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Diurnal variation of the adenylyl cyclase type 1 in the rat pineal gland

(cyclic AMP/circadian rhythms/norepinephrine/calmodulin)

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ABSTRACT **Nocturnal melatonin production in the pineal gland is under the control of norepinephrine released from superior cervical ganglia afferents in a rhythmic manner, and of cyclic AMP. Cyclic AMP increases the expression of serotonin *N*-acetyltransferase and of inducible cAMP early repressor that undergo circadian oscillations crucial for the maintenance and regulation of the biological clock. In the present study, we demonstrate a circadian pattern of expression of the calcium/calmodulin activated adenylyl cyclase type 1 (AC1) mRNA in the rat pineal gland. *In situ* hybridization revealed that maximal AC1 mRNA expression occurred at midday (12:00–15:00), with a very low signal at night (0:00–3:00). We established that this rhythmic pattern was controlled by the noradrenergic innervation of the pineal gland and by the environmental light conditions. Finally, we observed a circadian responsiveness of the pineal AC activity to calcium/calmodulin, with a lag due to the processing of the protein. At midday, AC activity was inhibited by calcium (40%) either in the presence or absence of calmodulin, while at night the enzyme was markedly (3-fold) activated by the calcium–calmodulin complex. These findings suggest (*i*) the involvement of AC1 acting as the center of a gating mechanism, between cyclic AMP and calcium signals, important for the fine tuning of the pineal circadian rhythm; and (*ii*) a possible regulation of cyclic AMP on the expression of AC1 in the rat pineal gland.**

In living organisms, development, maturation, or reproduction usually undergo circadian or seasonal variations. In dictating the adaptative response of the organism to this temporal program, the pineal gland plays a key role, functioning as a neuroendocrine transducer. During the night–day cycle, input information from the retina is perceived at the pinealocyte membrane level as a rhythmic nocturnal norepinephrine (NE) signal, via a multisynaptic modulatory pathway (1). This neural information is then converted into a hormonal message, the nocturnal melatonin production and release, a phenomenon mediated essentially by the cyclic AMP pathway (2–4).

The nocturnal rise in cyclic AMP amount (5) and the subsequent increase in cyclic AMP-responsive element-binding protein (CREB) phosphorylation (6) account for the nocturnal profile of melatonin production, by stimulation of both the transcription and the activity of serotonin *N*-acetyltransferase (NAT), the rate limiting enzyme in melatonin synthesis (7–9). Additionaly, cyclic AMP controls the expression of immediate early genes capable of further modulating cyclic AMP-responsive element (CRE) responsiveness (10–12). In particular, the mRNA of inducible cyclic AMP early repressor (ICER), a known cyclic AMP-inducible repressor of cyclic AMP-dependent transcription (13, 14), obeys a dramatic circadian pattern of expression, suggesting the existence of a feedback control between CREB activation and CRE-responsive gene transcription (11, 12).

NE increases the amount of cyclic AMP through both β- and α1- noradrenergic receptors. This effect involves a synergistic action of G proteins on the one hand, and PKC or Ca^{2+} signaling pathways on the other hand (6, 15, 16). In various systems indeed, intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) operates as a modulator of cyclic AMP signaling by (*i*) regulating Ca^{2+}-dependent phosphodiesterases and phosphatases (17, 18), (*ii*) phosphorylating CREB via calcium/calmodulin-dependent kinases (19–21), or (*iii*) regulating adenylyl cyclase (AC) activity (22).

In the present study, we have focused on the possible involvement of AC1, a cyclase isoform already known to be implicated in several examples of such convergent regulation. Indeed, AC1 is activated by calcium/calmodulin and has been postulated as a "coincidence detector" of G protein-mediated and calcium-mediated signal transduction (23, 24). We demonstrate here that AC1 expression obeys a strict circadian rhythm controlled by the photoperiodic environment and constitutes an important element in the regulation of pineal gland function.

EXPERIMENTAL PROCEDURES

Animals. Male, adult, Wistar rats (200–250 g) were used. They were kept under a standard 12-h light/12-h dark cycle (light on from 6:00 to 18:00) and were adapted to this rhythm for at least 1 week before any manipulation. The animals were quickly decapitated at the indicated time points under ambient light conditions.

