

Selective Adrenergic/Cyclic AMP-Dependent Switch-Off of Proteasomal Proteolysis Alone Switches on Neural Signal Transduction: An Example from the Pineal Gland

Christof Schomerus, Horst-Werner Korf, Elke Laedtke, *Joan L. Weller, and *David C. Klein

Dr. Senckenbergische Anatomie, Institut für Anatomie II, Johann Wolfgang Goethe-Universität Frankfurt, Frankfurt/Main, Germany; and *Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, U.S.A.

Abstract: The molecular processes underlying neural transmission are central issues in neurobiology. Here we describe a novel mechanism through which noradrenaline (NA) activates its target cells, using the mammalian pineal organ as a model. In this neuroendocrine transducer, NA stimulates arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), the key enzyme regulating the nocturnal melatonin production. In rodents, AANAT protein accumulates as a result of enhanced transcription, but in primates and ungulates, the AANAT mRNA level fluctuates only marginally, indicating that other mechanisms regulate AANAT protein and activity. These were investigated in cultured bovine pinealocytes. AANAT mRNA was readily detectable in unstimulated pinealocytes, and levels did not change following NA treatment. In contrast, NA increased AANAT protein levels in parallel with AANAT activity, apparently through a cyclic AMP-mediated mechanism. Immunocytochemistry revealed that the changes in AANAT protein levels occurred in virtually all pinealocytes. Inhibition of AANAT degradation by proteasomal proteolysis alone was found to switch-on enzyme activity by increasing AANAT protein levels five- to 10-fold. Accordingly, under unstimulated conditions AANAT protein is continually synthesized and immediately destroyed by proteasomal proteolysis. NA appears to act via cyclic AMP to protect AANAT from proteolytic destruction, resulting in accumulation of the protein. These findings show that tightly regulated control of proteasomal proteolysis of a specific protein alone can play a pivotal role in neural regulation. **Key Words:** Arylalkylamine *N*-acetyltransferase—Biological rhythms—Neural signaling—Noradrenaline—Pineal gland—Proteasomal proteolysis.

J. Neurochem. **75**, 2123–2132 (2000).

response, the production of the neurohormone for darkness, melatonin. NA is released from intrapineal sympathetic nerve endings at the onset of darkness (Drijfhout et al., 1996) in response to neural signals from the endogenous oscillator in the hypothalamic suprachiasmatic nucleus (Klein and Moore, 1979). NA stimulates pinealocytes through α_1 - and β -adrenergic receptors (Klein, 1985; Chik and Ho, 1989). The critical molecular target of NA is arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), the key regulatory enzyme in melatonin biosynthesis (Klein et al., 1997). AANAT activity is low during day and high during night. The rhythm in AANAT activity gives rise to the daily rhythm in circulating melatonin. This role of AANAT as a molecular transducer—one that converts regulatory input into changes in melatonin—is highly conserved among vertebrates; however, remarkable species-to-species differences exist in the molecular mechanisms through which NA acts on AANAT (Klein et al., 1997).

Several studies indicate that AANAT activity closely mirrors AANAT protein levels (Coon et al., 1995; Klein et al., 1997; Zatz et al., 2000). Two regulatory mechanisms can control the latter. In rodents, but not in ungulates and primates, transcriptional regulation plays an essential role in the switch-on of AANAT at night. NA controls AANAT transcription through a cyclic AMP mechanism involving activation of protein kinase A and phosphorylation of cyclic AMP response element (CRE)-binding protein (CREB) (Roseboom and Klein,

Received June 5, 2000; revised manuscript received July 7, 2000; accepted July 7, 2000.

Address correspondence and reprint requests to Dr. H.-W. Korf at Dr. Senckenbergische Anatomie, Institut für Anatomie II, Johann Wolfgang Goethe-Universität Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt/Main, Germany. E-mail: korf@em.uni-frankfurt.de

Abbreviations used: AANAT, arylalkylamine *N*-acetyltransferase; CRE, cyclic AMP response element; CREB, cyclic AMP response element-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NA, noradrenaline; SUMDENS, product of inverted gray values and area (number of pixels).

