocytes. These sympathetic fibers constitute the last part of a multisynaptic pathway linking the

hypothalamic circadian master clock in the suprachiasmatic nucleus to nocturnal release of norepinephrine from sympathetic nerve terminals in the pineal gland. In the pineal gland, norepinephrine elevates the levels of cyclic AMP, which in turn induces a nighttime increase in AANAT enzyme activity [20]. In the rodent pineal gland, the nocturnal increase in AANAT activity is regulated at transcriptional and posttranslational levels. There is a prominent night-day rhythm (>100-fold) in AANAT transcript abundance [22]. This rhythm is mediated by *cis*-regulatory cyclic AMP responsive elements (CREs) in the *Aanat* promoter [23]; transcription is activated by the CRE-binding protein (CREB) upon cyclic AMP-dependent phosphorylation by protein kinase A [24]. Daily rhythms in the pineal transcriptome are broadly driven by this norepinephrine-cyclic AMP mechanism [10]. At the posttranslational level, AANAT activity is controlled by nocturnal cyclic AMP-dependent phosphorylation by protein kinase A; the phosphorylated AANAT reversibly binds 14-3-3 protein and represents the stable and activated form of AANAT [20].

Preceded by the developmental appearance of transcripts encoding the above mentioned melatonin enzymes (Fig. 2), melatonin synthesis is first detectable at P5 in the rat pineal gland [25]. However, day-night rhythms in pineal melatonin synthesis depend on both structural and molecular maturation of the circadian system [26,27], including establishment of the projections terminating with the sympathetic innervation of the pineal. In the rat, sympathetic fibers have been shown to penetrate the pineal parenchyma at P5 [28]; this time point coincides with the first rhythmic expression of the *Aanat* gene [29] as well as rhythmic AANAT enzyme activity [30]. The amplitude in AANAT activity rapidly increases, and rhythmic pineal melatonin synthesis is detectable between P8 and P11 [31,32], thus marking the ontogenetic establishment of mature pineal physiological function.

In addition to control of daily rhythmicity, the sympathetic innervation of the pineal may also have direct developmental implications on the mammalian pinealocyte. Light sensing properties of the neonatal pineal gland are supported by experiments showing that neonatal pinealocytes in culture display rhodopsin immunoreactivity [33] and that exposure to light inhibits melatonin production [34]. Including norepinephrine in the culture medium, thereby mimicking the sympathetic innervation, abolishes the apparent photosensitive capacity of the neonatal pinealocyte [33,34]. This set of observations suggests that the sympathetic innervation of the gland suppresses early photoreceptor-like characteristics of the immature pinealocyte and may be essential for proper pinealocyte maturation.

3. Evolution of the mammalian pinealocyte: pineal and retinal similarities

The capacity to synthesize melatonin appears to have evolved in an ancestral photodetector cell very early in chordate evolution as a detoxification pathway [35,36]. This ancestral photodetector cell is thought to have diverged into two lineages in vertebrates, one being the retinal photoreceptor optimized for efficient phototransduction and the other being the pinealocyte optimized for melatonin synthesis.

The pineal gland of sub-mammalian vertebrates, e.g. fishes, amphibians, reptiles and birds, is a complex photoreceptive organ located directly beneath the skull or as in amphibian and reptilian species with an extracranial location referred to as “the third eye” [37,38]. This extracranial portion, also known as the frontal or parietal organ, is connected to the brain via a pineal nerve and tract. In most sub-mammalian species, the pineal organ is composed of cells endowed with both light-sensing properties [39,40] and nocturnal melatonin production guided by daily oscillations in AANAT activity [41,42]. Light sensing properties of the pineal gland of non-mammalian vertebrates have been interpreted as an earliest phylogenetic evidence of a common ancestral origin of the pinealocyte and the retinal photoreceptor.

