

Published in final edited form as:

J Neurochem. 2011 October ; 119(2): 262–274. doi:10.1111/j.1471-4159.2011.07405.x.

Crx broadly modulates the pineal transcriptome

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Abstract

Cone-rod homeobox (Crx) encodes Crx, a transcription factor expressed selectively in retinal photoreceptors and pinealocytes, the major cell type of the pineal gland. Here, the influence of Crx on the mammalian pineal gland was studied by light and electron microscopy and by use of microarray and qRT-PCR technology, thereby extending previous studies on selected genes (Furukawa *et al.* 1999). Deletion of *Crx* was not found to alter pineal morphology, but was found to broadly modulate the mouse pineal transcriptome, characterized by a >2-fold downregulation of 543 genes and a >2-fold upregulation of 745 genes ($p < 0.05$). Of these, one of the most highly upregulated (18-fold) is *Hoxc4*, a member of the Hox gene family, members of which are known to control gene expression cascades. During a 24-hour period, a set of 51 genes exhibited differential day/night expression in pineal glands of wild-type animals; only eight of these were also day/night expressed in the *Crx*^{-/-} pineal gland. However, in the *Crx*^{-/-} pineal gland 41 genes exhibit differential night/day expression that is not seen in wild-type animals. These findings indicate that *Crx* broadly modulates the pineal transcriptome and also influences differential night/day gene expression in this tissue. Some effects of Crx deletion on the pineal transcriptome might be mediated by *Hoxc4* upregulation.

Keywords

Crx, *Hoxc4*, microarray; pineal gland; transcriptome profiling; gene expression

INTRODUCTION

Cone-rod homeobox (*Crx*) is a homeodomain transcription factor and member of the *Otx* family; *Crx* has been thought to play a critical role in determining and maintaining the phenotype of both pinealocytes and retinal photoreceptors (Chen *et al.* 1997; Furukawa *et al.* 1997; Rath *et al.* 2006; Rath *et al.* 2007). The selective expression of *Crx* in these cell types

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The authors have no conflicts of interest.

reflects their common evolutionary origin (Klein 2004; Mano and Fukada 2007). In the mammalian retina, *Crx* is essential for the normal development and maintenance of cones and rods (Furukawa *et al.* 1999) and regulates expression of the network of genes that characterize the retina (Hsiau *et al.* 2007). Elimination of *Crx* by disruption of the homeobox domain results in loss of the image-forming visual system (Furukawa *et al.* 1999) but not the non-image-forming visual system controlling circadian rhythms (Panda *et al.* 2003; Rovsing *et al.* 2010). *Crx*^{-/-} mice exhibit suppressed circadian rhythms in locomotor activity, suggesting that *Crx* may directly or indirectly alter retinal ganglion cell output, the suprachiasmatic nucleus, other structures controlling locomotor activity, or a combination (Rovsing *et al.* 2010).

Whereas it is clear that the retina is severely impacted in the *Crx*^{-/-} animal, the full impact of *Crx* deletion on the pineal gland is less clear. Studies on mouse pineal gland and on gene expression have indicated that *Crx* does not appear to alter pineal morphology, but does moderately modulate the abundance of *Aanat* transcripts (Li *et al.* 1998; Furukawa *et al.* 1999); *Aanat* is of special importance in the pineal gland because it encodes the enzyme that regulates the daily rhythm in melatonin production in vertebrates (Klein 2007). Studies in the chicken indicate that *Crx* is also involved in the control of expression of the last enzyme in melatonin synthesis, *Asmt/Hiomt* (acetylserotonin O-methyltransferase/ hydroxyindole O-methyltransferase) (Bernard *et al.* 2001).

In the current report, we have pursued the goal of determining the full impact of *Crx* deletion on the pineal gland using anatomical methods and microarray technology, which has not been used previously to study the adult mouse pineal gland. Our results indicate that *Crx* plays a very broad role in modulating the pineal transcriptome and that a link exists between *Crx* and the homeobox gene *Hoxc4*.

MATERIALS AND METHODS

Animals

Crx^{-/-} mice were provided by Dr. Connie Cepko. The *Crx*^{-/-} mice were made on a 129sv background (Furukawa *et al.* 1999), 129sv mice were used as wild-type controls in the current study. Genotypes were identified by use of primers detecting *Crx* (Table 1) and primers detecting the *neo* cassette in *Crx*^{-/-} mice, which amplify a 470 bp fragment not detected in the wild-type mouse. Wild-type and *Crx*^{-/-} mice (male and female, > two months of age) were bred and kept in a 12L:12D light cycle with food and water *ad libitum*. To collect tissue, animals were euthanized with CO₂ and decapitated at ZT (zeitgeber time) 6 or ZT20. The skull cap was carefully removed with the intention not to alter the position of the superficial pineal gland. The gland was then located on the skull, removed and cleaned of extraneous tissue. Dim red light was used when animals were euthanized at ZT20. For microarray analysis, pineal glands were immediately frozen on Dry Ice, then stored at -80°C in pools of eight glands; for qRT-PCR, pools of three to eight glands were prepared similarly. For radiochemical *in situ* hybridization histology, pineal glands from five wild-type animals and five *Crx*^{-/-} animals were used. For Affymetrix GeneChip analysis and qRT-PCR, three pools were analyzed per time point. All animal experiments were performed in accordance with the guidelines of EU Directive 86/609/EEC (approved by the Danish Council for Animal Experiments) and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Microarray

RNA was purified using the RNeasy micro kit (Qiagen, Valencia, CA) according to the manufacturer's protocol (including DNase treatment); high quality was verified using the

Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). A commercially available kit (Nugen Ovation RNA amplification system V2, NugenTech, San Carlos, CA) was used to convert 100 ng RNA to cDNA following the manufacturer's protocol. Double stranded cDNA was SPIA[®] amplified using the same kit followed by purification (instead of using the washing buffer, the cDNA was washed twice in freshly prepared 80% ethanol) (Zymo Research, Orange, CA). cDNA (3.75 µg) was used for fragmentation and labeling (FL-Ovation cDNA Biotin Module V2, NugenTech, San Carlos, CA). Labeled cDNA was mixed with control oligonucleotides B2, 20× eukaryotic hybridization controls (Affymetrix, Santa Clara, CA), herring sperm DNA, acetylated BSA, 2× hybridization buffer, and DMSO according to the Affymetrix protocol. This was used for analysis with the Affymetrix mouse GeneChip 430_2 microarray chip. The labeled cDNA was allowed to hybridize for 18 to 24 hours at 45°C before processing according to Affymetrix protocols. GeneChips were scanned on an Affymetrix 3000 Scanner. The microarray data are available at the Entrez Gene Expression Omnibus, National Center for Biotechnology (Edgar *et al.* 2002), and are accessible through GEO series accession number GSE24625 (ncbi.nlm.nih.gov).

Data analysis—The data files (.CEL) were analyzed with ChipInspector V2.0 software (Genomatix Software Inc., Munich, Germany); using this program gene expression in wild-type glands at ZT6 (n=3 groups) was compared to that in wild-type glands at ZT20 (n=3 groups). *Crx*^{-/-} ZT6 (n=3 groups) and *Crx*^{-/-} ZT20 (n=3 groups) were compared in a similar manner. In addition, a comparison of wild-type (n=6 groups) and *Crx*^{-/-} (n=6 groups) gene expression was done independently of sampling time. In all analyses, the following filters were chosen: exhaustive matching, a False Discovery Rate (FDR)=0, cut off= 1, region size = 300 bp and a minimum of 4 and 5 significant probes (depending on the number of genes). Differences of p< 0.05 were considered to be statistically significant.

Bioinformatics—Networks and canonical pathways were identified using Ingenuity Systems (IPA; Redwood, CA). Analysis of consensus sequences and transcriptional function were performed with Genomatix Pathway System (GePS), Genomatix Gene2Promoter and Genomatix RegionMiner.