A central issue in neurobiology is the nature of mechanisms involved in neural regulation of intracellular processes, critical for specific functions of neuronal cells. An excellent model to study this is the mammalian pineal organ, which transduces a neuronal signal, the release of the neurotransmitter noradrenaline (NA), into a specific

1995; Tamotsu et al., 1995; Maronde et al., 1999*a,b*). Phosphorylated CREB activates expression via CREs in the AANAT promoter and increases AANAT mRNA levels > 150-fold (Borjigin et al., 1995; Coon et al., 1995; Roseboom et al., 1996; Baler et al., 1997; Klein et al., 1997; Burke et al., 1999; Maronde et al., 1999*a*). The increase in AANAT mRNA content is followed by increases in AANAT protein levels and activity (Klein et al., 1997).

The second regulatory mechanism is NA/cyclic AMP-dependent inhibition of proteasomal proteolysis of AANAT, reversal of which causes the switch-off of AANAT activity, seen when adrenergic stimulation is blocked in the rat (Gastel et al., 1998). It is not yet clear whether this mechanism also regulates the NA-induced switch-on of AANAT early in the night; analysis of this issue in the rat is confounded by the essential role of transcription in this species.

In ungulates and primates, the AANAT mRNA level fluctuates only marginally (Coon et al., 1995; Klein et al., 1997), suggesting that in these species NA/cyclic AMP-dependent inhibition of AANAT proteolysis might be the primary mechanism switching-on AANAT. This was studied here using bovine pinealocytes, which express the AANAT gene constitutively. Our results provide the first example of a system in which NA/cyclic AMP-inhibited AANAT proteolysis alone may switch-on AANAT activity. This demonstrates that a neurotransmitter can have profound influence simply by inhibiting proteolysis of a single critical protein.

MATERIALS AND METHODS

Materials

Drugs and chemicals. NA, dibutyl cyclic AMP, puromycin, actinomycin D, and poly-L-lysine were purchased from Sigma (Deisenhofen, Germany). Forskolin, lactacystin, MG-132, calpain inhibitor I, and calpain inhibitor III were obtained from Calbiochem (Bad Soden, Germany).

Antisera. Polyclonal rabbit anti-S-antigen serum NEI 04111083 was a gift from Dr. I. Gery (National Eye Institute, Bethesda, MD, U.S.A.). Polyclonal rabbit anti-serotonin serum was purchased from Incstar (Stillwater, MN, U.S.A.). Polyclonal anti-ovine AANAT₁₋₂₅ 3343 serum was generated by immunizing rabbits with a synthetic peptide corresponding to amino acids 1–25 of ovine AANAT. The peptide was conjugated to cationized bovine serum albumin before immunization. This antiserum (1:25,000) strongly detects a single (~24-kDa) band of protein on immunoblots of ovine and bovine pineal glands and of bovine pinealocyte preparations.

Cell culture

Bovine pineal glands were obtained between 0700 and 1000 h from male and female animals at an abattoir in the vicinity of Frankfurt/Main, Germany, within 15 min after death and brought to the laboratory on wet ice. Pinealocytes were dissociated (Schaad et al., 1993) and seeded into 96-well multiwell plates (200,000 cells per well; Nunc, Wiesbaden, Germany). After 5 days, cells were treated as indicated, harvested, and further processed.

Immunocytochemistry

To characterize the bovine pineal cell preparation by immunocytochemical demonstration of pinealocyte-specific markers, isolated pineal cells were cultured on poly-L-lysine-coated coverslips for 5 days, fixed with 4% paraformaldehyde for 10 min, and incubated with anti-S-antigen serum NEI 04111083 [1:1,000 (Korf et al., 1985, 1998)] or anti-serotonin serum (1:5,000). Binding of the primary antibodies was visualized using the ABC method with a biotin-conjugated anti-rabbit IgG (Sigma) as the second antibody, a horseradish peroxidase-conjugated streptavidin complex (Sigma), and 3,3'-diaminobenzidine as the chromogen.