Based on ultrastructural studies on the pineal organ of various vertebrates, Collin [43] divided the cells of the pinealocyte lineage into three classes: 1) The *true pineal photoreceptor* found in anamniotes bear an outer segment consisting of numerous discs connected to an inner segment via a cilium, thus closely resembling the retinal photoreceptor; furthermore, the inner somal part with the perikaryon of the true pineal photoreceptor forms synapse with intrapineal neurons connecting the pineal photoreceptors to the brain via the pineal nerve [44]. 2) The *rudimentary pineal photoreceptor*, found in most reptile and avian species, is characterized by a less regular light-sensing outer segment and does not synapse with intrapineal neurons. However, secretory granules are often seen in the basal part of these photoreceptors. 3) The *mammalian pinealocyte sensu strictu* lacks outer and inner segments, but a cilium can be found in some pinealocytes [43,45]. These morphological characteristics presumably reflect a gradual evolutionary transformation of a sensory cell line, like the retinal photoreceptor, into the mammalian secretory pinealocyte without photoreceptive properties [43]. A later revision of the theory of pineal evolution stresses the importance of changes in molecular regulatory mechanisms within the pineal field of the neural plate during vertebrate evolution restricting the development of pineal photoreceptors in mammals [46]; this implies changes in cell fate restriction rather than transformation within a single cell lineage [47].

The close relationship between the retinal photoreceptor and the pinealocyte is also supported by the results of ontogenetic studies in mammals. In neonatal rats, a subset of the developing pinealocytes transiently exhibits photoreceptor characteristics, e.g. rudimentary outer segments [48]. This is in accord with gene expression studies showing that the pineal gland of neonatal rats expresses all of the molecular components required for establishing functional phototransduction [49]. The transient existence of a pineal nerve in fetal mammals connecting the pineal and dorsal diencephalon, homologous to the pineal tract and nerve in other vertebrates, also supports a probably rudimentary sensory property of the early mammalian pineal gland [50,51].

Further evidence for a common origin of the pinealocyte and retinal photoreceptor comes from studies showing that numerous gene products otherwise involved in retinal phototransduction are also expressed in the adult mammalian pineal gland (Fig. 2; Fig. 3). These include rhodopsin kinase [52], S-antigen [53], phosducin [54], recoverin [55], interphotoreceptor retinol-binding protein [56], aryl-hydrocarbon-interacting protein-like 1 [10], phosphodiesterase 6B [10] and uncoordinated protein 119 [10]. Whereas rhodopsin expression is either regarded as diffuse or absent in the adult pineal gland [57], the expression of shortwave opsin is similar to that in the retina [10]. Similarly, the retina expresses genes dedicated to melatonin synthesis, albeit at distinctly lower levels than in the pineal gland; the capacity to synthesize melatonin in small quantities is seen in all vertebrates [58–60]. Retinal *Aanat* expression is localized in photoreceptors [61,62] and exhibits a day-night rhythm [63], but as opposed to the sympathetic neural regulation in the pineal gland, retinal *Aanat* transcription is driven directly by the endogenous retinal circadian clock via E-boxes in the *Aanat* promoter [64].

The most parsimonious explanation of pineal-retinal similarities is that they are due to expression of a common set of homeobox genes, which direct developmental regulatory functions and tissue-specific expression of genes involved in melatonin synthesis and phototransduction in both the retina and the pineal gland (Fig. 2; Fig. 3).

3. Homeobox genes in pineal development

Homeobox genes including members of the Pax- and Otx-families as well as *Rax* are detectable in the pineal gland. Among these, *Otx2* and *Pax6* are essential for development of the murine pineal [65,66].

3.1 Pax genes in pineal gland development

Pax6 is extensively expressed in the developing central nervous system [67]. An intriguing aspect of *Pax6* biology is its association with eye development throughout the animal kingdom [68]; *Pax6* exhibits a diversity of regulatory functions in several cell lineages of the eye [69]. *Pax6*-deficient mice exhibit an early arrest of eye morphogenesis with a severe reduction in proliferation of retinal progenitor cells [70–72]. Further, *Pax6* maintains the multipotent state of retinal progenitors and controls the timing of differentiation [73], thus showing an early developmental role of *Pax6* in the retina.

