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Analysis of daily and circadian gene expression in the rat pineal gland

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Abstract

The mammalian pineal gland is an important component of the circadian system. In the present study, we examined the expression of roughly 8000 genes in the rat pineal gland as a function of time of day under light-dark (LD) cycles and in constant dark (DD) using oligo DNA microarray technique. We identified 47 and 13 genes that showed higher levels at night and day, respectively, under LD. The same patterns of expression were also observed in DD. About half of the genes that peaked at night have a known biological function, i.e., transcription factors and proteins that are involved in signaling cascades, whereas 14 are expressed sequence tags and 8 have an unknown biological function. Twelve of the genes that are up-regulated at night were also up-regulated after 1 h NE stimulation, thus suggesting that the expression of these genes is controlled by adrenergic mechanisms. Of the 13 genes that were up-regulated in the daytime, 6 coded for proteins that are involved in intracellular signaling pathways. The results obtained with microarray analysis were well correlated with data obtained using real time quantitative RT-PCR. The present results provide new materials to dissect and understand the pineal physiology.

Keywords

microarray; circadian rhythms; pineal; rat; genomic

1. Introduction

In mammals, melatonin is synthesized by the pineal gland and its synthesis is under direct control of the central circadian pacemaker that is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1997).

Previous studies have shown that at night, norepinephrine (NE) is released from the sympathetic nerve endings and activates the adrenergic receptors located in the pineal gland, the activation of adrenergic receptors leads to the transcriptional activation of the *arylalkylamine N-acetyltransferase* (*Aanat*, the rate limiting enzyme of melatonin synthesis) gene *via* CRE (cyclic AMP response element) and thus to the activation of circadian melatonin synthesis (Baler et al., 1997; Maronde et al., 1999). The adrenergic mechanisms also regulate circadian *Period1* gene expression (Takeida et al., 2000; Fukuhara et al., 2002), further

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suggesting that the SCN via the adrenergic mechanism is responsible for circadian events in the pineal gland.

In the recent years, the DNA microarray technique has been successfully used to study circadian gene expression in the SCN, liver, heart, kidney, and fibroblasts (Grundschober et al., 2001; Akhtar et al., 2002; Duffield et al., 2002; Kita et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002). The picture that is emerging from these studies indicates that in each tissue or organ a certain number of genes (approximately 2-10 %) are under circadian control.

In a previous study using DNA microarray, we reported that in the rat pineal NE stimulation affects regulation of several genes; 44 and 29 genes were up- or down-regulated more than 2.5-fold, respectively (Fukuhara et al., 2003). The purpose of the present study was to investigate the overall gene expression in 12 h light: 12 h dark (LD) and in constant darkness (DD) in the pineal gland using Affymetrix GeneChip array (Rat Genome U34A). Our data demonstrate that in the pineal gland a limited number of genes (about 2 %) are rhythmic in LD and DD conditions.

2. Materials and methods

Male rats of the Wistar strain (Charles Rivers Laboratories, Wilmington, MA, USA), 6-week-old at purchase, were adapted to 12 h-12 h (lights on, 07.00-19.00 h) LD cycles for one week before the start of the experiments. Animals were allowed free access to food and water throughout the experiments. The animals were divided into two groups: one was kept in LD cycles, and the other was transferred to constant darkness for 2 days before sacrifice. Light onset was defined as zeitgeber time (ZT) 0, and offset was ZT12. Circadian time (CT) was used for the rats sacrificed in DD. The pineal glands were removed from animals at ZT6 or ZT18 in LD cycles, or at CT6 or CT18 in DD. When the pineal glands were removed in the dark, the procedures were performed under a dim red light. The pineal samples were immediately frozen using dry ice, and kept at -80 °C.

RNA samples for microarray analysis were prepared using procedures previously described (Fukuhara et al., 2003). Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Biotin-labeled cRNA probes were generated according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). In brief, 10 μg each RNA sample was used to synthesize the microarray RNA targets. RNA was reverse-transcribed into double-stranded cDNA with a T7 promoter-containing primer using Superscript II, RNase H, and DNA polymerase (Invitrogen). After extraction with phenol-chloroform and ethanol precipitation with ammonium acetate, the cDNA was used as a template to generate biotin-labeled cRNA probe (Enzo Bioarray, Affymetrix, Santa Clara, CA, USA). Resulting target cRNA was collected on RNeasy columns (QIAGEN, Valencia, CA, USA), and then fragmented in alkaline buffer for hybridization to the microarray GeneChips.