For immunocytochemical demonstration of AANAT, immobilized bovine pinealocytes were cultured for 5 days and stimulated with NA (100 nM) for 6 h or left untreated. The cells were fixed with 4% glutaraldehyde for 10 min and then incubated with anti-ovine AANAT₁₋₂₅ antiserum 3343 (1:25,000). Binding of the primary antibody was visualized using the ABC method as described above. Immunocytochemical controls were performed by omitting the primary AANAT antibody or by incubating sections with the AANAT antibody that had been preabsorbed with an excess of different peptides of the ovine AANAT. All control preparations did not contain any immunoreactive signal.

Northern blot analysis

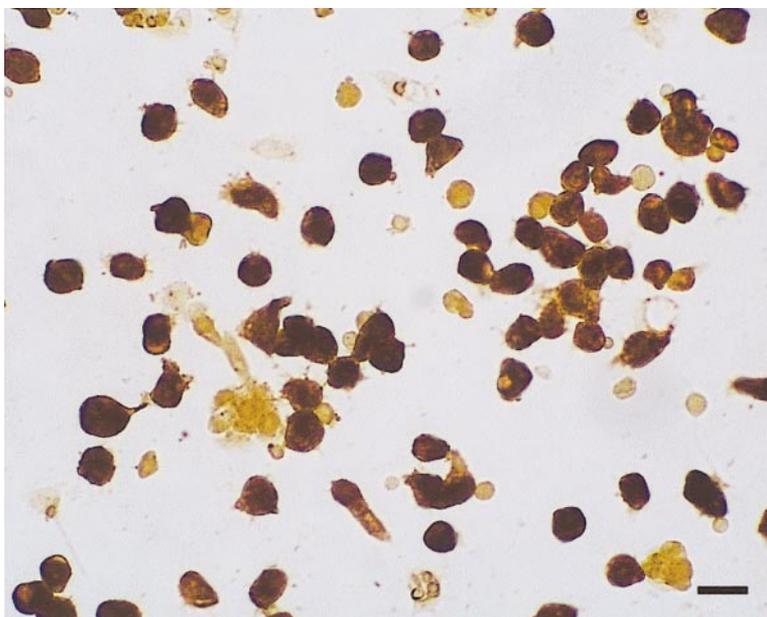
Probes for northern blot analysis were prepared by RT-PCR. For this, total RNA was extracted from cultured bovine pinealocytes using the RNeasy kit (Qiagen, Hilden, Germany) and reverse-transcribed with oligo(dT) primer and Superscript II reverse transcriptase (GibcoBRL, Karlsruhe, Germany). PCR was performed using *Taq* polymerase (Qiagen) over 30 cycles on a thermocycler (1-min denaturation at 95°C, 1-min annealing at 53°C, 1-min elongation at 74°C). PCR primers (MWG Biotech, Munich, Germany) for AANAT cDNA amplification were designed against bp 120–141 and 408–429 of the bovine AANAT sequence [GenBank accession no. AD000742; sense, 5'-CCGAGCATCCACTGCCTGAAAC-3'; antisense, 5'-CCTGAGTAAGTCTTTCCTCGTC-3' (Craft et al., 1999)]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification primers were designed against bp 830–846 and 1,058–1,077 of the rat sequence (GenBank accession no. X02231; sense, 5'-TGATGACATCAAGAAGGTGG-3'; antisense, 5'-TTTCTACTCCTTGGAGGCC-3'). The PCR products were verified by standard procedures and finally used as templates for RNA analysis.

Total RNA (12 µg per lane) was prepared from cultured bovine pinealocytes, separated on 1% agarose denaturing formaldehyde gels, blotted to Hybond-N nylon membranes (Amersham Pharmacia, Freiburg, Germany) using capillary forces, and immobilized by UV cross-linking. DNA probes were labeled with [α -³²P]dCTP (3,000 mCi/mmol; ICN, Eschwege, Germany) using a random primer labeling kit (Amersham Pharmacia). Membranes were prehybridized at 42°C for 8 h and hybridized with the radiolabeled 309-bp AANAT probe generated by RT-PCR (see above) for 16 h. To normalize the abundance of AANAT transcripts for loading, northern blots were stripped and reprobed with a 247-bp rat GAPDH PCR product. The signals were analyzed semiquantitatively by determining their SUMDENS values (product of inverted gray values and area) according to the method of Wicht et al. (1999).