Detailed quantitative *in situ* hybridization analyses of *Pax6* expression in the developing rat pineal gland from E16 into adulthood have revealed a strong prenatal expression peaking around E18 (Fig. 2) [8]. *Pax6* transcripts are detectable in the dorsal roof of the diencephalon and the adjacent pretectal area with a remarkable signal in the pineal recess (Fig. 4A), which also exhibits a strong PAX6 immunoreactivity (Fig. 4B), establishing PAX6 as an early marker of pineal development. *Pax6*-deficient mice exhibit a lack of diencephalic dorsal midline structures, including the pineal recess [66]; mutations in *PAX6* results in the absence of the pineal gland in human subjects [74,75]

The close relationship between the retina and the pineal gland, as well as the known function of *Pax6* in retinal development, makes it reasonable to compare the expression pattern of *Pax6* in the pineal gland to that of the rat retina [76], in which *Pax6* expression peaks in undifferentiated retinal progenitor cells during the last intrauterine days (Fig. 3). At the time of *Pax6* dominance in the developing gland, the pineal cells are undifferentiated pinealoblasts [8,18], suggesting that pineal *Pax6* in line with the situation in the developing retina controls proliferation and maintenance of differentiation potential of immature pinealocytes in early pineal gland development (Fig. 5A).

In both the pineal gland and retina, the prenatal *Pax6* expression peak is followed by a rapid perinatal decline (Fig. 2; Fig. 3), which is in line with a terminated developmental role. Notably, *Pax6* expression persists in the inner retina [76] and a limited number of pinealocytes are also PAX6-immunopositive in the adult pineal gland (Fig. 4C); however, adult functions of *Pax6* in these non-renewable tissues are currently unknown. The PAX6-immunopositive pinealocytes of the mature pineal gland may represent a population of pinealocyte precursors.

The extensive diversity of the pineal transcriptome has been revealed by microarray analysis [10]; among the detected transcripts were *Pax4*, which is closely related to *Pax6*, and is essential for development of mature insulin-producing β -cells in the pancreas [77]. Ontogenetic analysis of *Pax4* in the rat pineal revealed an expression pattern, which is in marked contrast to that in the pancreatic islets. Pineal *Pax4* expression starts around birth and persists into adulthood [8], whereas the gene is strongly expressed early in prenatal pancreatic development [77]. Accordingly, it appears that whereas *Pax4* primarily has a developmental role in the pancreas, a regulatory role in the differentiated pinealocyte is more likely in the pineal gland (Fig. 5A). A regulatory function in the mature pinealocyte is supported by the observation that the developmental appearance of pineal *Pax4* transcripts is coincident with that of genes required for core pineal functions, e.g. melatonin synthesis (Fig. 2). A similar pattern of expression has been reported in the developing retina of the rat

(Fig. 3), where expression is restricted to postnatal photoreceptor cells [76]. Studies on the pancreas have emphasized the antagonizing *trans*-repressing functions of PAX4 on promoters that are otherwise activated by PAX6. [78]. In mature pinealocytes and retinal photoreceptors, the antagonizing actions of PAX4 therefore may counteract the developmental role of PAX6 to ensure maintenance of the fully differentiated phenotype. Studying the specific function of *Pax4* in the rodent pineal gland has been frustrated by the perinatal death of the *Pax4* knockout mouse [77] and also the difficulties in analyzing *Pax4* in the mouse pineal gland due to very low expression levels, which suggest a limited degree of conservation in pineal *Pax4* biology among rodents.

3.2 *Otx* genes in pineal development

Members of the mammalian *Otx*-family expressed in the pineal gland include *Otx2* and *Crx* [7,79–81]. *Otx2* is expressed from embryonic pre-gastrulation stages and in the developing central nervous system becomes restricted to the pros- and mesencephalic brain regions [79]; *Otx2* knockout mice fail to develop rostral head structures [82,83]. As is the case for *Pax6*, a broad involvement in brain development is accompanied by a central role in eye development [82]. However, within the retina, *Otx2* acts at later developmental stages than the progenitor-promoting *Pax6* to steer the progenitors towards a photoreceptor fate [65,84]. This division of labor is reflected by the delayed temporal expression of *Otx2* as compared to *Pax6* (Fig. 3). In contrast to the broad involvement of *Otx2* in development of the central nervous system, *Crx* expression and function is strongly restricted to the pineal gland and retinal photoreceptors [81,80,85]. The *Crx*-deficient retina displays a reduction in cellular integrity of the outer nuclear layer and absence of photoreceptor outer segments [85]. OTX2 is known to *trans*-activate *Crx* [65], which is expressed later in the retinal photoreceptors (Fig. 3) [86] and is believed to induce terminal differentiation of the photoreceptor cells [65,84].