Treatments. Isoproterenol at a dose of 5 mg/kg was administered at 10:00 and rats were killed 2 h later. Propranolol at a dose of 20 mg/kg was administered just before the onset of the dark phase, and rats were killed at 0:00. Both drugs were dissolved in 0.9% NaCl and injected s.c. in a volume of 0.5 ml. Control animals were treated with vehicle alone, under the same conditions. For some experiments, rats were maintained under constant light (LL) conditions with no other treatment, and were killed after various time periods of extra-light (i.e., 6 h, 18 h, 30 h)

AC Assay. Pineal glands were dissected at 4°C and placed in ice-cold homogenization buffer of the following composition: 20 mM Tris·HCl (pH 8), 1 mM EDTA, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride (Sigma). For each experiment, pineal glands of at least 20 rats were pooled and homogenized in 10 times the volume of the above buffer. Preparation of crude membrane fractions and AC assay were prepared as described (25). Briefly, membranes (15–30 μg protein) were incubated at 37°C for 10 min in 60 μl of an assay

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Abbreviations: AC, adenylyl cyclase; NAT, serotonin N-acetyltransferase; ICER, inducible cyclic AMP early repressor; NE, norepinephrine; CREB, cyclic AMP-responsive element-binding protein; CRE, cyclic AMP-responsive element; LL, constant light.

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medium with the following composition: 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 1 mM cAMP, and 100 μM ATP containing 10^6 cpm [α-^{32}P]ATP, in a regenerating system consisting of 5 mM creatine phosphate and 250 μg/ml creatine kinase. The reaction started with the addition of the membranes and was stopped in 500 mM HCl. The amount of the [α-^{32}P]cAMP formed was measured after separation of the cAMP from the ATP on alumina columns. To assess the calcium and calmodulin sensitivity of AC, 1 mM EGTA was added in all assay mixtures. Cyclase activity was assayed in the presence or absence of 950 μM calcium and 2.4 μM calmodulin, or of the calmodulin inhibitor W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide HCl; Sigma]. Protein amount was determined using the Bio-Rad reactif assay.

***In Situ* Hybridization Histochemistry.** Brains were quicky removed and frozen gradually in a dry-ice/isopentane preparation. Coronal sections (14 μm) containing the pineal gland mounted on gelatin-coated slides and fixed in 4% paraformaldehyde were hybridized at 42°C according to standard protocols (26) with a ^{33}P-labeled oligonucleotide probe (ICN) specific for an individual AC isoform (AC1 or AC5). For control sections, a 50-fold excess of unlabeled probe was added to the hybridization and prehybridization solutions.

The precise sequences for the oligonucleotide probes used are as follows: 5′-TCC TCA AAG CCC ATC CTC TTC ATT TCC TTG GAC ACC TGG TTG CCA CCC AA-3′ for AC5, and 5′-ATG TTC AGG TCT ACT TCA GTA GCC TCA GCC ACG GAT GTG ATG GTA TCG AT-3′ for AC1. These probes have been previously used in *in situ* hybridization studies for the localization of the respective ACs (27, 28). Under our conditions, the *in situ* hybridization patterns obtained in brain sections were identical to those reported in the respective initial studies. In Northern blot analysis, each oligonucleotide detected only one mRNA band corresponding to the appropriate size. For AC1 in particular, the sequence has been chosen in the first cytoplasmic loop, near the putative calmodulin binding domain of the AC1, and corresponds to a domain that is highly specific.

Quantification of *in Situ* Hybridization Histochemistry Results: Statistics. Autoradiogram quantification was performed by using a Biocom (Paris) image analyzer. To normalize variations between different animals, the results are expressed as the ratio OD pineal/OD cortex. For this purpose, the zone of the retrosplenial cortex adjacent to the pineal gland (29) was quantified for each section. Statistical analysis was performed using the GENERAL LINEAR MODEL package of the SAS software (30). Values of $P < 0.01$ were considered significant. Each experimental group consisted of at least three animals, except when otherwise stated.

RESULTS

AC Activity Is Differentially Regulated by Calcium/Calmodulin at Day and at Night. Midday (rats decapitated at 12:00) and midnight (rats decapitated at 0:00) AC activity was measured in pineal membrane preparations and its responsiveness to calcium/calmodulin was analyzed at both time points (Table 1). We found no difference between midday and midnight basal AC activity, but the enzyme was differentially regulated by calcium/calmodulin at midday and midnight [$F(1,25) = 33.3$, $P < 0.001$] (Table 1). At midday, AC was inhibited (40%) by calcium either in the presence or absence of calmodulin, while at night the enzyme was markedly (3-fold) activated by the calcium–calmodulin complex. At both time points, AC activity was stimulated by 100 μM noradrenaline or 100 μM isoproterenol, in the presence of 10 μM GTP, to the same extent (1.5- to 1.8-fold) (data not shown). Parallel assays of the cortex AC revealed no circadian variations (data not shown).