For microarray analysis, biotin-labeled cRNA probes were used for hybridization with Rat Genome U34A array GeneChip probe arrays (Affymetrix). This GeneChip array consists of 8784 probe sets, contains approximately 7000 full-length sequences and approximately 1000 expressed sequence tag (EST) cluster genes from *Rattus norvegicus*. ESTs are mapped to the National Center for Biotechnology Information (NCBI) UniGene database. Hybridization was performed overnight at 45 °C, and the samples were processed to washing and detection of signals. Detailed procedures for preparation of RNA samples, hybridization, and data analysis were described in the previous study (Fukuhara et al., 2003). After normalization, only the genes whose intensities were above threshold at least at one time point in both LD and DD

were subjected to further analysis. A significant difference was evaluated only if the changes in mRNA levels were more than 2.0 fold between day and night values.

Detailed method of real time quantitative RT-PCR (Q-PCR) using iCycler (BioRad, Hercules, CA, USA) is described in previous studies (Fukuhara et al., 2003; Fukuhara et al., 2004). In brief, pineal total RNA was extracted using Trizol reagent following sonication, and DNA was degraded by DNase I. Forty cycles of amplification was carried out following 30 sec of denaturation at 95 °C. The samples were incubated at 95°C for 15 sec, the temperature was decreased to 60 °C, maintained for 45 sec, and raised to 72 °C for 30 sec. After PCR reaction was complete, the PCR products were run on the agarose gel to verify the quality of products (i.e., the specificity of the amplified product). The amount of amplified RNA was estimated from standard curves using the iCycler software version 3. Each mRNA level was normalized using *Gapdh* mRNA levels. In each experimental set, maximum value was represented 100, and each value was normalized relative to the maximum value. Fold changes were obtained by dividing higher value by lower value between day and night values. Comparisons between different groups/treatments were performed using parametric statistics (*t*-test). Primers used are: GenBank Accession number U179791 (AAACCGATTCCAAGATGTGC and ACACCATCTGCCAAAAGTCC); M80545 (CATGAGACCAGTGGTGTTGG and CGTGCTTACTGGGGTTGTTT); AF055477 (GAGCCTGCATTAGTATAGTGAATTG and AGGATGCAGCAGCAGTCCGTA); L01624 (AATGGCGGAGAGCTGTTCTA and TGTGCTCGATGTTCTCCTTG); M64301 (AGCCTATTTCCAGCCATCCT and TCGGTGACATCAGACAGAGC); L26986 (GTCACTTGGGTGGCTATGAC and CCGCTAGTCTGGGTATGAGC).

3. Results

In each GeneChip 35.1 % (ZT6), 34.5 % (ZT18), 36.9 % (CT6), and 34.9 % (CT18) of the genes showed a significant hybridization signal. A total of 60 genes showed more than 2-fold day-night differences in LD and DD (Table 1). Forty-seven of these genes had higher levels at night (ZT18 or CT 18), while 13 showed higher levels in the day (ZT6 or CT6). Of the 47 genes that have higher levels during the night, 25 genes have a known biological function, whereas 14 are ESTs and 8 have an unknown biological function (Fig. 1). Of the 13 genes that were up-regulated in the daytime, 6 code for proteins that are involved in intracellular pathways (Fig. 1) (Table 2).

To confirm the results obtained using array technique, we measured mRNA levels of several genes by using Q-PCR method. Fold changes obtained using array and Q-PCR techniques were well correlated (Fig. 2, r =0.88, $P < 0.001$, n=11). The Q-PCR data for two genes (*Erk3* and *Ptps2*) that showed circadian expression with high levels in the day or night are shown in Figure 3. *Erk3* mRNA levels were high at night, and showed 5.28- and 10.8-fold differences between day and night in LD ($P < 0.05$) and DD conditions ($P < 0.05$), respectively (Fig. 3A). Another example *Ptps2* showed higher levels in the daytime LD ($P< 0.001$) and DD ($P < 0.001$) conditions (Fig. 3B).