Otx2 expression is detectable in the rat pineal gland at all developmental stages (Fig. 2)[7]; this pattern is in accord with the involvement of *Otx2* in both development of the pineal gland [65,87] and maintenance of the pinealocyte phenotype later in life (Fig. 5). A conditional knockout mouse, in which the *Otx2* gene is specifically inactivated in pinealocytes and retinal photoreceptor cells, has made it possible to investigate the specific role of *Otx2* in these tissues [65]; this mouse exhibits a total lack of pinealocytes and retinal photoreceptors, indicating an essential role for *Otx2* in development of both cell types. In this model, the *Crx* promoter was used to drive tissue-specific *Otx2* deletion [65]; since pineal *Crx* expression in the rat pineal is initiated at E18 [7], well after establishment of the *Otx2* expressing pineal recess, the role of *Otx2* in initiation and early development of the pineal is not established. However, based on the high expression levels during early pineal development (Fig. 2), it seems appropriate to suggest a role for *Otx2* in early pinealocyte specification (Fig. 5A), albeit at a later developmental stage than *Pax6*. Furthermore, the postnatal lack of pineal gland in the *Otx2* conditional knockout indicates a role of *Otx2* in promoting survival of immature pinealocytes.

The pineal gland of the *Crx* knockout develops normally [85,88], suggesting that *Crx*, in contrast to the situation in the retina, is not essential for pineal gland development. However, double knockout studies suggest that the apparent integrity of the pineal gland is due to complementation by *Otx2* [87]. As indicated above, pineal expression of *Crx* starts and peaks later than that *Otx2* (Fig. 2); this is in agreement with the *trans*-activating function of OTX2 on the *Crx*-promoter [65] and a later role of *Crx* in pineal development as compared to *Otx2*. *Crx* expression peaks around birth and remains high in the first postnatal weeks supporting a role in terminal differentiation of the mature pinealocyte (Fig. 2; Fig. 5), as is the case in the retinal photoreceptor. Notably, CRX itself *trans*-activates and confers tissue-specific expression of a number of genes involved in phototransduction and melatonin

synthesis in the pineal gland and retina [80,81,85,88–91] (see section 4). In both tissues, the developmental appearance of *Crx* transcripts immediately precedes that of melatonin- and vision-related gene products with clearly defined physiological roles (Fig. 2; Fig. 3).

The similarity in sequential expression patterns of each homeobox gene in the pineal gland and retina indicates that these genes may have conserved roles in development of each tissue. In this regard, the appearance and peak of *Pax6* transcripts before *Otx2* and *Crx* support current knowledge on *Pax6* functions in retinal progenitors, the role of *Otx2* in promoting photoreceptor precursors as well as *Otx2* and *Crx* in differentiating photoreceptors. In general, the appearance and peak in homeobox gene expression is delayed in the retina as compared to the pineal gland (compare Fig. 2 and Fig. 3); this is consistent with the observation that mature pineal function, e.g. melatonin synthesis, is developed prior to functional retinal phototransduction in the rodent [25,92]. This difference in physiological maturation is reflected by the developmental expression patterns of genes involved in melatonin synthesis and phototransduction in the pineal gland and retina, respectively.

3.3 *Rax*: pineal developmental aspects

Rax is another homeobox gene widely expressed in the developing forebrain controlling development of rostral brain structures [93,94]. *Rax* is essential for eye morphogenesis and maintains proliferative activity of retinal progenitors [95]; however, dual roles also involving regulation of postnatal photoreceptor-specific gene expression have been reported [96,97].