Table 1. Day versus night AC activity in the rat pineal gland

	Day	Night
EGTA (1 mM) plus calmodulin (2.4 μM)	126 ± 18	77 ± 3
$CaCl_2$ (950 μM) plus EGTA (1 mM) plus calmodulin (2.4 μM)	71 ± 23	219 ± 21*

Analysis of the responsiveness of AC to calcium and/or calmodulin. To assess the calcium and calmodulin sensitivity of AC, 1 mM EGTA was added in all assay mixtures. Cyclase activity was assayed in the presence or absence of 950 μM calcium and 2.4 μM calmodulin. Results are expressed in pmoles cyclic AMP formed/min/mg protein and represent means ± SD of two individual experiments run in triplicates. Parallel assays of the cortex adenylyl cyclase revealed no circadian variations (data not shown).
*$P < 0.001$ compared with day.

AC1 mRNA Expression in the Rat Pineal Gland Follows a Pronounced Circadian Pattern. The mRNA expression of the calcium regulated AC isoforms, AC1 and AC5, was investigated during the day–night cycle in the rat pineal gland. AC1 mRNA showed a dramatic circadian pattern of expression (Fig. 1) as evidenced by *in situ* hybridization autoradiograms (Fig. 1*A*) showing the distribution of AC1 mRNA in the pineal gland of rats decapitated either at 12:00 (day) or at 0:00 (night). The marked circadian variation of the AC1 signal was specific for the pineal gland [$F(5,11) = 16.41$, $P < 0.0001$], while in the cortex this signal remained constant [$F(5,11) = 5.14$, $P > 0.01$]. Therefore, results are expressed as the ratio OD pineal/OD cortex (see *Experimental Procedures*). The time-course of the relative AC1 mRNA expression in the pineal gland indicates a one-peak pattern of rhythmicity with a maximal AC1 expression at midday (12:00–15:00) and a minimal signal at night (0:00–3:00) (Fig. 1*B*). AC5 was not expressed either at midnight or midday (data not shown).

AC1 mRNA Expression in the Rat Pineal Gland Is Controlled by NE and Environmental Light Conditions. Most circadian oscillations between noradrenergic receptor activation and melatonin production in the pineal gland are controlled by upstream clock elements. NAT expression and activity (7–9), β-adrenergic receptor amount (31), ICER ex-

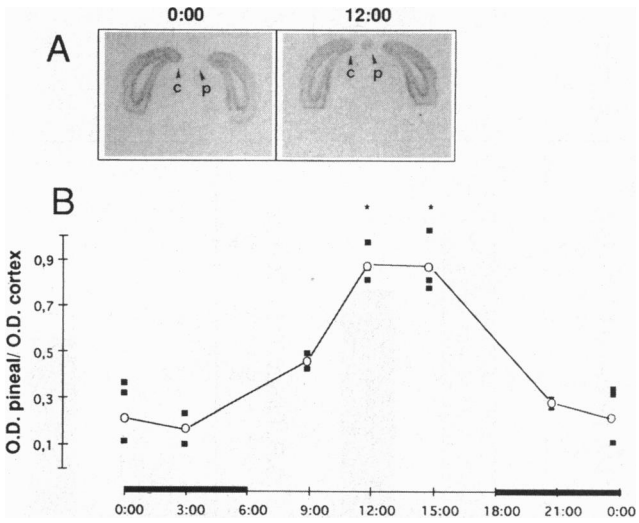

FIG. 1. Circadian variation of the AC1 mRNA expression in the rat pineal gland. (*A*) *In situ* hybridization autoradiograms showing the distribution of AC1 mRNA in the pineal gland (p) and cerebral cortex (c) of rats decapitated either at 12:00 (day) or at 0:00 (night). (*B*) Time course of the relative AC1 mRNA expression in the pineal gland. To normalize variations between different animals, the results are expressed as the ratio O.D. pineal/O.D. cortex (see *Experimental Procedures*). Solid squares, values of independent experiments; open circles, the calculated means; *, $P < 0.001$ compared with the nocturnal (0:00, 3:00) expression signals.