Quantitative real-time RT-PCR analysis

RNA was purified using an RNeasy micro kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's protocol. cDNA was synthesized from 350 ng of DNase-treated RNA using random primers and SuperScript III (Invitrogen, Taastrup, Denmark). Quantification of each gene was performed by use of a gene-specific internal standard curve of known copy number. For quantification, standard curves were produced by serial dilution (10¹ – 10⁷ copies/ µl) of each PCR target; target sequences had been cloned into a plasmid (for details see molecular cloning). Standard curves were prepared for each qRT-PCR analysis.

qRT-PCR (LightCycler 1.5;Roche, Hvidovre, Denmark) reactions were carried out in a 20 µl volume with 1 µM primers (for sequences see Table 1), 1× SYBR green master mix (SABiosciences, Copenhagen, Denmark) and a 2 µl sample of a 3.5-fold dilution of cDNA. The program included an initial step of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 30 sec, extension at 72°C for 30 sec. Product specificity was confirmed initially on an agarose gel, thereafter by melting curve analysis after every qRT-PCR run.

Housekeeping gene selection for normalization was performed using 2 µl of 3.5-fold diluted samples of cDNA from wild-type and *Crx*^{-/-} pineal glands. Seven genes (*Gapdh*, *Ap1g1*, *β-actin*, *B2m*, *Snap25*, *Rps27a* and *Ube2d2*) were analyzed to select those which exhibited the smallest time-dependent difference in expression. Crossing points were used for analysis by

GeNorm (Vandesompele *et al.* 2002). The genes selected were *Gapdh*, *Snap25*, *Ap1g1* and *Ube2d2*.

Data analysis—Two-way ANOVA was used to test for the influence of Crx on the genotype using Graph Pad Prism V4 (GraphPad software, USA). The four conditions were (129sv ZT6, 129sv ZT20, *Crx*^{-/-} ZT6 and *Crx*^{-/-} ZT20) compared with respect for genotype.

For each genotype, Student's two-tailed 't' test with Welch's correction was used on log(2) transformed data to determine if genes were differentially expressed at a day/night basis. For each time point, the data are presented as the mean and standard error of mean (SEM) of 3 samples. A p-value < 0.05 was considered to represent a statistically significant difference (GraphPad Prism V4).

Molecular cloning of standards used for qRT-PCR and of overlapping fragments of *Hoxc4*

Target sequences for molecular cloning were generated by standard PCR reactions using pineal cDNA prepared from DNAase-treated RNA (primer sequences are listed in Table 1). PCR products were isolated by gel electrophoresis and gel extraction (Qiagen, Sollentuna, Sweden) and cloned into pGEM-T Easy vectors (Promega, Madison, WI) according to the manufacturer's instructions. Plasmids were selected and amplified in DH5- α cells (Invitrogen, Hvidovre, Denmark). In all cases, insert identity was confirmed first by EcoRI digestion and agarose gel analysis followed by sequencing (DNA Technology, Århus, Denmark). The qRT-PCR products ranged in size from 101 bp to 159 bp.

Transmission electron microscopy

The pineal glands from both *Crx*^{-/-} mice and wild-type mice were fixed by immersion in cold 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 60 minutes. The sections were then dehydrated in a series of ethanol (30%, 50%, 70%, and 96%), block-stained in 1% uranyl acetate in absolute ethanol for 1 hour, rinsed twice in absolute ethanol, and embedded by propylene oxide in Epon[®]. Two-micrometer-thick survey sections were cut and counterstained with toluidine blue. Ultrathin sections, with a gray interference color, were cut from preselected areas and poststained in uranyl acetate and lead citrate. They were then viewed in a Philips EM 208 transmission electron microscope operated at 80 kV.

Radiochemical *in situ* hybridization histological detection of *Hoxc4* transcripts

Cryostat sections (12 μ m) were mounted on Superfrost Plus slides and hybridized as previously described (Møller *et al.* 1997; Rath *et al.* 2007) with a mixture of three ³⁵S-labelled 38-mer antisense DNA probes directed against mouse *Hoxc4* mRNA (NM_013553.2): 5'-CATAAAGCCCTCCTACTAGCTAGCGACCCTGTAAAGTT-3' (278-241), 5'-CGAATTGCCAGGCCCTGGAGACTGGTGCAGCTATACT-3' (562-525), 5'-TTCACCCAAACCAGACCATCACACCTTGCAATATATAA-3' (1519-1482). Sections from five wild-type and five *Crx*^{-/-} mice were prepared. The hybridized sections were exposed to X-ray film for three weeks and developed.

RESULTS

Morphology of the pineal gland

Examination of the *Crx*^{-/-} pineal (Fig. 1) revealed that the morphology is not different from the wild-type gland. Rather, the gland exhibits normal features, evident from light and electron microscopic examination, characterized by a perivascular space with myelinated

nerve fibers and interstitial cells surrounded by a parenchyma of pinealocytes; the shape, density and organelle content of pinealocytes in the *Crx*^{-/-} and wild-type pineal gland (Upson *et al.*, 1976; Møller *et al.*, 1978) are indistinguishable.

Crx has broad modulatory effects on gene expression in the pineal gland

Comparison of gene expression in the wild-type and *Crx*^{-/-} pineal gland indicated that 512 genes were significantly ($p < 0.05$) downregulated in *Crx*^{-/-} pineal gland: 23 >8-fold, 65 4- to 8- fold (Table 2) and 424 2- to 4-fold (supplemental Table S1). In addition, 714 genes were significantly ($p < 0.05$) upregulated in the *Crx*^{-/-} pineal gland: 10 > 8-fold, 33 4- to 8-fold (Table 2), and 671 2- to 4- fold (supplemental Table S2).

Among the genes downregulated in the *Crx*^{-/-} mouse pineal gland, the most affected gene other than *Crx* was *Cbln1*, which decreased by nearly 20-fold; *Cbln1* encodes a protein that releases norepinephrine via adenylate cyclase/PKA-dependent signaling pathway (Albertin *et al.* 2000).

The most highly upregulated transcript in the *Crx*^{-/-} pineal gland was identified as AK140080, which maps to a location adjacent to the ras-GTPase-activating protein SH3 domain binding protein (*G3bp1*) and is therefore assumed to be the 3'-extension of this gene. The second most upregulated gene was *Hoxc4* (~18-fold). *Hoxc4* encodes a homeodomain transcription factor required for development of the oesophagus and spinal cord (Geadia *et al.* 1992).

Analysis of networks by IPA (Ingenuity Systems) revealed Crx influences genes involved in cellular assembly and organization, signaling and cell morphology (Table 3), among others. The indication that Crx affects genes involved in morphology is interesting because, as indicated above, the morphology and cell composition of the *Crx*^{-/-} pineal gland appears normal (Fig. 1). Along with the ephrin receptor pathway, the GABA pathway was also observed to be affected by deletion of *Crx*.

An *in silico* analysis with Genomatix software revealed that among the 1288 genes that were dysregulated more than 2-fold in the *Crx*^{-/-} mouse, 433 (represented by 772 transcripts) have predicted binding affinity for Crx (core consensus, TAATC) in the 500 bp promoter region upstream of the first transcriptional start site (TSS). Analysis of the genes regulated > 4-fold with a Crx consensus sequence, revealed that 9 had a transcriptional regulatory function (Table 2). This suggests that some effects seen in the *Crx*^{-/-} pineal gland might be mediated by changes in one or more of these transcription factors.

Crx and Otx2 consensus sequences are similar and belong to the photoreceptor conserved element (PCE) family (Kikuchi *et al.* 1993). It was found that 194 of the dysregulated genes (representing 294 transcripts) had an Otx2 regulatory sequence (core consensus TAATCC/T) but only 54 genes could theoretically be regulated by both Crx and Otx2, thereby providing *in silico* evidence that expression of these genes might reflect the direct action of these transcription factors (Table 2). *Aanat* was one of these genes, having consensus sequences for both Crx and Otx2, are previously reported (Li *et al.* 1998). It was also found that that in some cases one transcript of a gene has a Crx and/or Otx2 regulatory sequence where another does not; this is seen with *Tia1* and *Mpp3*, and may reflect use of alternative start sites.