Rax expression has been reported in the rat pineal gland [9,10,98]. The developmental profile of *Rax* in the pineal gland shows a late onset in its expression (Fig. 2) as compared to expression in the retina (Fig. 3)[9]. Also, whereas *Otx2* and *Pax6* are widely expressed in the dorsal mesencephalic and diencephalic areas with expression being progressively restricted to the pineal [7,8], *Rax* expression is strongly initiated at E20 in the pineal gland well after establishment of the pineal anlage (Fig. 1). Therefore, it seems unlikely that *Rax* is required for early development of the pineal gland, as is the case in the retina; rather, it would appear that *Rax* is involved in terminal differentiation and maintenance of pinealocyte phenotype (Fig. 5). *RAX* has been shown to *trans*-activate *Otx2* during retinal photoreceptor determination [99]; however, given the temporal expression profiles of these genes in the rodent pineal (Fig. 2), a similar relationship in immature pinealocytes is unlikely. The *Rax* knockout mouse fails to develop the forebrain [93]; thus making analysis of the pineal gland impossible. However, a recently developed conditional knockout mouse, in which *Rax* is specifically deleted in the pineal gland and retinal photoreceptors [99,100], may shed light on the function of *Rax* in pineal development. It has previously been pointed out that a truly pineal-specific and pineal-defining homeobox gene has not been identified [101]; in this regard, the temporal sequence rather than the final composition of homeobox gene products may be the defining feature of the mammalian pinealocyte restricting the cell towards a pinealocyte fate rather than a photoreceptor phenotype. This interpretation would be in line with the previously mentioned proposed theory of pinealocyte evolution depending on cell fate restriction in the pineal area of the neural plate [46,47]. Thus, the absence of a developmental initiator, such as *Rax*, early in pineal development may prevent the immature pinealocyte from establishing photoreceptor features.

4. Homeobox genes in the mature pinealocyte

As indicated in the introduction, gene products encoded by a number of homeobox genes have been identified in the mature pineal gland [7–11]. Among these, members of the *Otx*-

family are involved in conferring tissue-specific melatonin synthesis, whereas other homeobox genes also display daily variations.

4.1 Tissue-specific melatonin synthesis

Melatonin synthesis is restricted to pinealocytes and the retinal photoreceptor cells. Pineal and photoreceptor-specific gene expression is directed by *cis*-regulatory photoreceptor conserved elements (PCE; consensus TAATT/C[102]); several PCE copies are found in the promoters of mammalian pineal and photoreceptor-specific genes [81,89,91,103,104]. Different subtypes of PCEs bind both OTX2/CRX and RAX proteins though with slightly different preferences and affinities [96]. In the zebrafish, PCEs are essential for the CRX-related OTX5 transcription factor in mediating expression of pineal specific genes including *Aanat* [105,106]. Thus, in regulation of pineal gene expression the PCE motif conferring tissue-specificity seems to be a highly conserved feature from fishes to mammals, whereas the norepinephrine-responsive CRE is a mammalian feature for generating rhythmic pineal gene expression replacing the clock-driven E-box in non-mammalian vertebrates (Fig. 5B). Notably, in regulation of tissue-specific *Aanat* expression in the mammalian pineal gland, an E-box sequence has been shown to inhibit ectopic *Aanat* expression, thus introducing another level of tissue-specificity control [107].

OTX2 and CRX are persistently expressed in the pineal gland of the adult rat (Fig. 2; Fig. 6) [7]; this is also the case in retinal photoreceptor cells [86]. OTX2 and CRX have been shown to be involved in transcriptional regulation of the two last enzymes in melatonin synthesis [88]; direct *trans*-acting PCE-dependent links, e.g. promoter binding and activation, have been identified between OTX2 and *Asmt* [108] as well as CRX and *Aanat* [85,88,89] and *Asmt* [90]. Since both transcription factors seem to act by binding the same PCEs, the division of labor between CRX and OTX2 is unclear, and is further complicated by the fact that both transcription factors *trans*-activate *Crx* itself (Fig. 5B) [65]. The *Crx* knockout displays only a reduced pineal expression of *Aanat* [88], suggesting that OTX2 or other factors compensate for the lack of CRX. Notably, OTX2 and CRX are present in both the superficial and deep pineal gland (Fig. 6). The molecular machinery for melatonin synthesis, that is expression of *Aanat* and *Asmt* [109,110], is also present in the deep pineal gland (Fig. 6), which is in line with the common ontogenetic origin of these structures (Fig. 1).