AANAT activity assay

Cells harvested from single wells of a 96-well multiwell plate were briefly sonicated in phosphate-buffered saline, and

FIG. 1. Immunocytochemical demonstration of S-antigen in bovine pineal cells cultured for 5 days. Most of the cells are S-antigen-immunoreactive and thus represent pinealocytes. The intensity of the immunoreaction varies on a cell-to-cell basis. Bar = 20 μm .



AANAT activity was determined as described (Fleming et al., 1999) using 1 mM [$1\text{-}^{14}\text{C}$]acetyl-CoA (55 $\mu\text{Ci}/\mu\text{mol}$; New England Nuclear, Köln, Germany) and 1 mM tryptamine as substrates. Radiolabeled reaction products were verified by TLC using authentic *N*-acetyltryptamine as a marker. Synthesis of *N*-acetyltryptamine was totally blocked by the specific AANAT bisubstrate inhibitor CoA-*S*-acetyltryptamine (100 μM ; Research Biochemicals International, Natick, MA, U.S.A.) (Khalil and Cole, 1998). Activity values were normalized against the amount of total protein (Bradford, 1976).

Immunoblot analysis

Total cell homogenates were prepared by sonication of the cells in sodium dodecyl sulfate sample buffer. Immunoblotting and immunodetection were performed as described (Schomerus et al., 1999) using the above-described AANAT antiserum 3343 (1:25,000). Semiquantitative analysis of the immunosignals was performed by determining their SUMDENS values (Wicht et al., 1999).

Statistics

Data from three individual experiments were statistically analyzed using an ANOVA with subsequent Dunnett's test with $p < 0.05$ as the criterion of significance and expressed as mean \pm SEM values.

RESULTS

The pinealocyte is the dominant cell type in the bovine pinealocyte culture system

Cultured bovine pineal cell preparations were characterized by immunocytochemical demonstration of the S-antigen (arrestin), a marker for retinal photoreceptors and pinealocytes (Korf et al., 1985, 1998). More than 90% of the cells were S-antigen-immunoreactive (Fig. 1). S-antigen immunoreactivity was evenly distributed in the cytoplasm. The intensity of the immunoreaction varied on a cell-to-cell basis. Similar results as regards the cellular composition of the culture were obtained with an

antiserum against serotonin, which is converted to *N*-acetylserotonin, the melatonin precursor (data not shown). Accordingly, it appears that the pinealocyte is the dominant cell type in the culture system used in this study.

AANAT mRNA content in bovine pinealocytes does not change following adrenergic stimulation

To investigate the role of transcriptional control of bovine AANAT, total RNA extracted from unstimulated and NA-stimulated pinealocytes was analyzed by northern blot analyses. In untreated cells, AANAT transcripts ~ 1 kb in size were readily detectable (Fig. 2a). The AANAT mRNA level did not change significantly on treatment with 100 nM NA, as shown by semiquantitative analysis of autoradiographic images from northern blots probed for AANAT mRNA and corrected for the GAPDH signal. After NA treatment for 1, 3, or 6 h, the intensities of the AANAT signals were 94, 110, and 109%, respectively, of that in unstimulated preparations.

AANAT protein levels and activity in bovine pinealocytes are increased by adrenergic/cyclic AMP stimulation

The effects of NA on AANAT enzyme activity were studied in a radiochemical assay. A basal AANAT activity was readily detectable in unstimulated cell preparations (Fig. 2c), and this was increased approximately fourfold by NA treatment. The effects of NA were dose- and time-dependent. Half-maximal effects were elicited at a NA concentration of 20 nM (data not shown); 100 nM NA was found to evoke maximal effects. An increase in AANAT activity induced by 100 nM NA was first observed after 60 min; after 8 h, AANAT activity reached maximal values (Fig. 2c).