The finding of a large upregulation of the transcription factor *Hoxc4* encouraged us to investigate *in silico* if some of the most dysregulated genes in the *Crx*^{-/-} mouse had regulatory sequences for Hoxc4 (core consensus TAATTA). Three hundred and fifteen transcripts, encoded by 205 different genes, were found to have an Hoxc4 consensus

sequence. None of the transcripts that were upregulated >4-fold had a transcriptional role, thereby providing no reason to suspect that effects of *Hoxc4* were mediated by any of these transcription factors. The *Hoxc4* co-cited network generated by Genomatix Pathway System (GePS)(Figure 2) has only a single gene that is dysregulated in the *Crx*^{-/-} pineal gland, *Hoxc5* (2-fold). The absence of any other dysregulated gene in this co-cited network suggests that the genes influenced by *Hoxc4* in the pineal gland differ from those regulated by *Hoxc4* in other tissues.

Crx influences differential day/night expression of genes in the mouse pineal gland

In the wild-type pineal gland, 51 transcripts were differentially expressed on a night/day basis (>2-fold, $p < 0.05$, Table 4); amongst these the abundance of 34 (67%) decreased at night and that of 17 (33%) increased at night (Table 4). The abundance of ten transcripts changed more than 8-fold and that of 35 transcripts changed 4-to 8-fold (Table 4); the transcript with the largest nocturnal increase was *Aanat*. The transcript exhibiting the largest nocturnal decrease (1/9.2) was encoded by *Nrxn3*, which is thought to be involved in synaptic plasticity (Kelai *et al.* 2008).

In the *Crx*^{-/-} mouse, 49 transcripts displayed differential night/day expression (Table 4), of which 7 are not annotated. Among these 49 differentially expressed transcripts, 22 (45%) decreased at night and 27 (55%) increased at night (Table 4). The transcripts that were day/night expressed in the *Crx*^{-/-} pineal gland differed from those in the wild-type mouse; only 8 genes had a different day/night expression in both groups (Table 4).

Of special note was the observation that *Aanat* increased in the *Crx*^{-/-} mouse pineal at night compared to day time (~8.5-fold; Table 4). The transcript that exhibited the largest decrease at night was *Nr1d1/Rev-erba*, which functions as a transcriptional repressor and plays a role in circadian systems (Burriss, 2008; Meng *et al.* 2008).

Genes that were differentially expressed on a day/night basis in the wild-type mouse were associated with networks dedicated with behavior, development and function as defined by IPA; the primary canonical pathways include dopamine receptor signaling, GABA receptor signaling and β -adrenergic signaling (Table 5), the last two of which are involved in melatonin synthesis (Klein *et al.* 1971; Sun *et al.* 2002).

The genes that are differently expressed on a day/night basis in the *Crx*^{-/-} mouse were found to be involved in networks committed to behavior, development and function; the same networks as observed in the wild-type mouse (Table 5). Different canonical pathways were observed to be altered in the *Crx*^{-/-} mouse versus the wild-type animal: the phototransduction pathway, protein kinase A signaling, cAMP-mediated signaling and circadian rhythm signaling exhibit daily change in the *Crx*^{-/-} mouse (Table 5). These pathways are all involved in the production of melatonin or the control of this process. The β -adrenergic signaling pathway was the only canonical pathway to exhibit differential night/day expression in the pineal glands of both the wild-type and *Crx*^{-/-} mice (Table 5).

Using Genomatix software it was found that 24 of the 49 genes exhibiting differential day/night expression in the *Crx*^{-/-} mouse had a *Crx* consensus sequence (Table 4), only five genes had binding affinity for both *Crx* and *Otx2*. Six of the upregulated genes had a *Hoxc4* consensus sequence, indicating that these are candidates for upregulation by *Hoxc4*. Moreover, it is possible that the transcription factor *Cry1*, might mediate effects of *Hoxc4* by virtue of a *Hoxc4* consensus sequence in the *Cry1* promoter. In addition, it was investigated that the *Aanat* 500 bp upstream promoter did not had a *Cry1* consensus sequence indicating that *Cry1* is not responsible for the upregulation of *Aanat* at night in the *Crx*^{-/-} mouse in the absence of *Crx*.

qRT-PCR validation of microarray data

Six genes that exhibited differential night/day expression by microarray in either wild-type or *Crx*^{-/-} pineal glands (*Aanat*, *Gng4*, *Cpm*, *Pvr*, *Pde10a*, *Cry1*) were tested with qRT-PCR; a daily rhythm was detected in all encoded transcripts in glands from wild-type animals (Fig. 3) and in 5 genes from *Crx*^{-/-} animals. In addition, the differential wild-type/*Crx*^{-/-} expression patterns seen in four transcripts (*Hoxc4*, *1700042O10Rik*, *Gm626* and *Rasgrf1*) were confirmed by qRT-PCR.

In the case of *Pde10a*, a set of primers directed against the 3' region of the transcript confirmed the results of microarray which interrogated this region (Fig. 3). However, a set of primers directed against the 5' region did not confirm the results of microarray; rather qRT-PCR with these primers revealed a 1/3.5 -fold ZT6/ZT20 inverse rhythm ($6.2 \times 10^3 / 1.8 \times 10^3$ transcripts) in the wild-type (p-value=0.02) and 1/1.4 -fold ZT6/ZT20 inverse rhythm ($3.8 \times 10^3 / 2.7 \times 10^3$) in the *Crx*^{-/-} mouse (p-value=0.1), indicating at least two *Pde10a* transcripts are expressed in the pineal gland and that they are differentially regulated.

Studies on *Hoxc4*

The marked effects of *Crx* deletion on *Hoxc4* was studied in greater detail because homeobox genes are known to broadly control cascades of gene expression. Three overlapping portions of the *Hoxc4* transcript were cloned from *Crx*^{-/-} pineal mRNA; sequencing confirmed their identity (ORF >99% identical to NM_013553.2), thereby indicating that a full-length *Hoxc4* transcript is likely to be present in the *Crx*^{-/-} pineal gland.

Hoxc4 expression in the *Crx*^{-/-} mouse pineal gland was also confirmed using radiochemical *in situ* hybridization histology (Fig. 4), which failed to detect expression of the gene in surrounding tissues or in the wild-type gland.

DISCUSSION

This study was primarily designed to extend previous studies on the pineal gland of the *Crx*^{-/-} mouse; these results, including the broad modulatory effects of *Crx* on the pineal transcriptome, will be addressed below. In addition, since this is the first report of microarray analysis of the adult mouse pineal gland, the results of this study provide new understanding of gene expression in this tissue; this topic will also be discussed. It should be noted that microarray has been used to study the neonatal mouse pineal gland (Munoz *et al.* 2007). There are large differences in the genes expressed in the neonatal and adult pineal gland, consistent with evidence that marked developmental changes occur at about this time in the rodent, including a marked decrease in cell division and an increase in expression of genes linked to melatonin synthesis and visual signal transduction (Quay W.B. 1979; Klein *et al.* 1981). However, a thorough discussion of the developmental differences in gene expression in the pineal gland as revealed by both microarray studies is beyond the scope of this report.

Night/day differences in transcript abundance in the pineal gland are less prevalent in the mouse as compared to the rat

The pineal transcriptome has previously been studied by microarray in the rat (Tosini *et al.* 2008; Bailey *et al.* 2009), chicken (Bailey *et al.* 2003) and zebrafish (Toyama *et al.* 2009) but not in the mouse. Here we have found that 51 genes in the wild-type mouse pineal gland exhibit a daily rhythm (>2-fold). These 51 genes were found to be elements of canonical pathways that impact melatonin synthesis (Table 5). In a similar study of the rat pineal,

Bailey et al., (2009) found >600 genes were different day/night expressed >2-fold. The rat versus mouse difference in the number genes differentially expressed on a night/day basis is striking. Further, the amplitude of changes appears to be greater in the rat, in which 130 genes exhibited a >4-fold night/day difference. In the wild-type mouse there are only 35 genes with a night/day difference of this magnitude; in addition, only *Aanat* and *Pvr* show a difference in both rat and mouse.

Differences in day/night gene expression between the rat and mouse may reflect fundamental biological differences between these species as are known to exist between other mammals. For example, the large night/day difference in *Aanat* transcript number seen in the rat is not seen in the Rhesus monkey or sheep, which exhibit little or no night/day difference in AANAT mRNA whereas changes in AANAT activity do occur (Coon *et al.* 1999; Johnston *et al.* 2004; Klein 2007; Coon *et al.* 2002), presumably as a function of posttranslational mechanisms (Klein 2007).