4. Discussion

DNA microarray technique is useful for analyzing gene expression. In the present study, we compared overall gene expression in the rat pineal gland under LD and DD using oligo DNA microarray and Q-PCR methods.

The present study shows that 34.5-36.9 % of the approximately 8,000 genes present on the GeneChip probe array (Rat Genome U34A Array) are present in the pineal gland. Surprisingly, only a small number (about 2.0 %) of these genes' expression showed significant difference

in the expression levels between day and night under LD and DD conditions (Tables 1 and 2). These results are consistent with previous studies, which reported that 40 % of genes are expressed in the rat pineal gland (Fukuhara et al., 2003), and only a modest number (2-3%) are rhythmic (Humpries et al., 2002). The present study expands a previously published array study in which diurnal gene expression was examined for 1176 genes in LD (Humpries et al., 2002), since we examined over 8000 gene expressions both in LD and DD. To verify the results obtained with the microarray analysis, we measured mRNA levels for several genes using Q-PCR. In all cases, we observed a significant correlation between the data obtained using the two methods (Fig. 2 and Fig. 3). This result indicates that microarray analysis can be used to assess relative fold-changes in gene expression levels. Interestingly, we found no difference in the total number of genes that are rhythmically expressed in LD and in DD conditions, thus indicating that the mechanisms controlling pineal gene expression, at least for the ones present on the U34A GeneChip, are under strict circadian control (i.e., by the SCN) and the photic environment does not significantly affect gene expression independently from the SCN.

A recent study investigated overall gene expression in the chicken pineal (Bailey et al., 2003). In this investigation the authors reported that a larger percentage of genes was rhythmically expressed in the chicken pineal since they found that 377 genes showed a day/night variation (> than 2-fold) in LD and 126 genes in DD. At first sight, it may appear that such a result is in sharp contrast with our own results, both in terms of the total number of genes expressed and in terms of the considerable difference between the numbers of genes that are rhythmically expressed in LD and DD. However, it must be noted that the chicken pineal is photosensitive whereas the rat (adult) pineal is not (Tosini et al., 2000). Therefore, it may be possible that the observed lower in the number of rhythmically expressed genes in the rat pineal may be a direct consequence of the loss of these two important functions. Furthermore, it is worthwhile to mention that in our study we investigate only two time point (ZT6-18 and CT 6-18) and therefore it is likely that differences in gene expression may become apparent at other times.

A series of recent studies investigated the circadian patterns of gene expression in the SCN, liver, heart in mouse and rat, as well as rat fibroblasts. Each study has employed a different plat form, data analysis methods, and screened different numbers of genes. In general, it appears that about 10 % of the genes are circadianly expressed in the liver, heart, and kidneys (Akhtar et al., 2002; Kita et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002). Interestingly, in the fibroblasts it has been reported that fewer than 100 genes (or about 2% of the genes present in these cells) are rhythmically expressed (Duffield et al., 2002; Grundschober et al., 2001). The fact that fibroblasts showed much less rhythmic genes expression than the SCN and/or other tissues, but are similar to what has been observed in the pineal (Humpries et al. 2002, and this study) suggests that the reduction in the number of rhythmically regulated genes may be due to the reduction in metabolic and physiological activities in fibroblasts and pinealocytes in comparison with cells in other organs and tissues.

As we have already mentioned, 47 genes showed clear (more than 2-fold) day/night variations in LD and DD with high levels at night, thus suggesting that expression of these genes is regulated by the SCN via adrenergic mechanisms. We were surprised to find that only 12 genes are increased after 1-h NE stimulation and also up-regulated at night (Table 3 and (Fukuhara et al, 2003). Such a result suggests that the majority of the genes that are acutely regulated by NE are later down-regulated and thus did not show any significant variations in the middle (ZT-CT 18) of the night.

Among the genes that are up-regulated by NE and at ZT (CT) 18 are AA875032 (580 bp) which shares 100 % homology with *Fos*-related antigen (GeneBank accession number X98051) in 177 bp regions, and AA859996 and AI169756 showed no similarity with any other genes.

AI176710 showed a similarity with neuron-derived orphan receptor (NM_031628) (EST 614 bp: 318/326 bp, 97%), which is highly homologous to *ngfi-B/nur77*. The role of these genes in the regulation of pineal physiology is unknown.