RAX has been shown *in vitro* to at least partly exert its function by cooperating with CRX to ensure high expression of photoreceptor genes [96]. Although experimental data in pineal systems are lacking, we hypothesize that RAX acting on PCEs in pineal promoters may also generate high expression of pineal-specific genes in synergistic cooperation with CRX and OTX2 in the mature pinealocyte (Fig. 5B). From a developmental point of view, the expression of *Otx2*, *Crx* and *Rax* peaks perinatally, thus temporally correlated with the first detectable expression of genes involved in melatonin synthesis (Fig. 2); this supports a role for these transcription factors in controlling development of the physiologically mature pinealocyte (Fig. 5A).

In summary, the two-fold rhythms detected for *Crx*, *Otx2* and *Rax* in the pineal gland [7,9,10,89] do not imply that these genes are not involved in controlling the rhythmic nature of genes that display marked circadian rhythms in the pineal gland, such as *Aanat* exhibiting a 150-fold daily rhythm [22,20] (Fig. 7). Although the proposed synergistic action of CRX and RAX may generate a more than two-fold rhythm in PCE-based *trans*-activating potential, we suggest a model where homeodomain proteins acting on PCEs confer tissue-specificity, whereas the norepinephrine-induced increase in cyclic AMP generates CRE-based circadian rhythmicity (Fig. 5B).

4.2 Daily rhythms in pineal homeobox gene expression

A large number of transcripts display daily rhythms in the rat pineal gland [10]. *Pax4*, *Crx*, *Otx2* and *Rax* are rhythmic in the pineal (Fig. 7)[8,9], whereas *Pax6* is expressed at the same levels throughout the day [7,8]. *Rax* and *Crx* display moderate two-fold rhythms in the pineal gland and are both arrhythmic in the retina [9,89]; *Pax4* exhibits a larger six-fold rhythm in both tissues [8,76]. These are not clear-cut day-night phenomena as is the case for *Aanat* in the rodent pineal gland [22] (Fig. 7): *Pax4* expression peaks during daytime, *Rax* expression is high at the day-night transition, the peak in *Otx2* appear early in the night, and only the expression of *Crx* appears in the middle of the night preceding the nocturnal peak in *Aanat*. As indicated above, RAX and CRX/OTX2 acting through PCEs may in concert modulate, fine-tune or even boost the daily expression profile of pineal-specific genes displaying cyclic AMP/CRE dependent rhythms. This interpretation is consistent with the sequence of peaks in the daily expression of *Rax*, *Otx2*, *Crx* and *Aanat* (Fig. 7).

As described above, a multisynaptic pathway passing through the superior cervical ganglia controls pineal functions, including the daily rhythm in melatonin synthesis [12,13]. Removal of these ganglia blocks sympathetic neural stimulation of the gland. The rhythmic expression of *Pax4* in the pineal gland is abolished by ganglionectomy, but sustained high levels of the *Pax4* transcript are detectable [8]. The *Pax4* rhythm is driven by nocturnal suppression of transcript levels via norepinephrine acting through cyclic AMP. This inverse relationship between norepinephrine and *Pax4* transcript levels is in marked contrast to the inducing effect of adrenergic stimulation on pineal levels of the *Aanat* transcript, which is reduced to constitutive daytime values by superior cervical ganglionectomy [22]. On the other hand, midday and midnight pineal expression of *Otx2* is not affected by ganglionectomy [7]. These observations suggest that although the input of norepinephrine to the gland is important for rhythmic gene expression, the intrinsic non-rhythmic expression of homeobox genes in the pineal is not influenced by the sympathetic input to the gland.