pression (11) are regulated by NE and show a one-peak profile. Interestingly, the two-peak diurnal pattern reported for cyclic GMP production has been shown to be NE independent (32). As AC1 expression obeys a single day-night fluctuation program, we investigated the potency of NE in modulating its expression. Thus, we mimicked the physiological nocturnal innervation of the pineal gland by administering the β-adrenergic agonist isoproterenol in the middle of the light phase. This resulted in a reduced (30%) midday AC1 mRNA signal [$F(1,5) = 25.97, P < 0.01$ as compared with control; Fig. 2*A*]. In addition, blocking the physiological nocturnal NE elevation by administration of the β-adrenergic antagonist propranolol 2 h before the onset of the dark phase induced a marked increase (more than 2-fold) of the AC1 mRNA signal at midnight [$F(1,7) = 19.36, P < 0.01$ as compared with control, Fig. 2*B*].

Further evidence for the involvement of the photoperiodic environment in regulating AC1 mRNA fluctuations was provided when rats were kept under LL conditions (Fig. 2*C*). AC1 mRNA remained abundant at the following "midnight" point [animals decapitated after 6 h of extra light, $F(1,7) = 47.15$, $P < 0.001$ as compared with control], but was undetectable afterwards {rats decapitated after 18h [$F(1,9) = 94.83, P < 0.001$ as compared with control] and 30 h of extra light, respectively}.

As a control for the above experiments, we measured the *in situ* hybridization signal for ICER mRNA in the pineal gland (data not shown). Isoproterenol induced a 40% increase in the midday expression of ICER mRNA, while propranolol resulted in a pronounced decrease (more than 2-fold) in the ICER mRNA signal at midnight. When rats were kept under LL conditions, ICER expression switched off after 6 h of extra-light; at the same time-point the AC1 mRNA signal persisted at high levels. Overall ICER mRNA expression results are in agreement with the previous data of Stehle *et al.* (11).

DISCUSSION

In the present study, we demonstrate a dramatic circadian pattern of the regulation of AC activity by calcium in the rat pineal gland, with a marked nocturnal switch to a calcium/calmodulin activated isoform. By *in situ* hybridization we have identified a circadian expression of the mRNA for AC1, a major neural isoform (27, 33) also implicated in various paradigms of learning and cellular adaptation (33–35). Interestingly, calcium/calmodulin-stimulated AC activity predominates at night, whereas the mRNA of the calcium/calmodulin activated AC1 is maximal during the day. Such a time lapse is not surprising for the processing of a functional membrane protein. The variation of β-adrenergic receptors in the pineal