Inbreeding might also be responsible for the small number of genes expressed differentially on day/night basis in the mouse pineal gland. This is consistent with the observation that the genes encoding the melatonin synthesis enzymes *Aanat* and *Asmt/Hiomt* are nonfunctional in most laboratory mice because of mutations (Ebihara *et al.* 1987; Roseboom *et al.* 1998). Accordingly, a global difference in the daily pattern of gene expression might be caused by a mutation in a gene with a broad impact on gene expression, e.g. a transcription-related factor.

These findings provide further reason to question whether conclusions drawn from analysis of gene expression patterns in tissues of one species are necessarily applicable to other species.

The broad pleiotropic effect of *Crx* on the pineal transcriptome

The current study identified more than a thousand genes that are dysregulated in the *Crx*^{-/-} mouse, more than half of which were upregulated. It is surprising that differential expression of such a high number of genes is not reflected in a major morphological change in the pineal gland (Fig. 1) as for example seen in retina of this mouse. It is of interest that several of the dysregulated transcripts in the *Crx*^{-/-} mouse are part of the ephrin pathway, which controls cell morphology and cell communication; in view of this, it is surprising that morphological changes are not apparent.

An *in silico* analysis of *Hoxc4* consensus sequences revealed that some of the most upregulated genes (Table 2) had at least one *Hoxc4* consensus sequence; the upregulation of these genes might therefore be explained by a direct reflection of the upregulation of *Hoxc4*. It was observed that of the upregulated genes having a *Hoxc4* consensus sequence, none had a transcriptional regulatory function and therefore were not responsible for upregulation of other genes. The finding of “genetic disorder” pathway as one of the networks most affected reflects *Crx*'s proven involvement in several retinal diseases (Furukawa *et al.* 1997; Furukawa *et al.* 1999).

In contrast to the absence of marked morphological changes in the pineal gland, the retina of *Crx*^{-/-} mice is grossly affected as evident from the loss of photoreceptor outer segments (Furukawa *et al.* 1999; Rovsing *et al.* 2010). A large number of genes are also dysregulated in the retina of the *Crx*^{-/-} mouse with major morphological changes to follow (Hsiau *et al.* 2007; Furukawa *et al.* 1999; Rovsing *et al.* 2010). Some of the genes which are downregulated in the *Crx*^{-/-} pineal gland are also downregulated in the *Crx*^{-/-} retina including *rhodopsin* (*Rho*) and *cone opsin* (*Opn1sw*) (Hsiau *et al.* 2007; Furukawa *et al.* 1999). A comparison of the most affected genes in both tissues (data from Hsiau *et al.*,

2007) indicates that only a few are affected in both tissues. For example, *Cbln1* is downregulated ~19-fold in the pineal gland but is not affected in retina, indicating that this gene is regulated by different mechanisms in these tissues. This is consistent with the explanation that Crx acts in concert with other transcription factors to control transcription (Nishida *et al.* 2003); apparently, the combination of factors in the retina differs from that in the pineal gland.

Here we found that the amplitude of the daily rhythm in *Aanat* transcripts is reduced 50% in the pineal gland of the *Crx*^{-/-} mouse, in confirmation of previous observations (Furukawa *et al.*, 1999). This indicates that a functional Crx is required for the normal pattern of expression of *Aanat*. It is not clear whether the absence of Crx appears to reduce *Aanat* expression by lowering the maximum level of expression or by shifting the timing of the peak in expression. The expression of *Aanat* is of special interest because it is thought that multiple Crx consensus sequences in the *Aanat* promoter play a conserved role in *Aanat* expression (Appelbaum and Gothilf 2006; Klein 2007). However, it is clear that Crx is not an absolute requirement for expression of *Aanat*. This does not, however eliminate the role of the PCE sites. It is possible that the role of Crx might be redundantly played by the related transcription factor Otx2 which binds to the Otx variant of the PCE site. Otx2 is essential for pineal development (Nishida *et al.* 2003) and has a distinct postnatal role in the mouse retina (Koike *et al.* 2007). *Otx2* is expressed in the adult rodent pineal gland and retina in the presence and absence of Crx (Rath *et al.* 2006; Supplemental Table S3 and S5 in Hsiau *et al.* 2007). Otx2 and Crx have similar binding preferences and exhibit functional redundancy (Li *et al.* 1998; Bobola *et al.* 1999; Bernard *et al.* 2001; Dinet *et al.* 2006). Accordingly, it is reasonable to suspect that Otx2 substitutes for Crx in the pineal gland of the *Crx*^{-/-} mouse in a redundant manner to control expression of *Aanat* and perhaps other genes. In the case of genes with consensus binding sequences for both, as is the case with *Aanat*, expression may only reflect the interaction of Otx2 with the Otx2 consensus binding sequence. Whereas it is also possible that upregulation of *Otx1* or *Otx2* might compensate for decreased *Crx* expression, our microarray analysis failed to detect a change in expression of either *Otx1* or *Otx2*, thereby providing no reason to entertain such a compensatory explanation.

The reduction of the amplitude of *Aanat* expression in the *Crx*^{-/-} mouse might also reflect reduction of expression of *Cbln1*, which is thought to modulate norepinephrine release (Albertin *et al.* 2000). *Cbln1* might be normally released by pinealocytes into the extracellular space, where it could enhance norepinephrine release from sympathetic nerve endings. In addition, *Adcy3*, adenylate cyclase 3 is downregulated 5.3-fold in the *Crx*^{-/-} mouse. The product of this gene catalyzes the formation of cAMP (Linder 2006). *Cbln1* does not appear to be directly regulated by Crx because promoter analysis fails to reveal the presence of either a Crx or an Otx2 consensus sequence. Other possible scenarios include the impact of Crx elimination on visual function and control of *Aanat* levels and temporal organization of the transcription factor system controlling expression.

The results of this investigation also point to a role of Crx in circadian biology of the pineal gland, because there was no evidence in the *Crx*^{-/-} pineal gland of a day/night pattern of expression of 43 genes seen in the wild-type animal. This may reflect temporal disorganization of the transcription factor system which controls gene expression, including *Cry1* and *Nr1d1/Rev-erba*. It is also possible that this is linked to the less robust nature of circadian locomotor activity seen in the *Crx*^{-/-} mouse (Rovsing *et al.*, 2010), which provides evidence for a role of Crx in circadian rhythms, separate from phenotype determination in the pineal gland and retina. Effects on circadian biology may reflect *Crx*-dependent changes in expression of a subset of the >1000 genes that are dysregulated in the *Crx*^{-/-} mouse pineal gland, some of which are involved in signal transduction (for example *Gng4*, *Rasgrfl*

and *Rho*). It is also possible that Crx-dependent effects on the pineal gland contribute to the circadian amplitude of locomotor activity and temperature, both of which are reduced in the *Crx*^{-/-} mouse (Rovsing et al., 2010). It can be speculated that these effects are mediated by an unidentified pineal-dependent mechanism. It is unlikely that melatonin is involved because the capacity to synthesize melatonin is markedly reduced in the 129sv strain (Roseboom *et al.* 1998), which, as indicated above, is true of most laboratory mice (Ebihara *et al.* 1987).

As discussed above for *Aanat*, It is not clear if changes in differential day/night gene expression represent a true shift from constant non-day/night expression to day/night expression or if genes that appear to become expressed at a day/night basis in the *Crx*^{-/-} mouse are also expressed at a day/night basis in wild-type animals with different phasing; and, that changes in the circadian system combined with the limitations imposed by a two point sampling experimental design resulted in the detection of these rhythms. Future studies involving more frequent time samplings should provide a better profiling of the influence of Crx on the temporal nature of gene expression in the pineal gland.

The adult pineal gland is not known to be photosensitive. The expression of phototransduction genes in this tissue appears to reflect the common origin of photoreceptors and pinealocytes (Klein 2007). If the transcripts are translated, the encoded proteins could function indirectly in G-protein coupled receptor signaling. For example, photoreceptors could form heterodimers with the adrenergic receptors which control pineal function. Formation of receptor heterodimers is known to modify function and specificity (Prinster *et al.* 2005).