A set of genes that is highly expressed at night, but not acutely affected by 1 h NE stimulation, also deserves further consideration (Tables 1 and 3). Although it has been shown that glutamate inhibits melatonin synthesis in rat pinealocytes (Yamada et al., 1998), a specific role of mGluR1 in such inhibition or melatonin synthesis has not been elucidated. *Type II iodothyronine deiodinas*e expression has been shown to be rhythmic under LD cycles and affected by adrenergic receptor stimulation in the rat pineal gland (Murakami et al., 1997;Kamiya et al., 1999). An earlier study has reported the presence of *Erk1* and the absence of *Erk2* and *Erk3* in the rat pineal gland (Kiyama et al., 1994). Our data indicate that *Erk1* (GeneBank accession number M61177) and *Erk2* (M64300) are not present at any time of the day under LD and DD conditions, whereas *Erk3* shows robust day-night variations under both conditions (Table 1 and Fig. 3A). Such a discrepancy may be due to different sampling (i.e., the timing) procedures, e.g., *Erk3* is expressed very low levels (below threshold) during the daytime (Table 1). The role of ERKs in melatonin synthesis or any physiological functions in the mammalian pineal gland has not been reported.

An earlier study reported a diurnal rhythm in dopamine content in the rat pineal gland (Miguez et al., 1998) and our data indicate that a *dopamine D4 receptor* mRNA is present in the rat pineal gland (Table 1). Previous studies have shown that levels of retinal melatonin are partially regulated by dopamine that acts through a D4 receptor (Jaliffa et al., 2000; Tosini and Dirden, 2000). D4 receptors are negatively coupled with adenylyl cyclase (i.e., their activation decreases the activity of adenlylyl cyclase and thus the levels of cAMP) and therefore is likely that the D4 receptors present in the pineal are also involved in the regulation of pineal melatonin levels. Inositol-1,4,5-triphosphate receptor protein is highly expressed in the rat pineal gland (Sharp et al., 1993). Since inositol-1,4,5-triphosphate receptor is a calcium channel, day-night variations in the receptor mRNA levels may contribute to regulation of calcium levels, which control circadian regulation of a series of gene expression as well as the activation and inactivation of intracellular signaling cascades. Diurnal rhythms in cyclic nucleotide phosphodiesterase activity levels have been shown in the rat pineal gland (Minneman and Inversen, 1976); taken with the present results, it may suggest that such rhythms in the activity levels are regulated partly by a transcriptional mechanisms. Since cAMP levels are a critical parameter in regulation of melatonin synthesis (Klein et al., 1997), it is likely that the mechanisms regulating phosphodiesterase activity levels need to be finely controlled.

In the present study, we also found that 13 genes are up-regulated during the daytime (Table 2). Six of the 13 genes are involved in signaling cascades; particular interests are calreticulin and L-type calcium channel beta 2 subunit that are involved in regulation of calcium levels. Calreticulin is a calcium sensor, which binds to Ca^{2+} in the endoplasmic reticulum (Groenendyk et al., 2004). A recent study has revealed that calreticulin possesses high-affinity melatonin binding sites and that is involved in genomic regulation (Macias et al., 2003). Function of L-type calcium channel(s) in regulation of melatonin synthesis has been suggested in previous studies; inhibitory modulation, based on the observations that entry of Ca^{2+} through L-type calcium channel(s), at least in part, triggers microvesicle-mediated glutamate exocytosis in rat pinealocytes (Yamada et al., 1996), and glutamate inhibits NE-dependent melatonin synthesis (Yamada et al., 1998). Therefore, L-type calcium channel(s) may be one of the critical regulators that control circadian melatonin synthesis. AI072634 (629 bp) shows 99 % similarity with rat *guanylate cyclase 1, soluble, alpha 3* (GeneBank accession number NM_017090) in 626 bp regions.

The mechanisms regulating gene expression in the pineal gland are rather complex since gene transcription in this organ is controlled by both the central clock in the SCN *via* the NE pathway as well as *via* circadian clocks present in the pineal gland (Abe et al., 2002; Fukuhara et al., 2005). Based on our previous and present studies, it is apparent that a sub-group of gene expression is directly triggered by NE (Table 3) (Fukuhara, et al., 2003); whereas a second set of genes (i.e., the ones that peaked during the day or later at night) may be regulated by the circadian clock or by pathways that are indirectly activated by NE.