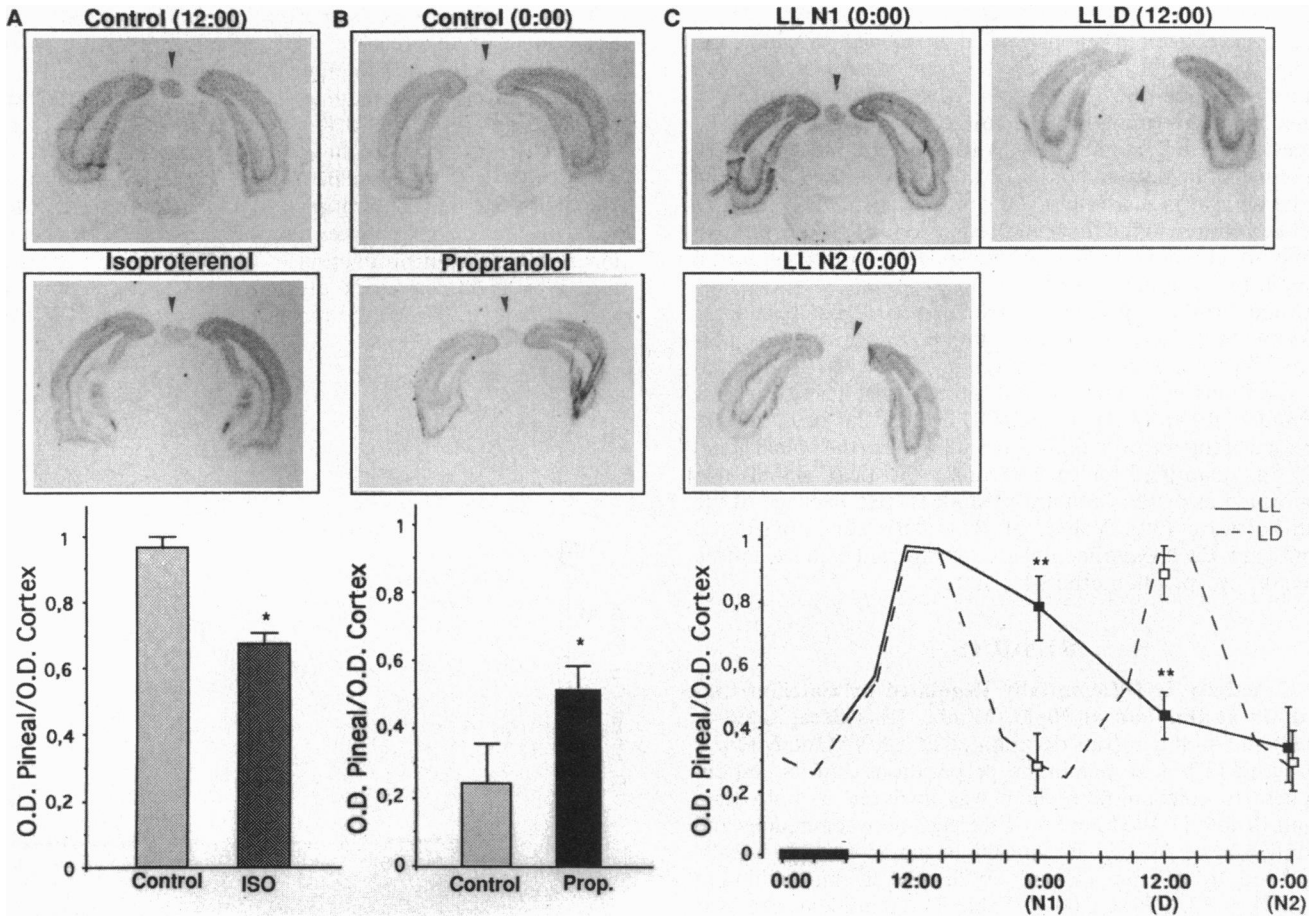

FIG. 2. The expression of AC1 mRNA in the pineal gland is under the control of the NE system and of environmental light conditions. Effects of isoproterenol (*A*), propranolol (*B*), and constant light (*C*) on AC1 mRNA expression are evidenced by representative *in situ* hybridization autoradiograms and relative OD of the AC1 hybridization signals. (*A*) Rats received a s.c. injection of isoproterenol (ISO) (5 mg/kg) at 10:00 and were killed 2 h later. (*B*) Rats received a s.c. injection of propranolol (PROP) (20 mg/kg) just before the onset of the dark phase and were killed at 0:00. In both cases, control animals were treated with saline. (*C*) Rats were maintained under LL conditions and were killed after various time periods of extra-light [i.e., 6 h (N1), 18 h (D), 30 h (N2)]. Control animals were kept under normal periodic light conditions (LD) and were killed either at 12:00 (day, D) or at 0:00 (night, N). All graphics present means ± SD of at least three individual experiments. *, $P < 0.01$ compared with control (saline); **, $P < 0.001$ compared with control (LD).

gland follows a similar pattern: their mRNA appears elevated at night (36), while their amount is increased during the day (31). In contrast, the end target of the signaling pathways in the pineal gland, namely NAT, the rate limiting enzyme in melatonin synthesis, is regulated in a different manner. Since its mRNA and the enzyme protein itself have a very small half-life, their diurnal variation occurs nearly simultaneously (8, 9), with a maximum at midnight.

We found no change in the enzyme responsiveness to NE. This is in apparent contradiction with the fact that the amount of β-adrenergic receptors in the pineal gland has been reported to follow a circadian pattern (31). However, the amplitude of the variation is small (less than 50% of peak-through-mean) (37). Indeed, as shown by Post *et al.* (38), the number of AC molecules is actually the main limiting step in the receptor/$G_{s\alpha}$/effector cascade. This has been recently shown in rat sweat glands, where a 50% decrease in β-receptor levels did not alter the stimulation of cyclic AMP production or its basal level (39).