Hoxc4 in the pineal gland

These studies have revealed a strong upregulation of *Hoxc4* in the *Crx*^{-/-} mouse pineal gland (Fig. 3 and 4), which is not seen in the wild-type or *Crx*^{-/-} retina by microarray (Hsiao *et al.* 2007) or qRT-PCR (data not shown). It seems reasonable to consider that the low levels of *Hoxc4* expression in the wild-type pineal gland normally influences the transcriptome profile by influencing the expression of the genes which are upregulated in the *Crx*^{-/-} pineal gland. It is of interest to find that analysis of the *Hoxc4* co-citation network (Figure 2) indicates that to a large degree, the genes linked to *Hoxc4* in other systems are not apparently linked to *Hoxc4* in the pineal gland. This suggests that Hoxc4 networks differ markedly on a tissue-to-tissue basis.

Summarizing, it appears that Crx plays a broad role in controlling the pineal transcriptome by weakly to mildly enhancing or suppressing expression of ~1200 genes. However, the change in the transcriptome is not associated with a remarkable change in cellular composition of the gland or in dramatic loss of expression of pinealocyte marker genes. This indicates that Crx is not an absolute requirement for expression of the pinealocyte phenotype. Some of the changes observed in the absence of Crx expression could possibly be mediated in part by a marked increase in the downstream expression of the homeobox gene *Hoxc4*, which may normally play a less obvious role in shaping the pineal transcriptome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the Lundbeck Foundation, the Danish Medical Research Council (grant number 271-07-0412), the Novo Nordisk Foundation, the Carlsberg Foundation, Fonden til Lægevidenskabens Fremme, Simon Fougnier Hartmanns Familiefond, the Danish Eye Health Society (Værn om Synet) and the Intramural Research Program of the The *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health. The authors wish to express their appreciation to Dr. Connie Cepko for the generous gift of the *Crx*^{-/-} mice.

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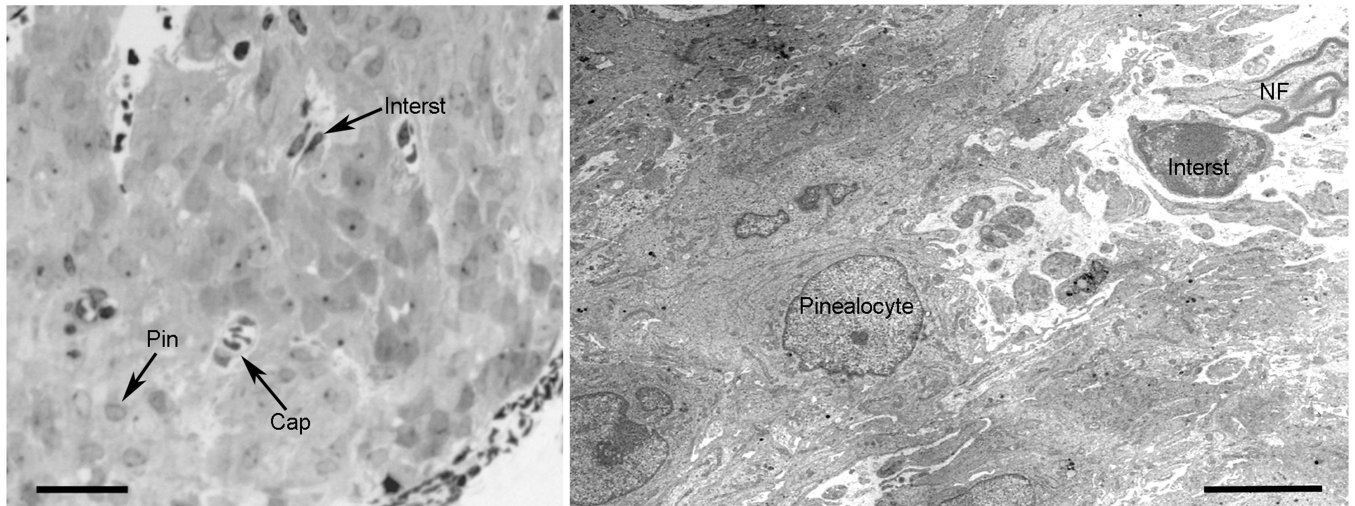


Fig. 1. Transmission electron microscopic analysis of the pineal gland of the *Crx*^{-/-} mouse (left) A Epon-embedded section (2 μ) of a *Crx*^{-/-} mouse pineal tissue stained with Toluidine blue. The gland exhibits a normal morphology with pinealocytes (Pin), interstitial cells (Interst) and capillaries (Cap). Bar = 25 μ m. (right) Electron micrograph of the pineal gland of the *Crx*^{-/-} mouse. A perivascular space with a myelinated nerve fiber (NF) and interstitial cell (Interst) is seen surrounded by pinealocytes with normal shape, density and organelle content. Bar = 5 μ m.

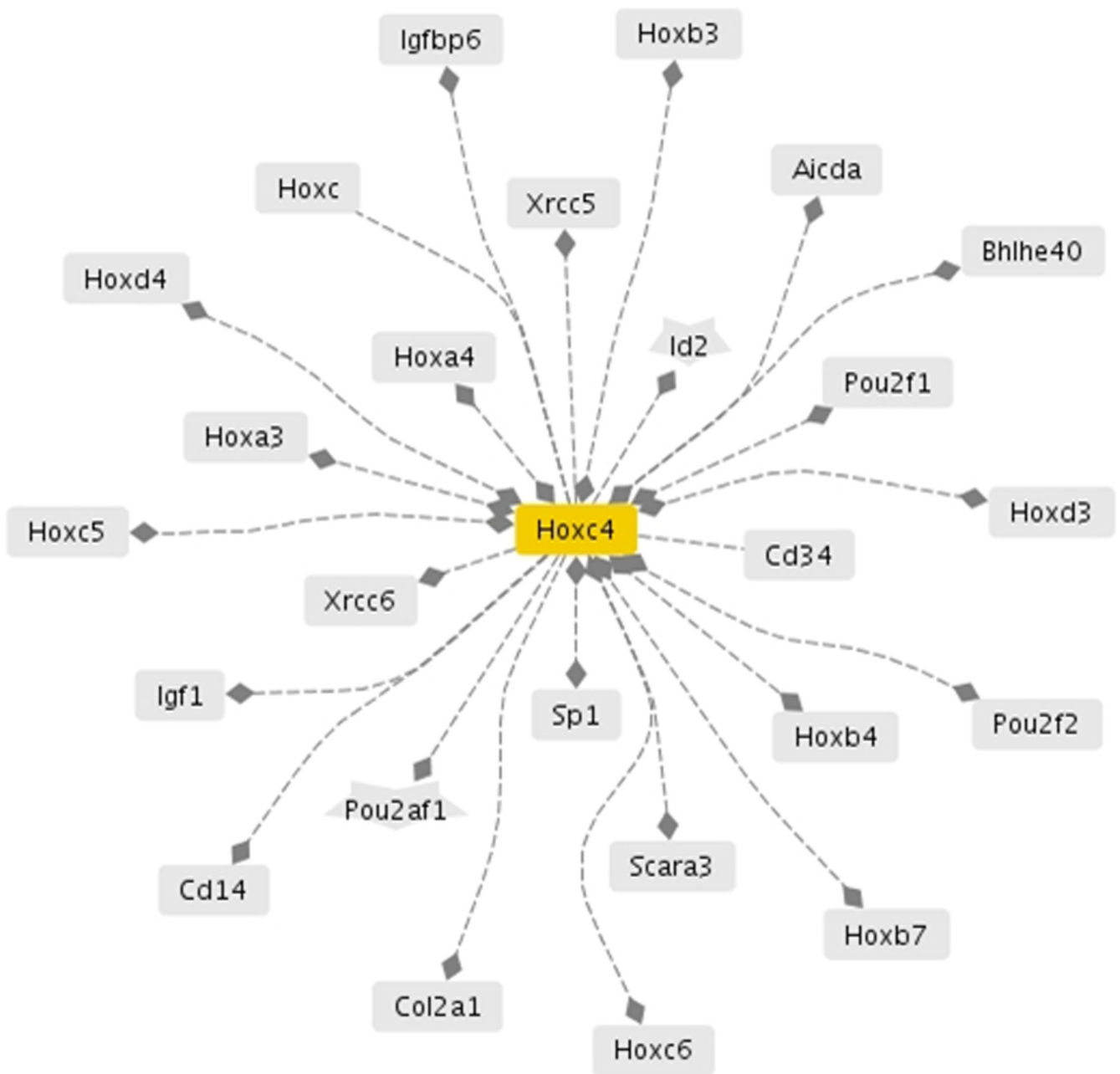


Fig. 2. Genes co-cited with Hoxc4

The dashed line indicates co-citation of the connected genes in the literature; a diamond indicates the promoter of gene B (the gene with the diamond) has a consensus sequence for the transcription factor encoded by gene A. An additional filled diamond at the other end of the line means that the promoter of gene A has a consensus sequence for the transcription factor encoded by gene B. The only gene from this network that exhibited a change in expression in the *Crx*^{-/-} mouse other than *Hoxc4* is *Hoxc5* (2-fold). (Figure generated by the Genomatix Pathway System).