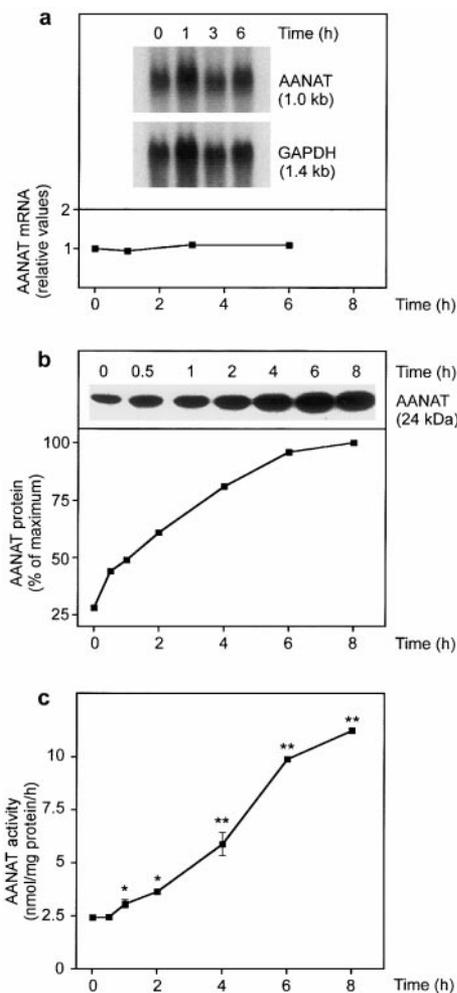


FIG. 2. Time-dependent effects of NA (100 nM) on bovine pinealocytes. **a:** Northern blot analysis of AANAT mRNA. The abundance of the AANAT transcript was analyzed semiquantitatively by determining the product of the sum of the gray values and the area of a given signal (Wicht et al., 1999). This product is normalized against corresponding values for GAPDH mRNA to correct for variations in loading. Similar results were obtained in three different experiments. **b:** Immunoblot analysis of AANAT. AANAT protein levels were calculated semiquantitatively as described (Wicht et al., 1999) and expressed as the percentage of the maximal signal obtained after NA treatment for 8 h. The molecular mass of the immunosignal (24 kDa) corresponds to the estimated molecular mass for bovine AANAT deduced from the published nucleotide sequence [GenBank accession no. AD000742 (Craft et al., 1999)]. Similar results were obtained in three different experiments. **c:** Radiochemical determination of AANAT enzyme activity in pinealocyte homogenates. The AANAT activity levels represent mean \pm SEM (bars) values determined in duplicate in different preparations. * $p < 0.05$, ** $p < 0.01$, versus untreated cells.

The effects of NA on AANAT protein levels were examined in immunoblot experiments with a highly specific AANAT antibody. In homogenates from unstimulated cells, we detected a 24-kDa signal (Fig. 2b) whose molecular mass corresponds to the molecular mass of AANAT deduced from the nucleotide sequence (Craft

et al., 1999) (GenBank accession no. AD000742). Semiquantitative analyses of the immunoreactive signals showed that treatment with 100 nM NA induced a four-fold increase in AANAT immunoreactivity (Fig. 2b; see also Figs. 4a and c and 5a and g). The effect of NA was time-dependent. An increase in AANAT protein levels was first evident after 30 min and reached maximal levels after 8 h (Fig. 2b).

Immunocytochemical analyses revealed that the NA-induced changes in AANAT protein levels occurred in the majority of bovine pinealocytes. Unstimulated cells displayed a weak AANAT immunoreactivity (Fig. 3a); treatment with 100 nM NA for 6 h induced a strong increase in AANAT immunoreactivity in nearly all cells (Fig. 3b). AANAT immunoreactivity was confined to small granules evenly distributed in the cytoplasm of most pinealocytes, whereas the cell nuclei were immunonegative. These immunocytochemical stainings visualize for the first time the subcellular distribution of a vertebrate AANAT. The intensity of the AANAT immunoreaction varied from cell to cell, as seen with other pineal-specific marker proteins (Korf et al., 1998); the functional significance of this variation remains unknown.

To determine whether cyclic AMP was involved in regulating AANAT activity and protein levels, cells were treated with cyclic AMP antagonists for 6 h. Forskolin (5 μ M) and dibutyryl cyclic AMP (1 mM) increased the AANAT protein level, albeit to a lesser degree than 100 nM NA (73 and 64%, respectively; Fig. 4a). Moreover, treatment with forskolin (5 μ M) and dibutyryl cyclic AMP (1 mM) enhanced AANAT activity to a similar extent as did stimulation with NA (Fig. 4b). Elevation of the intracellular Ca^{2+} concentration by various drugs, e.g., ionomycin, KCl, and phenylephrine, did not affect AANAT protein levels (authors' unpublished data).