Earlier investigations have reported that the transcription of clock-controlled genes can be activated by the circadian transcription factors BAML1 and CLOCK via the E-box (i.e., a DNA sequence to which these transcription factors can bind) present on the promoter region of these genes (Jin et al., 1999; Chen and Baler, 2000). However, it is important to mention that in the pineal gland BMAL1:CLOCK is not capable to activate *Aanat* transcription (Chen and Baler, 2000, Fukuhara and Tosini, 2003) and therefore a promoter analysis without a functional assay will only provide data of limited value.

Previous studies have also shown that circadian clock genes, such as *Period1*, *Cryptocrome*, *Bmal1* and *Clock*, are rhythmically expressed (Namihira, et al., 1999; Takekida et al., 2000; Fukuhara et al., 2002, Simonneaux et al., 2004). In our study we did not detect any circadian rhythm in the clock genes since most of these genes are not present on the Rat Genome U34A array GeneChip.

In conclusion, our study demonstrated that 60 genes in the pineal gland have a day/night variation (more than 2-fold) under LD and DD conditions. The majority of the genes peak during the night but we also identified a new and smaller set of genes that shows higher levels during the day (ZT-CT 6). The present results provide new materials to dissect and understand the pineal physiology.

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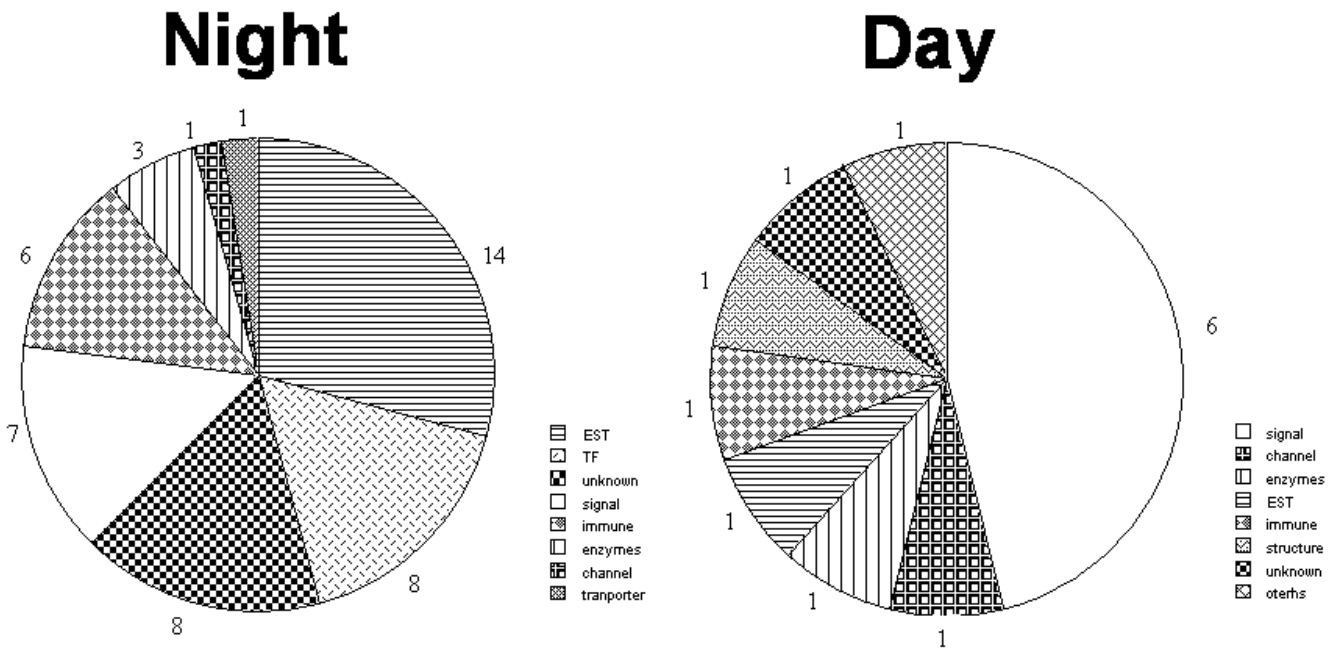

Fig. 1.

