Supplemental Experimental Procedures

Organ culture: The culture medium used for organ culture (NICHD) was BGJ_b medium (Fitton Jackson modification; Invitrogen, Carlsbad, CA) containing 1 gm/L bovine serum albumin fraction V, 25 mM HEPES buffer, pH 7.3, 2 mM glutamine, 0.1 mg/ml ascorbic acid and 0.1 ml/ 10 ml antibiotic-antimycotic solution (Sigma-Aldrich Corp., St. Louis, MO). Glands were incubated (37°C, 95% O_2, 5% CO_2) in 0.2 ml of culture medium on nylon screens in 24-well culture plates at the gas/liquid interface (1) for a 48 h control period; medium was changed at 24 and 36 h. After this control incubation period, glands were transferred to a top-loading tabletop incubator that allows addition of drugs and transfer of glands with minimal disturbance of pH, gas composition or temperature. Glands were incubated (1 gland / well) with fresh media containing NE (1 µM) , dibutyryl cAMP (DBcAMP; 0.5 or 1 mM) or forskolin (10 µM) (Sigma-Aldrich Corp.). Following a 6 hour treatment, glands were placed in microtubes on solid CO_2 and then stored at -80°C for the microarray analysis (experiment C) or were placed in RNA*Later* (Ambion) at room temperature, stored at 4°C and then shipped at room temperature for qRT-PCR analysis (Kings College London).

RNA isolation, labeling and hybridization: Total RNA was isolated (Trizol; Invitrogen); the amount and quality were assessed using a BioRad spectrophotometer (BioRad, Hercules, CA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was subjected to DNase treatment using Turbo DNA-free (Ambion, Austin, TX) to remove contaminating genomic DNA. Purified total RNA was converted to biotinylated cRNA using the GeneChip® One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA) The cRNA (20 µg) was then fragmented and hybridized to a microarray for 18 h at 45°C; the microarrays were stained and washed using Affymetrix protocols. The same protocols were used for all three experiments except that for experiments A and B (RG_U34A and RAE230 microarrays) 10 µg of total RNA was used as starting material for the GeneChip® Labeling Kit, and for experiment C (Rat230_2 microarray) 4 µg of total RNA was used and the hybridization time was 16 h (45°C).

Radiochemical in situ hybridization histology: Sagittal sections (12 µm) of frozen rat brains were prepared and stored at -80°C. For *in situ* hybridization histology (2,3), the sections were thawed and fixed for 5 min in 4% paraformaldehyde in phosphate buffered saline, washed 2 X 1 min in phosphate buffered saline, and acetylated (0.25% acetic anhydride in 0.9 % NaCl containing 0.1 M triethanolamine; 10 min). The sections were then dehydrated in a graded series of ethanols and delipidated in 100% chloroform (5 min). They were partially rehydrated in 100% and 95% ethanol (1 min each) and allowed to dry. For hybridization of the cryostat sections, [^{35}S]-labeled 38-mer oligonucleotide probes (Supplemental Data Table S1) were diluted in the hybridization buffer (10 µl labeled probe/ml hybridization buffer) consisting of 50% (v/v) formamide, 4 X SSC (150 ml mM NaCl, 15 mM sodium citrate, pH 7.0), 1 X Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 10% (w/v) dextran sulfate, 10 mM dithiothreitol, 0.5 mg/ml salmon sperm DNA and 0.5 mg/ml yeast tRNA. A 150 µl sample of the hybridization solution was placed on each section. The sections were then covered with Parafilm® and incubated in a humid chamber overnight at 37°C. After hybridization, the slides were washed in 1 X SSC for 4 X 15 min at 55°C, 2 X 30 min at room temperature, and rinsed twice in distilled water. The sections were dried and either exposed to X-ray film for 1-2 weeks or dipped into an Amersham LM-1® emulsion and exposed for 2 to 4 weeks at 4°C. The *in situ* hybridization images presented in Figure 2 are available at high resolution at http://sne.nichd.nih.gov/galleries/day_night/index.html.