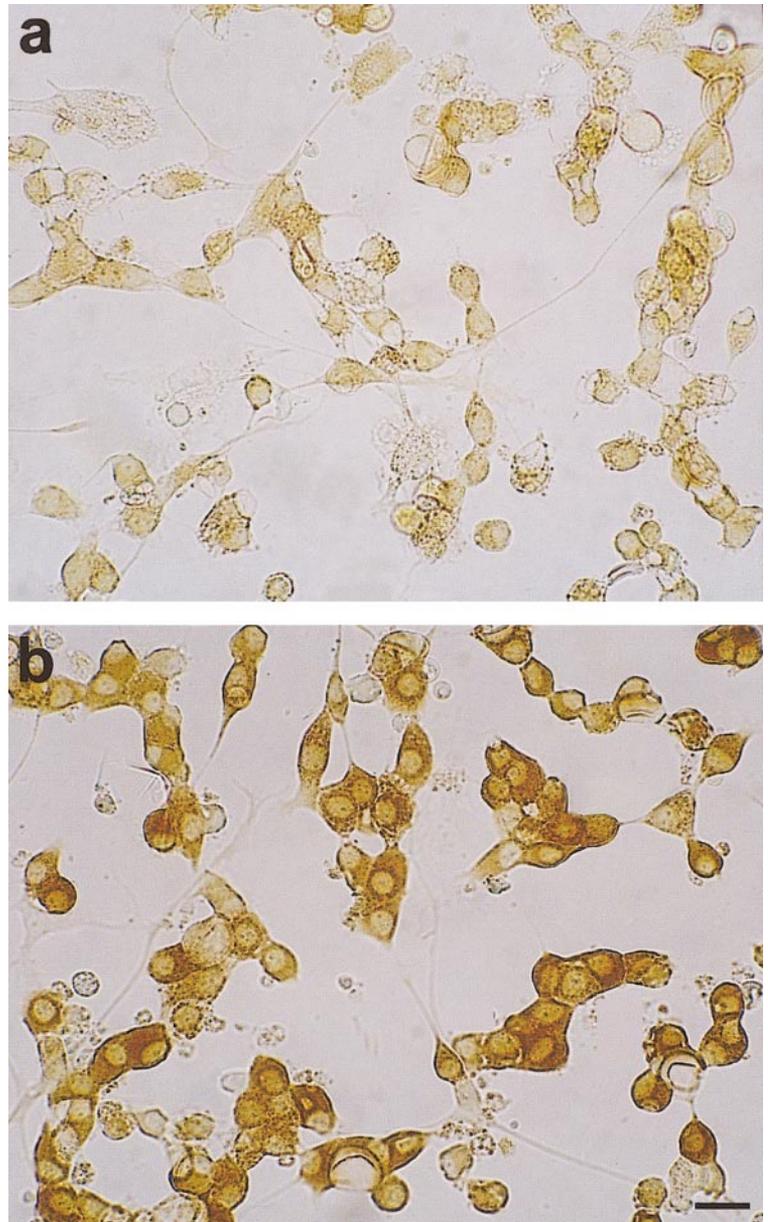
Adrenergic elevation of AANAT levels requires new protein synthesis—not new gene expression

As indicated above, direct analysis of AANAT mRNA content indicated that NA did not alter this parameter. To determine whether the NA-evoked increases in AANAT protein levels and activity require the new expression of any gene, pinealocytes were exposed to 30 μ g/ml actinomycin D for 0.5 h followed by treatment with a combination of 30 μ g/ml actinomycin D and 100 nM NA for 6 h. This treatment did not abolish the NA-induced rise in AANAT protein levels (Fig. 4c) and enhanced the NA-induced increase in AANAT activity (Fig. 4d). In contrast, inhibition of protein synthesis (by exposing cells to 50 μ g/ml puromycin followed by treatment with a combination of 50 μ g/ml puromycin with 100 nM NA for 6 h) dramatically reduced the NA-induced increases in AANAT protein levels and activity to levels below those found in unstimulated preparations (Fig. 4c and d).