Functional classification of the genes that peaked during the night (47 genes) or during the day (13 genes) in LD and DD in the rat pineal gland. The gene products are classified on the basis of known and unknown functional classes. Numbers represent the number of genes that belong to each class.

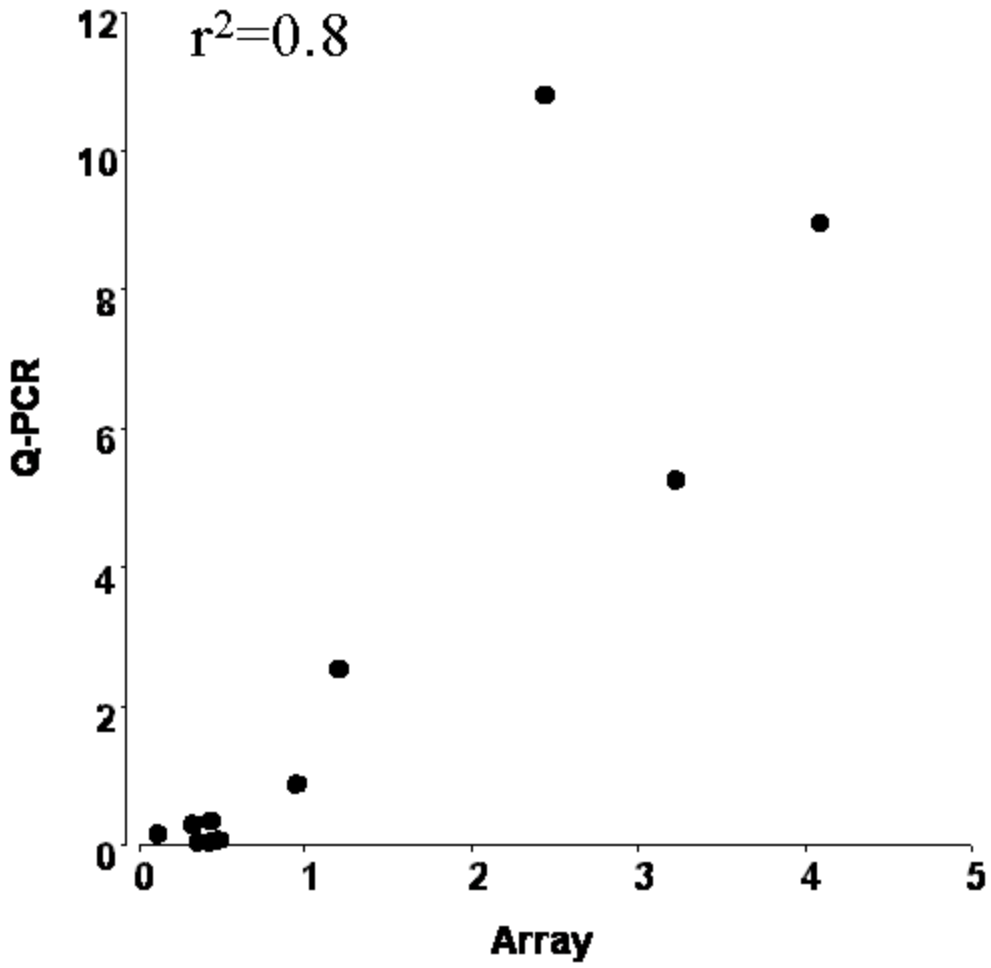

Fig. 2.

Regression analysis between values obtained using DNA microarray and real time quantitative PCR (Q-PCR) methods. The values obtained with the two methods were highly correlated (r=0.88, $P < 0.001$, n=11). Each value represents fold-change of night value relative to day value.