In the pineal gland, the AC1 mRNA oscillation fits in a set of parallel or opposite circadian variations of the different components of the cyclic AMP pathway. A convergent action of the β- and α1-adrenergic receptor pathways has been proposed as a key-factor in mediating melatonin production in pinealocytes (6, 15, 16). The fact that AC1 is the main AC type in the pineal gland, subject to a dramatic diurnal variation, is interesting since this isoform is stimulated by the calcium/calmodulin complex (22) and by protein kinase C (40) and since it has been postulated as a coincidence detector of intracellular information flowing in parallel via different signaling pathways (23, 24). Thus, in the pineal gland, the increase in cyclic AMP could occur via activation of the AC1 enzymatic activity by calcium/calmodulin specifically during the night. This mechanism involves the coincidence of a calcium-dependent and of a G protein-dependent signal and is in agreement with the synergistic effects of β- and α1-noradrenergic stimuli in the pineal gland (15, 16). AC1 would, therefore, integrate not only the β- and α1-NE responses but also additional modulatory messages mediated by other neurotransmitters that act in the pineal gland and that are known to regulate the intracellular calcium concentration (3). Of course, other AC isoforms should exist in the pineal gland, to account for the calmodulin-independent activity present in the day. They are not known at present, neither are their possible variations.

The AC1 mRNA level is directly regulated by NE and by the photoperiodic environment. The same systems control the expression of ICER mRNA (11), suggesting a common modulatory mechanism. Administration of exogenous isoproterenol at 10:00 leads to an increase of the ICER signal and to a decreased AC1 mRNA signal at 12:00. On the opposite, administration of propranolol at the onset of the dark-phase results in a decreased ICER mRNA signal and in an increased AC1 mRNA expression at midnight. Thus, ICER and AC1 mRNAs are controlled by the same system in an antiparallel manner. ICER belongs to the "immediate early response genes," its expression being driven through the P_2 intronic promoter within the cyclic AMP-responsive element modulator gene. It functions as a repressor of the cyclic AMP-dependent transcription, and is itself induced by cyclic AMP (13, 14). Since ICER is a repressor of the cyclic AMP-dependent transcription, one could hypothesize the existence of a negative control of the cyclic AMP system on the AC transcriptional level. This hypothesis is supported by the circadian properties of ICER expression which could reflect on the circadian plasticity of the AC1 mRNA signal. Likewise, the transcription factor Fra-2 follows a circadian pattern of expression in the pineal gland, which depends on cyclic AMP (10). Fra-2 could then influence AP-1- or CRE-like-dependent gene expression (10). The importance of CRE in mediating nuclear circadian responses, for instance those for c-*jun*, NAT, and ICER, (8, 9, 14, 41) in the pineal gland, makes it a major candidate for the regulation of AC1 transcription.

The specific physiological regulation of AC1 mRNA expression during the biological rhythm reported in the present study provides a striking example for the involvement of an AC isoform in cellular signaling. The generation and maintenance of the pineal gland biological clock would be due to the periodic convergence of two pathways: (*i*) through the β-adrenergic receptor and (*ii*) via calcium/calmodulin. The periodic expression of the calcium/calmodulin activated AC1 identifies this AC type as a potential regulatory element of circadian signal transduction in the pineal gland.