The pineal hybridization signals on X-ray films were quantified using "Image 1.42" (Wayne Rasband, NIH). Optical density was converted to dpm/mg tissue using simultaneously exposed [^{14}C]-standards calibrated by comparison with [^{35}S] brain-paste standards. Results are

based on the analysis of pineal glands from at least four animals killed at night and at least four during the day. The means of *in situ* hybridization signals (dpm/mg tissues) in the rat superficial pineal gland were compared statistically by a two-sample *t*-test using log transformed data.

qRT-PCR: For data in Figure 4, three pools of two glands were used for each time point. Total RNA was extracted using the RiboPure RNA isolation kit (Ambion). The amount and quality of RNA were assessed using a BioRad spectrophotometer (BioRad) and an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was then subject to DNase treatment using TURBO DNA-free (Ambion) to remove contaminating genomic DNA. cDNA synthesis followed the Superscript II protocol (Invitrogen) using 1 μg of DNase-treated total RNA as starting material. For the data in Table 6 (and Supplemental Data Table S6), total RNA was extracted from three pools of three glands for each treatment group. The RNeasy Mini kit (Qiagen, Crawley, UK) was used to extract RNA; quality and quantity were measured as above. cDNA synthesis was done using the Quantitect Reverse Transcription kit (Qiagen).

qRT-PCR determinations were made using a LightCycler™ 1.2 (Roche Applied Sciences, Burgess Hill, UK) or a Rotor-Gene™ 6000 (Corbett Research U.K. St Neots, U.K). Reactions (10 μl volume) contained 0.5 μM primers (Table 2), QuantiFast SYBR Green PCR Kit (Qiagen, Crawley, U.K.) and cDNA (diluted 1:10) according to the manufacturer's instructions. All incubations included an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of 95°C for 10s, 20s annealing at 57°C and then extension at 72°C for 10s.

Product specificity was confirmed by ethidium bromide (EtBr)- agarose gel (2.2%w/v) electrophoresis of the amplified products and thereafter during every qRT-PCR run by melting curve analysis (Tm). Transcript number was determined using internal standards. PCR standards were prepared by amplification of a pool of 10-fold diluted pineal cDNA, followed by isolation of the single PCR product by EtBr-agarose gel electrophoresis. The band was then cut from the gel, spin column purified using a QIAquick gel extraction kit (Qiagen) and quantified by densitometry on an EtBr-stained gel with reference to known quantities of molecular weight markers. For all runs of all genes, a set of 10-fold serial dilutions of each internal standard ($10^1 – 10^7$ copies/2 μl) was used to generate a standard curve. All qRT-PCR assays were linear within this concentration range with correlation coefficients (r^2) >0.999. Transcript number was determined using a 2 μl sample of a 10-fold dilution of rat pineal cDNA prepared as described above. Values were normalized using a normalization factor calculated from the following four reference genes: *Actb , Gapdh, Hprt1, Rnr1* using GeNorm to calculate relative expression of the genes of interest (4). The normalization factor stays reasonably constant through 24 hrs indicating that changes in reported values of genes of interest do not reflect changes in the expression of the reference genes (data not shown).

Detection of cis-regulatory elements: The groups of genes analyzed by this method are described in the text. For analysis, each rat gene was converted to its human equivalent in Ensembl; only genes for which there was a clear homolog were included in the analysis. The relative enrichment of PWMs is given a weight score as described (5); PWM weight scores derived from ModuleMiner submissions of gene groups typically range between 0.005 and 1.0, with rare scores greater than 1.0 (see Supplemental Data Table S6). Larger values are indicative of greater PWM enrichment. In Supplemental Data Table S6, we have limited the presentation of weight scores to those greater than 1.0, thereby highlighting the most enriched PWMs in particular groups of genes.

ModuleMiner represents an advance over similar methods because it incorporates candidate gene filtering based on scoring of human-mouse conservation of non-coding sequences. However, although this approach selects for evolutionarily conserved features, filtering in this manner deselects particular genes if they don't exhibit such conservation and consequently

regulatory sequence features that occur in a particular rat gene, but not in the corresponding human and mouse gene are not detected. Details of individual PWMs were obtained from MatBase (6) (Genomatix Software Gmbh, Munich, Germany), TRANSFAC (http://www.gene-regulation.com/pub/databases.html) or TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

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