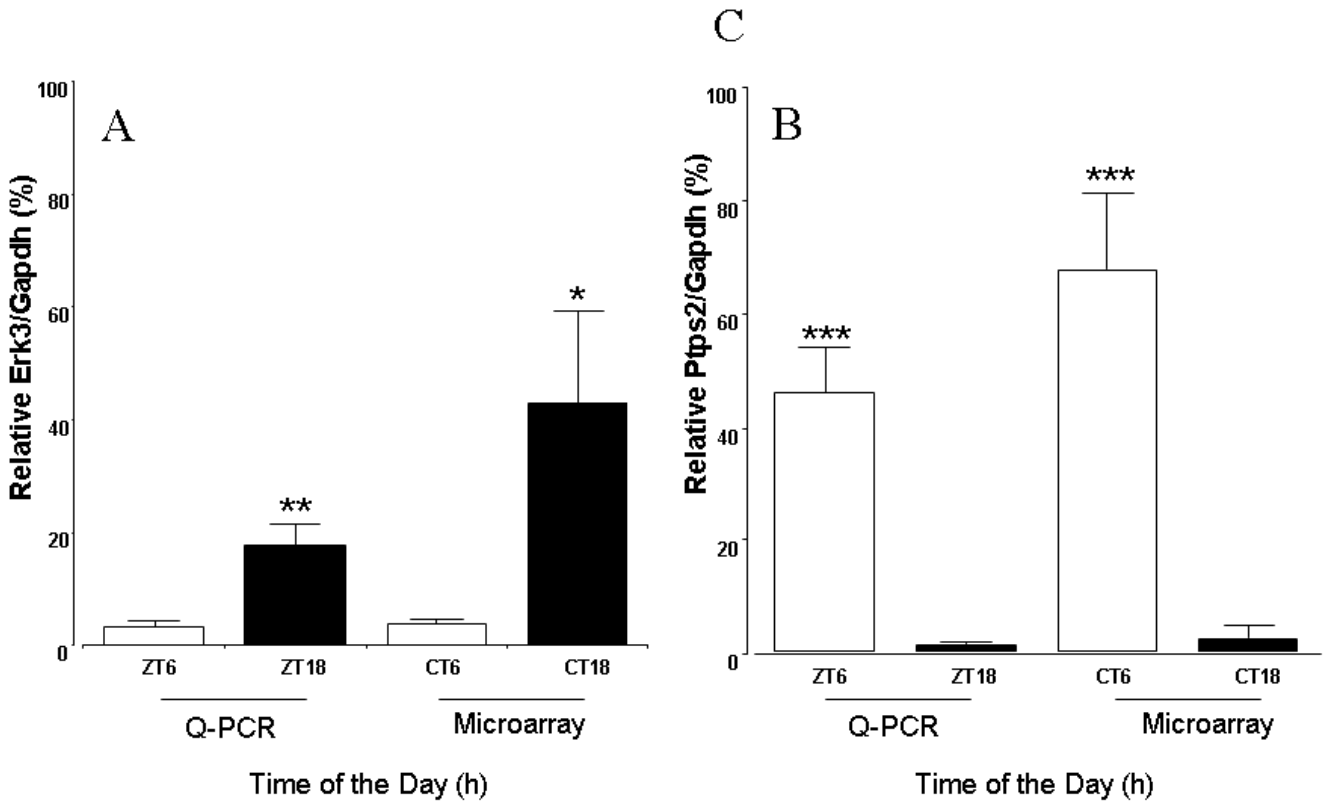

Fig. 3.

Day-night rhythms in *Erk3* (A) and *Ptp2* (B) mRNA levels in LD and DD conditions. *Erk3* and *Ptp2* mRNA levels showed higher levels at night and day, respectively. The data obtained using Q-PCR are consistent with those obtained using DNA microarray technique. Open bars represent daytime samples, and filled columns show nighttime samples. Values are means ± S.E.M. n=5 per group. * $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Table 1