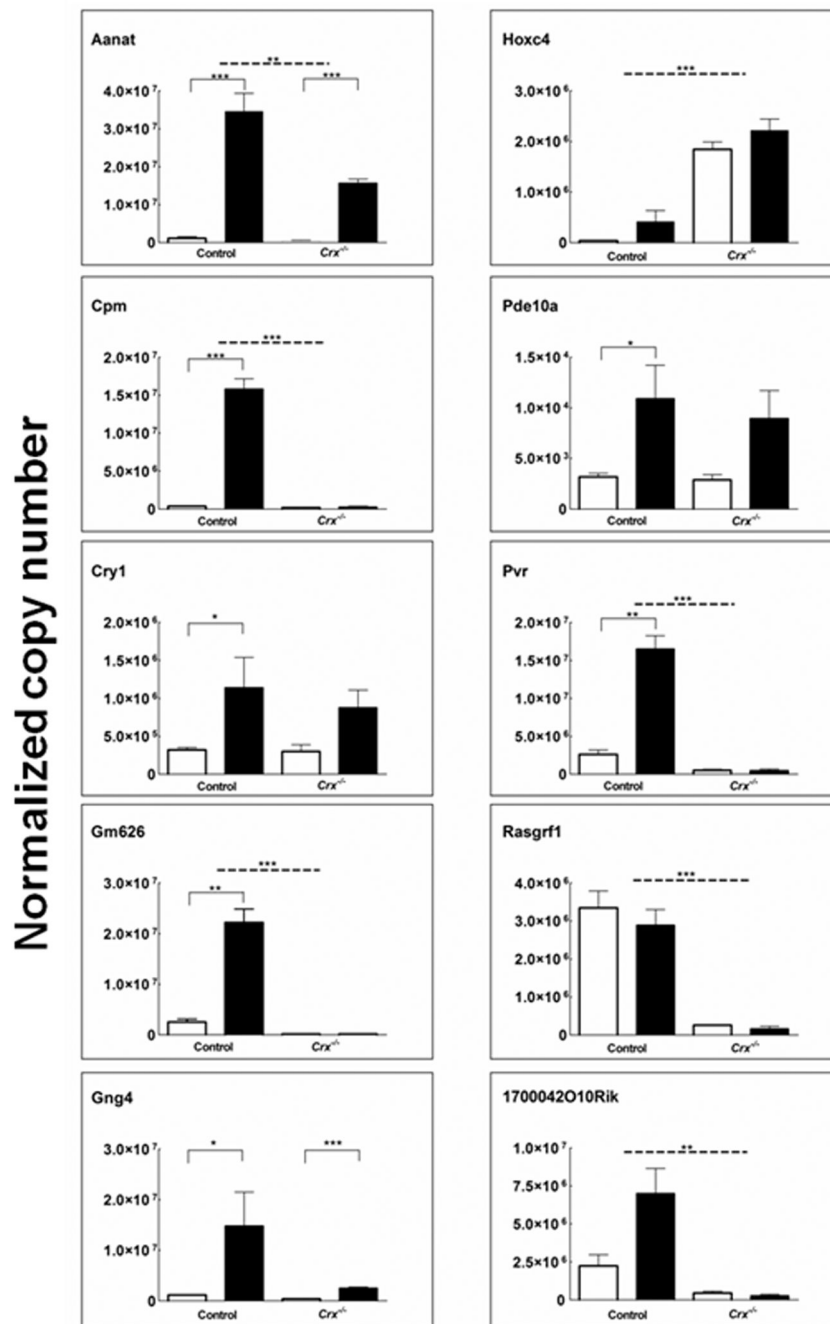


Fig. 3. qRT-PCR analysis of transcripts detected as either day/night expressed or differentially expressed in *Crx*^{-/-} mice as compared with wild-types
 Black bars, night samples; white bars, day samples. Transcripts are identified by gene symbols. Values were normalized to *Gapdh*, *Ube2d2*, *Snap25* and *Ap1g1*. Each value represents the mean \pm S.E.M of three independent analyses. * p-values < 0.05; ** p-values < 0.01; and, *** p-values < 0.001. For further details see the Materials and methods section.

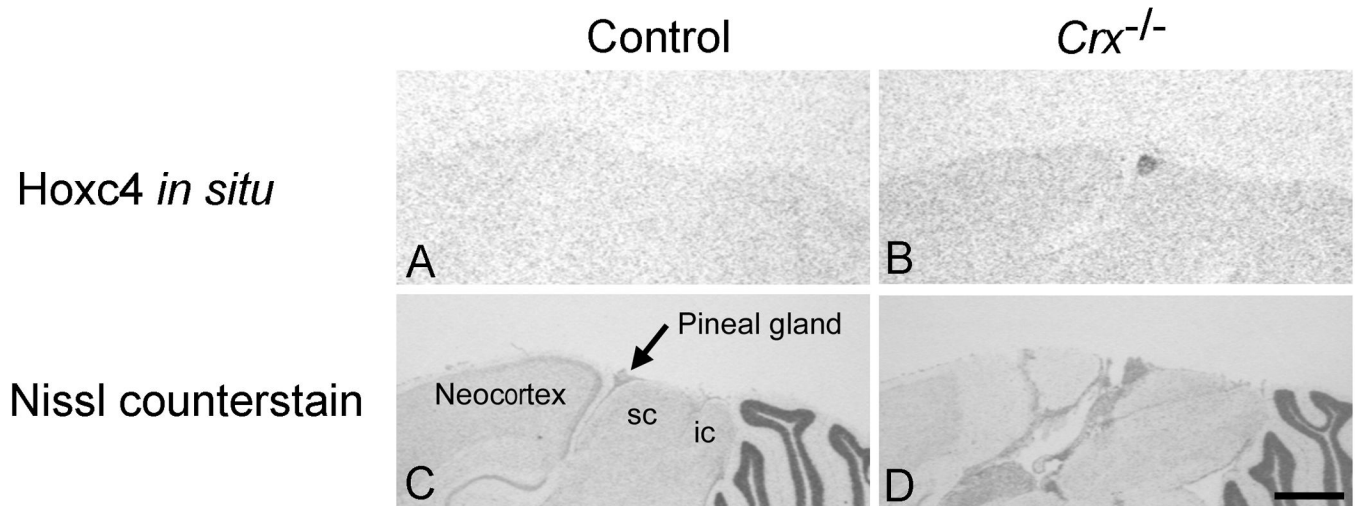


Fig. 4. Radiochemical *In situ* hybridization histochemical (ISH) detection of *Hoxc4* mRNA
 Panels A and C are sagittal brain sections from a wild-type mouse and panels B and D are sagittal brain sections from a *Crx*^{-/-} mouse. The upper panels are results from ISH and the lower panels are results of Nissl counter staining of the sections used for ISH analysis. A positive ISH signal is only seen in the superficial pineal gland of the *Crx*^{-/-} mouse (Fig B) and not in that of the wild-type (Fig A), in agreement with the results of microarray and qRT-PCR studies. A signal was not generated with a sense probe. Scale bar = 1 mm. For further details see the Materials and methods section. sc, superior colliculus; ic, inferior colliculus.

Table 1
Primer sequences for genotyping, quantitative real-time RT-PCR analyses and cloning

Where accession numbers are not available, Entrez Gene identifiers are given.