5. Future directions

A number of pineal homeobox genes, in addition to *Pax*, *Otx* and *Rax*, have been reported and analyzed to variable degree. Among these, the brain-specific homeobox (*Bsx*) is expressed in the pineal gland of the mouse [11], and the *Bsx* knockout exhibits a hypoplastic pineal gland, suggesting a cell proliferative or cell survival function of *Bsx* in rodent pineal gland development [111]. Contrarily, in the *Xenopus* pineal organ, *Bsx* promotes photoreceptor fate while restricting pineal cell proliferation [112], thus suggesting a relatively late role for this gene in development of the *Xenopus* pineal photoreceptor. Certain LIM homeobox (*Lhx*) genes also exhibit a conserved pineal-specific pattern of expression as evidenced by investigations in several vertebrate species including the mouse [113]. Other only sporadically described homeobox genes expressed in the rodent pineal gland include ALX homeobox 4 (*Alx4*) [98] and msh homeobox 1 (*Msx1*) [10]. Detailed comparative ontogenic analyses of these homeodomain transcription factors will help to elucidate the transcriptional network underlying pineal gland development and adult physiology.

The content of this paper has been restricted to homeobox genes. Drawing on knowledge on development of the retinal photoreceptor, other transcriptional regulators such as the basic helix-loop-helix transcription factors may in combination with homeodomain proteins also be important in development and mature function of the pineal gland. Among these, the basic helix-loop-helix transcription factor *NeuroD* has been shown to modulate transcription in both the developing and mature rodent pineal gland [114,115]. The zebrafish pineal organ has been used as a model for understanding neuronal cell fate determination; these studies have revealed the involvement of the BMP (bone morphogenic protein) signaling pathway

in defining pineal neuronal subtypes and positioning the epiphyseal expressing domain of the homeobox gene *floating head* [116,117], which is required for zebrafish pineal development [118]. In specification of pineal cell identity, *floating head* acts via basic loop helix downstream targets including *NeuroD* [119]. Similar mechanisms may be involved in development of the mammalian pineal gland.

Recent data show that the microRNA miRNA-483 suppresses pineal *Aanat* expression and exhibits an inverse developmental expression pattern with high levels early in development [120], thus suggesting that maturation of pineal physiology in addition to a specific composition of homeodomain transcription factors may also involve a timely controlled relief from suppressing microRNAs.

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Figure 1. Anatomy of the developing pineal gland

Nissl-stained sagittal sections of the brain including the epithalamus of rats at indicated developmental ages. E, embryonic day; P, postnatal day. Scale bar, 1 mm.

Figure 2. Ontogenetic expression of homeobox gene transcripts (upper part) and gene products involved in melatonin synthesis (middle part) and phototransduction (lower part) in the rat pineal gland

Curves are based on data from several studies on the developing rat pineal gland: **Aanat**, arylalkylamine N-acetyltransferase [29,30]; **Crx**, cone-rod homeobox [7]; **Asmt**, acetylserotonin O-methyltransferase or hydroxyindole O-methyltransferase [32,121]; **Otx2**, orthodenticle homeobox 2 [7]; **Pax4**, paired box 4 [8]; **Pax6**, paired box 6 [8]; **Pdc**, phosducin or MEKA [122]; **Rax**, retina and anterior neural fold homeobox [9]; **Rhok**, rhodopsin kinase or G protein-coupled receptor kinase 1 [123]; **Sag**, S-antigen or arrestin [122]; **Tph1**, tryptophan hydroxylase 1 [28,124].

Figure 3. Ontogenetic expression of homeobox gene transcripts (upper part) and gene products involved in phototransduction (lower part) in the rat retina

Curves are based on data from several studies on the developing rat retina: **Crx**, cone-rod homeobox [86]; **Irbp**, interphotoreceptor retinol-binding protein or retinol binding protein 3 [125]; **Otx2**, orthodenticle homeobox 2 [86]; **Pax4**, paired box 4 [76]; **Pax6**, paired box 6 [76]; **Pdc**, phosducin or MEKA [122]; **Rax**, retina and anterior neural fold homeobox [9]; **Rho**, rhodopsin or opsin [126]; **Rhok**, rhodopsin kinase or G protein-coupled receptor kinase 1 [123]; **Sag**, S-antigen or arrestin [122]