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1. Moore, R. Y. (1996) *Behav. Brain Res.* **73,** 125–130.
2. Axelrod, J. (1974) *Science* **184,** 1341–1348.
3. Reiter, R. J. (1991) *Trends Endocrinol. Metab.* **1,** 13–19.
4. Reiter, R. J. (1991) *Endocr. Rev.* **12,** 151–180.
5. Mikuni, M., Saito, Y., Koyama, T. & Yamashita, I. (1981) *J. Neurochem.* **36,** 1295–1297.
6. Roseboom, P. H. & Klein, D. C. (1995) *Mol. Pharmacol.* **47,** 439–449.
7. Buda, M. & Klein, D. C. (1978) *Endocrinology* **103,** 1483–1493.
8. Borjigin, J., Wang, M. M. & Snyder, S. H. (1995) *Nature (London)* **378,** 783–785.
9. Coon, S. L., Roseboom, P. H., Baler, R., Weller, J. L., Namboodiri, M. A. A., Koonin, E. V. & Klein, D. C. (1995) *Science* **270,** 1681–1683.
10. Baler, R. & Klein, D. C. (1995) *J. Biol. Chem.* **270,** 27319–27325.
11. Stehle, J. H., Foulkes, N. S., Molina, C. A., Simmoneaux, V., Pevet, P. & Sassone-Corsi, P. (1993) *Nature (London)* **365,** 314–320.
12. Sassone-Corsi, P. (1994) *Cell* **78,** 361–364.
13. Molina, C. A., Foulkes, N. S., Lalli, E. & Sassone-Corsi, P. (1993) *Cell* **75,** 875–886.
14. Lalli, E. & Sassone-Corsi, P. (1994) *J. Biol. Chem.* **269,** 17359–17362.
15. Sudgen, D., Vanecek, J., Klein, D. C., Thomas, P. T. & Anderson, W. B. (1985) *Nature (London)* **314,** 359–361.
16. Vanecek, J., Sudgen, D., Weller, J. & Klein, D. C. (1985) *Endocrinology* **116,** 2167–2173.
17. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508.
18. Bentley, J. K. & Beavo, J. A. (1992) *Curr. Opin. Cell Biol.* **4,** 233–240.
19. Sun, P., Lou, L. & Maurer, R. A. (1996) *J. Biol. Chem.* **271,** 3066–3073.
20. Matthews, R. P., Gutrie, C. R., Wailes, L. M., Zhao, X., Means, A. R. & McKnight, G. S. (1994) *Mol. Cell. Biol.* **14,** 6107–6116.
21. Dash, P. K., Karl, K. A., Colicos, M. A., Prywes, R. & Kandel, E. R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5061–5065.
22. Choi, E.-J., Wong, S. T., Hinds, T. R. & Storm, D. R. (1992) *J. Biol. Chem.* **267,** 12440–12442.
23. Wayman, G. A., Impey, S., Wu, Z., Kindsvogel, W., Prichard, L. & Storm, D. R. (1994) *J. Biol. Chem.* **269,** 25400–25405.
24. Impey, S., Wayman, G., Wu, Z. & Storm, D. R. (1994) *Mol. Cell. Biol.* **14,** 8272–8281.
25. Hanoune, J., Stengel, D., Lacombe, M. L., Feldmann, G. & Coudrier, E. (1977) *J. Biol. Chem.* **252,** 2039–2045.
26. Wisden, W., Morris, B. J. & Hunt, S. P. (1991) in *Molecular Neurobiology: A Practical Approach*, eds. Chad, J. & Wheal, H. (Oxford Univ. Press, Oxford), pp. 205–225.
27. Mons, N., Yoshimura, M. & Cooper, D. M. F. (1993) *Synapse* **14,** 51–59.
28. Glatt, C. E. & Snyder, S. H. (1993) *Nature (London)* **361,** 536–538.
29. Paxinos, G. (1995) *The Rat Nervous System* (Academic, San Diego).
30. SAS Institute Inc. (1989) SAS/STAT User's Guide (SAS Inst., Cary, NC), Vol. 1, Version 6.

31. Romero, J. A., Zatz, M., Kebabian, J. W. & Axelrod, J. (1975) *Nature (London)* **258,** 435–436.
32. Spessert, R., Albertowski, K., Reuss, S., Layes, E. & Vollrath, L. (1995) *Neurosci. Let.* **190,** 61–64.
33. Xia, Z., Choi, E.-J., Wang, F., Blazynski, C. & Storm, D. R. (1993) *J. Neurochem.* **60,** 305–311.
34. Mons, N. & Cooper, D. M. F. (1995) *Trends Neurosci.* **18,** 536–542.
35. Wu, Z.-L., Thomas, S. A., Villacres, E. C., Xia, Z., Simmons, M., Chavkin, C., Palmiter, R. D. & Storm, D. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 220–224.
36. Carter, D. A. (1993) *Endocrinology* **133,** 2263–2268.
37. Pangerl, B., Pangerl, A. & Reiter, R. J. (1990) *J. Neural Transm.* **81,** 17–29.
38. Post, S. R., Hilal-Dandan, R., Urasawa, K., Brunton, L. L. & Insel, P. A. (1995) *Biochem. J.* **311,** 75–80.
39. Habecker, B. A., Malec, N. M. & Landis, S. C. (1996) *J. Neurosci.* **16,** 229–237.
40. Choi, E.-J., Wong, S. T., Dittman, A. H. & Storm, D. R. (1993) *Biochemistry* **32,** 1891–1894.
41. Carter, D. A. (1992) *Mol. Brain Res.* **16,** 111–118.