Gene	GenBank Accession number	position	Forward primer 5'-3'	Reverse primer 5'-3'
Genotyping				
Crx	NM_007770.4	461–770	GCAGCGACAGCAGCAGAAACA	ATGACCTATGCCCCGCTTCT
Neo	-	274–975	ATGGATTGCACGCAGGTTCTC	GATCTGGACGAAGAGCATCAG
qPCR				
Aanat	NM_009591.3	4–105	TGCAGTCAGGAGTCTCAGCTT	AAGTGCTCCCTGAGCAACAG
Actb	NM_007393.2	414–550	CTAAGGCCAACCGTGAAAAG	GTCTCCGGAGTCCATCACAAT
Ap1g1	NM_009677.3	2489–2641	GAGCTAGACATGACGGACTTTG	CAGCTGTTGCTTCTGTGGAT
B2m	NM_009735.3	111–211	TATCCAGAAAACCCCTCAAAT	GAGGCGGGTGAAGTGTGTTA
Cpm	NM_027468	984–1139	AAGTGTTTCGATCAGAGTGGAGC	CGTGTCCAGGGACTGTAACAT
Cry1	NM_007771	872–1015	TCAATTGAGTATGATTCTGAGCCT	TCCGCCATTGAGTTCTATGATC
Gapdh	XM_001473623.1	77–178	TGGTGAAGGTCCGGTGTGAACG	AGGGGTCTGTTGATGGCAACAA
Gm626	268729	319–473	GGGAGCGAGAGTGACTGG	GATGAAAATCAGCTGAGGGC
Gng4	NM_010317	210–368	GGAGTGCAGGAATGAAGGAA	GCACGTGGGCTTCACAGTA
Hoxc4	NM_013553	1726–1867	GGAGGACAGCAAACAAAGCTA	TAACCACGATGAGGGTAGGG
Pde10a	NM_011866	372–518	GAAGGCTGACCGAGTGTTC	TGGTTTTCTCTTCAGCCAC
Pvr	NM_027514	454–606	TTCCCCAGAGGCAGTAGAAG	AGAGATTTCGTCAGGAGGGT
Rasgrf 1	NM_011245	1757–1911	GAGGGCTGTGAGATCCTCCT	GAATAGGAAACACTGGCGCT
Rps27a	NM_024277	232–376	GAAGACCTTACGGGGAAAA	GCCATCTTCAGCTGCTTAC
Snap25	NM_011428.3	93–244	GCTCCTCCACTCTTGCTACC	GCTCATTGCGCATGTCTGCG
Ube2d2	NM_019912.2	389–533	GGCTCTGAAGAGAATCCACAA	TACTCCACCTGATAGGGGC
1700042010RIK	73321	836–988	CTGCATAGATTTTGCACGGA	TAACCTGAGGGTTGATTGGGG
PCR for cloning				
Hoxc4	NM_013553	61–465	GGTGTGCAATGGTGAACACC	TGGAATCCCGATTCCCTGGT
Hoxc4	NM_013553	351–965	TGGACTCTAACTACATCGAT	TCTTCCATTTTCATACGACGGT
Hoxc4	NM_013553	861–1867	ACCGCTACCTGACCCGAAGG	TAACCACGATGAGGGTAGGG

*The primers generate a Neo derived product within this area.

Table 2

Genes dysregulated in the pineal gland of the *Crx*^{-/-} mouse

The pineal transcriptomes of the wild-type and *Crx*^{-/-} were compared independent of time of sampling. A set of 512 genes were found to be directly or indirectly downregulated by *Crx* deletion ($p < 0.05$); among these, 424 genes are downregulated only 2- to 4-fold; these genes are listed in supplemental data Table S1. A set of 714 genes were found to be directly or indirectly upregulated by *Crx* deletion ($p < 0.05$); among these, 671 genes are upregulated only 2- to 4- fold; these genes are listed in supplemental Table S2. All genes are assigned by ChipInspector V2 (Genomatix). A "+" symbol in the *Crx*, *Otx2* and/or *Hoxc4* columns indicate the gene has at least one *CRX*, *OTX2*, or *HOXC4* consensus sequence in the promoter region (500bp upstream of first TSS). Genes encoding transcription factors are identified by a "+" symbol in the column labeled TF. The 1/x convention is used to indicate downregulation by a factor of *x*. For further details see the Materials and methods section. N/A, information is not available.

Gene Symbol	Fold	<i>Crx</i>	<i>Otx2</i>	<i>Hoxc4</i>	TF
G3bp1	26.4				
Hoxc4	17.5				+
Sntnl2	15.6	+			
Tia1	15.5	+	+	+	
D430019H16Rik	13.5				
Chodl	12.0			+	
A330092A19Rik	10.3				N/A
Glra1	9.0				
Mpp3	9.0	+	+		
Kenip4	8.2	+		+	
Nefl	6.5				
C230022P04Rik	6.5				N/A
Ipcfl	5.9				
Trank1	5.8	+		+	
Isl1	5.7	+			+
Kctd4	5.6	+	+		
Lgi2	5.5		+		
Neurod2	5.5	+			+
Ngef	5.5				
Gria2	5.4	+			
Calm1	5.1	+		+	

Gene Symbol	Fold	Crx	Otx2	Hoxc4	TF
Tmem178	5.0	+		+	
Synpr	5.0	+		+	
A2bp1	4.9			+	
Grin2c	4.9				
Prox1	4.9	+		+	
Stl8	4.8				+
AI593442	4.8				
Crtam	4.7	+			
Cntn3	4.5	+	+	+	
Epha8	4.5				
Dusp26	4.4				
Hist1h3i	4.4				
Pou3f1	4.4				+
LOC100045707	4.4				
Atp13a5	4.4	+			
Kcnc2	4.4	+			
Cox6a2	4.3				
Slec6a1	4.3				
2900092D14Rik	4.3				
Lrtm1	4.2	+	+		
Nova1	4.1	+		+	
Nefn	4.1				
Cbln1	1/19.4				
1700042O10Rik	1/19.3	+			
4833423E24Rik	1/17.4	+			
Gm626	1/16.0	+	+	+	
Gm2595	1/16.0				N/A
Ptpn20	1/15.7	+			
Tal2	1/12.2				+

Gene Symbol	Fold	Crx	Otx2	Hoxc4	TF
Mycn	1/12.1				+
Tubb3	1/12.0				
Hk2	1/11.5	+			
Ankrd33	1/11.2				
Trim15	1/10.5				
Camkv	1/10.3	+			
Ng23*	1/9.9				
Rasgrf1	1/9.2	+			
Myog	1/8.9	+			+
Cyp2j13	1/8.8	+			
Odz2	1/8.8				
Panx2	1/8.6				
Rho	1/8.6	+			
17H6S56E-3	1/8.5				
Acen3	1/8.2	+	+	+	
Rpp25	1/8.1				
Gabrd	1/7.9				
Mst1r	1/7.9	+			
Arhgef15	1/7.7	+	+		
4930583H14Rik	1/7.6	+			
Gpmb	1/7.4				
Syng3	1/7.4				
Paps2	1/7.1	+		+	
3930402G23Rik	1/7.1				
Ptwil4	1/6.8	+		+	
Slc24a1	1/6.7				
Rpia	1/6.5				
Ube2t	1/6.3	+		+	
LOC100047829	1/6.3				

Gene Symbol	Fold	Crx	Otx2	Hoxc4	TF
Fhod3	1/6.1	+		+	
Nefh	1/6.1				
Gm2694	1/6.1				N/A
LOC100045304	1/6.1				
FLJ22297	1/6.1				N/A
FLJ22717	1/6.1				N/A
Rbpms	1/5.9	+			
Garnl3	1/5.8	+	+	+	
Myrn	1/5.8	+		+	
Npl	1/5.7	+	+		
Stc2	1/5.7				
Wdr66	1/5.6				
Recql	1/5.5	+			
Ret	1/5.4				
Adey3	1/5.3				
Sall4	1/5.3				
2310011E23Rik	1/5.3				
Als2	1/5.2	+	+	+	
Mpp4	1/5.2				
Mesp1	1/5.1			+	+
Cpm	1/5.0	+	+	+	
Golt1b	1/5.0				
Pvr	1/5.0	+		+	
C1ql3	1/4.9				
Gfra1	1/4.9				
2010107G12Rik	1/4.7	+	+		
Cenpo	1/4.6	+	+		
Gabbr1	1/4.6	+			
Lhfp15	1/4.6				