Figure 4. PAX6 is an early marker of pineal development and is only detectable at low levels in the adult

A) *In situ* hybridization for detection of Pax6 mRNA in a sagittal section of the rat brain at E16. Note the strong signal in the epithelium around the pineal recess (arrow). Di, diencephalon; Mes, mesencephalon; Rhomb, rhombencephalon; Tel, telencephalon. Scale bar, 1 mm. **B)** Immunohistochemical detection of PAX6 protein in the developing rat pineal gland at E17. Scale bar, 50 μ m. The fetal animal was not perfused; therefore, the very dark cells are erythrocytes with a high level of endogenous peroxidase. **C)** Immunohistochemical detection of PAX6 protein in the pineal gland of a perfusion fixed adult rat. Scale bar, 50 μ m. For methodological details, see [76].

Figure 5. Potential roles of the pineal homeobox genes

A) Development of the pinealocyte: The proliferation, differentiation and maturation processes of the mammalian pinealocyte are driven by homeodomain transcription factors acting sequentially at different developmental stages. **B)** Tissue-specific gene expression in the mature pinealocyte: Intrinsic pineal factors in the form of homeodomain proteins control pineal-specific gene expression by binding *cis*-acting photoreceptor conserved elements (PCEs). On top of this, extrinsic factors in the form of norepinephrine released from sympathetic nerve terminals during nighttime drive cAMP-dependent circadian rhythms in pineal gene expression. For simplicity, only one copy of each *cis*-element is displayed. The rat *Aanat* promoter contains at least three PCEs; two of these preferentially bind OTX/CRX, whereas the third might prefer RAX, as indicated by Genomatix promoter analysis. The roles of *Pax4* and *Rax* in the pinealocyte are not yet established, as indicated by question marks (?). CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; NE, norepinephrine; PCE, photoreceptor conserved element, this term refers a group of *cis*-elements with slightly different core sequences including both OTX2/CRX- and RAX-binding sequences [102].

Figure 6. Persistent presence of OTX2 and CRX proteins in the pineal gland of the adult rat Continuous expression is also detectable in the deep pineal gland, which is also a source of melatonin synthesis as indicated by expression of *Aanat* in this structure. **A)** Immunohistochemical demonstration of OTX2 protein in the rat superficial pineal gland. Scale bar, 100 μ m. **B)** Western blot analysis of CRX protein distribution in the central nervous system of the adult rat. Rat CRX protein (NP_068627) has a predicted molecular weight of 32.4 kDa. The antiserum was raised in rabbit against a peptide corresponding to position 284–299 on rat CRX protein. The lower blot represents a pre-absorption specificity-control. D, daytime sample; N, nighttime sample. **C)** Immunohistochemical demonstration of OTX2 protein in the rat deep pineal gland. Scale bar, 50 μ m. **D)** Anatomical demonstration of the deep pineal gland in a Nissl-stained coronal section of the adult rat brain. 3V, third ventricle; HC, habenular commissure; PC, posterior commissure. Scale bar, 500 μ m. **E)** Expression of *Aanat* in the deep pineal as demonstrated by radiochemical *in situ* hybridization. An autoradiograph of same section shown in (D) is displayed. For methodological details, see [7,10,76,86].

Figure 7. Daily expression patterns of rhythmic homeobox genes and *Aanat* in the rat pineal gland

Data are adapted from several studies. Lower horizontal bar reflects the daily lighting conditions (light, white part; darkness, black part). Data were obtained by radiochemical *in situ* hybridization followed by densitometric quantitation on sections of the rat pineal gland. ***Aanat***, arylalkylamine N-acetyltransferase (Rohde K, Møller M and Rath MF, in preparation); ***Crx***, cone-rod homeobox (Rohde K, Møller M and Rath MF, in preparation); ***Otx2***, orthodenticle homeobox 2 (Rohde K, Møller M and Rath MF, in preparation); ***Pax4***, paired box 4; ***Rax***, retina and anterior neural fold homeobox [9]. For methodological details, see [7,8,10].