List of genes that are up-regulated at night (ZT18-CT18) in the rat pineal gland

GenBank Accession number	Fold changes LD	DD	Description
D50664	133.64	274.32	Oligopeptide transporter
S66024	33.91	3.39	CREM
AI639378	15.28	3.69	cDNA clone rx01635
U38306	11.37	21.86	Arylalkylamine N-acetyltransferase
M21622	9.04	9.47	High-affinity IgE receptor (Fc-epsilon-R-I)
S81478	8.42	10.41	3CH134/CL100 PTPase
U17254	6.05	4.84	NGFI-B
AA892813	5.72	21.99	EST196616
Z75029	5.20	3.08	Heat shock protein 70
AA875032	5.00	14.65	cDNA
M65251	4.92	4.24	Angiotensinogen gene-inducible enhancer-binding protein 1
AA893658	4.78	2.78	EST197461
U04835	4.63	8.83	CREMdeltaC-G
AA799812	3.91	3.45	EST189309
AA891595	3.82	3.39	EST195398
Z34264	3.79	2.26	Potassium channel subunit
M61099	3.77	3.78	Glutamate receptor (MGLUR1)
U53505	3.75	58.79	Type II iodothyronine deiodinase
AA900505	3.59	2.17	cDNA
U49062	3.56	4.06	Heat sle antigen CD24
AI639417	3.47	2.95	cDNA clone rx02173
M64301	3.22	2.44	Extracellular signal-related kinase (ERK3)
M84009	3.22	5.31	Dopamine receptor D4 (RATD4)
L16764	3.03	3.39	Heat shock protein 70 (HSP70)
AA875042	3.01	5.08	cDNA
AA799729	3.00	4.57	EST189226
AA859996	2.88	3.16	cDNA
AI169756	2.80	3.49	EST215655
AI008131	2.78	2.40	EST202582
AA892259	2.76	3.00	EST196062
U14398	2.75	3.48	Synaptotagmin IV homolog
L29232	2.64	4.06	Insulin-like growth factor I receptor
AI104389	2.56	2.21	EST213678
J05510	2.55	2.51	Inositol-1,4,5-triphosphate receptor
AF023087	2.54	3.13	Nerve growth factor induced factor A
AF093267	2.47	2.75	Homer-1b
AI070783	2.45	3.62	cDNA
L27060	2.39	6.80	Phosphodiesterase
D50436	2.36	2.30	Adrenodoxin
AI176710	2.35	6.76	EST220303
AA893742	2.26	2.17	EST197545
J05499	2.22	5.06	L-glutamine amidohydrolase
AA800572	2.15	2.18	EST190069
L12025	2.13	2.66	Tumor-associated glycoprotein E4 (Tage4)
H33093	2.09	2.57	EST108772
AB007689	2.04	2.01	Vesl-2
AA892319	2.04	2.03	EST196122

Table 2

List of genes that are up-regulated during the day (ZT6-CT6) in the rat pineal gland

GenBank Accession number	Fold changes LD	DD	Description
X53363	19.98	2.23	Calreticulin
M80545	9.03	2.05	L-type calcium channel beta 2 subunit
M94919	4.28	3.26	Beta-globin
X51529	3.82	3.29	Platelet phospholipase A2
M94918	3.29	3.94	Beta-globin
X58465	3.05	2.90	Ribosomal protein S5
U17971	2.77	2.35	Protein tyrosine phosphatase 2E (PTP2E)
AB017711	2.72	2.28	RNA polymerase II
S65091	2.71	2.08	Cyclic AMP-regulated phosphoprotein
AB011528	2.46	2.09	MEGF2
AA849036	2.42	2.16	EST191798
L01624	2.34	3.10	Serum and glucocorticoid-regulated kinase (sgk)
AI072634	2.29	3.73	cDNA
AF097593	2.16	2.13	Testicular N-cadherin

Table 3

Genes that up-regulated by 1h-NE stimulation and peaked at night (ZT18- CT18) in the rat pineal gland. The table was generated by comparing the lists shown in Table 1 of the present study and those in our previously published data [7]

GenBank Accession number	Fold changes LD	DD	NE	Description
D50664	134.00	274.00	13.70	Oligopeptide transporter
S66024	33.90	3.39	37.30	CREM
U38306	11.40	21.90	31.40	Arylalkylamine N-acetyltransferase
M21622	9.47	9.04	9.20	Fc fragment of Ige
S81478	8.41	10.40	6.20	3CH134/CL100
U17254	6.05	4.84	8.80	NGFI-B
AA875032	5.00	14.70	4.50	cDNA
U04835	4.63	8.83	7.80	CREMdeltaC-G
AA859996	2.88	3.16	2.90	cDNA
AI169756	2.80	3.49	2.70	EST215655
U14398	2.75	3.48	4.20	Synaptotagmin IV homolog
AI176710	2.35	6.76	14.80	EST220303