Gene Symbol	Fold	Crx	Otx2	Hoxc4	TF
Rnf207	1/ 4,6				
LOC639211	1/ 4,6				
Accn1	1/ 4,5	+			
Bub1b	1/ 4,5		+	+	
Galnd2	1/ 4,5	+			
Sez6l	1/ 4,5	+			
Cng4	1/ 4,4				
Sell13	1/ 4,4				
Spsb4	1/ 4,3	+	+		
A930007K23Rik	1/ 4,3				N/A
D4Bwg0951e	1/ 4,3	+	+		
Acox1	1/ 4,2	+	+	+	
Cngt1	1/ 4,2		+	+	
Lrit1	1/ 4,2	+			
Akap6	1/ 4,1			+	
Bace2	1/ 4,1	+	+		
Bdkrb2	1/ 4,1				
Dnnt	1/4,1				
Kif22	1/ 4,1	+			
Mc1r	1/ 4,1				
Pde6g	1/ 4,1	+			
Rgs20	1/ 4,1		+	+	
LOC100044395	1/ 4,1				

* Gene name assigned by authors based on homology.

Table 3
Networks of genes and canonical pathways influenced by the deletion of Crx

Network and canonical pathway analysis was done using Ingenuity IPA software on the 50 most up- and 50 most downregulated genes.

Networks	Canonical pathways
Cell-to-cell signaling and interaction	Amyotrophic lateral sclerosis signaling
Cellular assembly & organization	GABA Receptor Signaling
Cellular function & maintenance	Ephrin Receptor Signaling
Neurological disease	Neuropathic pain signaling in dorsal horn neurons
Genetic disorder	
Nutritional disease	
Lipid metabolism	
Molecular transport	
Small molecule biochemistry	
Nervous system development & function	
Cellular compromise	
Organismal injury & abnormalities	

P-value <0.05.

Table 4

Transcripts differentially expressed ($p < 0.05$) on a day/night basis in the wild-type and/or *Crx*^{-/-} pineal gland. A. Genes upregulated at night; B. Genes downregulated at night

Ratios are given as transcript levels at ZT20/ ZT6. The columns labeled *Crx*, *Otx2* and *Hoxc4* indicate the presence of regulatory binding sequences for the indicated transcription factor in the promoter of the gene (500bp upstream of first TSS).

A. Genes upregulated at night						
Gene symbol	Wild-type	<i>Crx</i> ^{-/-}	<i>Crx</i>	<i>Otx2</i>	<i>Hoxc4</i>	
Aanat	14.6	8.5	+	+		
Gng4	6.9	6.9				
Slc6a5	7.4	5.0	+			
E2f8	13.5	-	+			
Evi2a	12.0	-	+			
Cpm	11.2	-	+	+		+
Pols	9.5	-				
Gm2788	9.3	-				
LOC100046261	9.3	-				
2810011L19Rik	9.2	-				
Pvr	8.9	-	+			+
3930402G23Rik	7.5	-				
Kene1	7.5	-	+			
Asphd2	7.4	-				
E130002L11Rik	6.0	-	+			
Gulo	5.7	-				
Frmpl1	5.6	-	+			
Pde10a	-	4.7				+
Lrit1	-	4.4	+			
Mitf	-	4.3	+	+		
Syt10*	-	4.2				
Vill	-	4.1				

A. Genes upregulated at night						
Gene symbol	Wild-type	Crx ^{-/-}	Crx	Otx2	Hoxc4	
Delk1	-	4.0	+			+
ENSMUT00000011414	-	3.7				
Ccdc109b	-	3.7		+		+
EG245190	-	3.7				
Rftn1	-	3.7	+			
Slc6a17	-	3.6				
Gis2	-	3.5				
Rho	-	3.4	+			
Odc1	-	3.4				
Adora1	-	3.2	+			
181004IL15Rik	-	3.2		+		
Clec4d	-	3.1	+			+
Bok	-	2.9	+			+
Nrp2*	-	2.8				
Cry1	-	2.8	+			+
643041K18Rik	-	2.6		+		
Clstn3	-	2.5				
Stard4	-	2.3				

B. Genes downregulated at night						
Gene symbol	Wild-type	Crx ^{-/-}	Crx	Otx2	Hoxc4	
Atg16l1	1/4.0	1/2.6	+	+		
Cacnb2	1/7.4	1/2.9	+			+
Hspa12a	1/4.8	1/3.3				
Nr1d1	1/4.4	1/5.9		+		
Pdc	1/5.9	1/3.7	+			
Nrxn3	1/9.2	-	+			
AK048867	1/6.6	-	+			

B. Genes downregulated at night						
Gene symbol	Wild-type	Crx ^{-/-}	Crx	Otx2	Hoxc4	
Cntn4	1/6.0	-	+	+	+	
Xpo7	1/5.9	-				
Gabrd	1/5.7	-				
BC027072	1/5.5	-	+			
Ctca3	1/5.4	-	+			
A330008L17Rik	1/5.4	-				
A730054J21Rik	1/5.4	-			+	
Ppp2r2b	1/5.3	-	+	+	+	
ENSPTRT00000066164	1/5.3	-				
Car8	1/4.9	-				
Ppfia2	1/4.9	-	+	+	+	
LOC676792	1/4.9	-				
Ubr1*	1/4.8	-				
EG665934	1/4.8	-				
Bai3	1/4.6	-	+		+	
Gabra1	1/4.5	-	+		+	
Fat3	1/4.4	-		+		
Slc8a1	1/4.4	-	+	+	+	
Csmc3	1/4.3	-	+	+	+	
4930414L22Rik	1/4.3	-				
Fmn1	1/4.2	-	+		+	
Mima	1/3.9	-	+	+	+	
Sphkap	1/3.9	-				
Sv2b	1/3.9	-		+	+	
4930526L06Rik	1/3.9	-				
Ddc	1/3.8	-	+		+	
Tmem90a	1/3.7	-				
Cdh8	-	1/4.0	+			
Gngt1	-	1/4.0		+	+	

B. Genes downregulated at night

Gene symbol	Wild-type	Crx ^{-/-}	Crx	Otx2	Hoxc4
Rprm	-	1/3.8			
Chrma6	-	1/3.7	+		
Ancy1	-	1/3.3			
Sgip1	-	1/3.1	+		+
ENST00000371039	-	1/3.1			
Sphk2	-	1/3.0			
Dbp	-	1/2.9	+		
A830039N20Rik	-	1/2.6	+		
C030014L02	-	1/2.6			
B3galt2	-	1/2.5			+
Cngb3	-	1/2.4	+	+	
Gjd2	-	1/2.4	+		
Myppn	-	1/2.4	+		+
Srpk3	-	1/2.3			+
Morn1	-	1/2.0			

* Gene name assigned by authors based on homology.

[†] Transcript is not differentially expressed at a day/night basis. For further details see the Materials and methods section.

Table 5
Assignment of genes differentially expressed on a day/night basis in the wild-type and *Crx*^{-/-} mouse to networks and canonical pathways

Network and canonical pathway analysis was done using Ingenuity IPA software.

	Networks	Canonical pathways
Wild-type	Behavior Nervous system development & function Gastrointestinal disease Neurological disease Psychological disorders Cell morphology Cellular development Tumor morphology Lipid Metabolism Small molecule biochemistry Cell death Organ morphology	Cardiac b-adrenergic Signaling GABA Receptor signaling Dopamine Receptor Signaling
<i>Crx</i>^{-/-}	Behavior Nervous system development & function Genetic disorder Cell-to-cell signaling & Interaction Cardiovascular disease Neurological disease Skeletal & Muscular disorders Cellular development Cellular growth & proliferation Embryonic development Organismal development Skeletal & muscular system development & function	Phototransduction Pathway Protein Kinase A Signaling cAMP-mediated Signaling Circadian Rhythm Signaling Cardiac b-adrenergic Signaling

P-value <0.05.