AANAT protein levels and activity are regulated via proteasomal proteolysis

The above evidence indicated that posttranscriptional processes are important for regulation of bovine

FIG. 3. Immunocytochemical demonstration of AANAT protein in bovine pinealocytes cultured for 5 days. **a:** Unstimulated cells. **b:** Cells stimulated with NA (100 nM) for 6 h. Treatment with NA induced strong AANAT immunoreactivity in basically all pinealocytes, whereas unstimulated cells were weakly immunoreactive. AANAT immunoreactivity was confined to small granules evenly distributed in the cytoplasm of most pinealocytes, whereas the cell nuclei were immunonegative. As is seen with other pineal-specific proteins (Korf et al., 1998), the intensity of the AANAT immunoreaction varies from cell to cell. Bar = 20 μ m.



AANAT. This led us to examine the issue of whether AANAT protein levels and activity were regulated by proteasomal degradation of AANAT. Cells were treated with three different selective proteasomal proteolysis inhibitors (6 h, 25 μ M): lactacystin, MG-132, and calpain inhibitor I. Treatment with these compounds alone increased AANAT protein levels (Fig. 5a) and activity (Fig. 5b) to a greater extent than a maximally effective dose of NA. Treatment with calpain inhibitor III, which specifically inhibits the protease calpain I but not the proteasome, was ineffective.

The effect of the most selective proteasomal protease inhibitor, lactacystin (Fenteany and Schreiber, 1998), was dose- and time-dependent; a maximal increase in AANAT protein levels was found at a concentration of

50 μ M lactacystin (Fig. 5e), and the increase in AANAT activity was maximal at concentrations ranging between 10 and 50 μ M lactacystin (Fig. 5f). On the basis of these dose-response relationships, a concentration of 25 μ M lactacystin was used in standard experiments throughout this study. A lactacystin-induced increase in AANAT protein levels was first evident after 1 h (Fig. 5c); after 6 h, AANAT protein levels were increased five- to 10-fold (Fig. 5a, c, e, and g). This time course resembles that seen with NA (Fig. 2b). A similar time course was found for the effects of lactacystin on AANAT activity (Fig. 5d).

The issue of whether NA and lactacystin control AANAT protein levels and activity through the same or different mechanisms was examined by stimulating cells with combinations of lactacystin (25 μ M) with NA (100

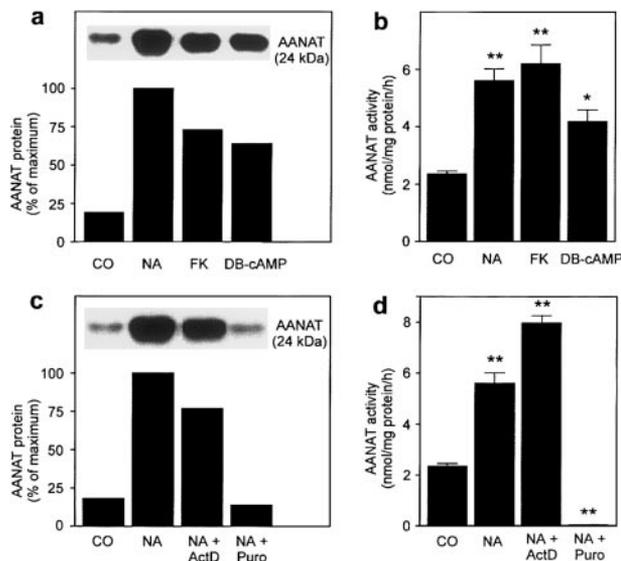


FIG. 4. Effects of selected drugs on adrenergic stimulation of AANAT protein levels and activity in bovine pinealocytes. **a** and **b**: Effects of cyclic AMP antagonists. Cells were left unstimulated (CO) or were exposed to NA (100 nM), forskolin (FK; 5 μ M), or dibutyryl cyclic AMP (DB-cAMP; 1 mM) for 6 h. **c** and **d**: Effects of inhibitors of transcription or translation. Cells were left unstimulated (CO), treated with NA (100 nM) for 6 h, or incubated with actinomycin D (ActD; 30 μ g/ml) or puromycin (Puro; 50 μ g/ml) for 0.5 h before treatment with combinations of ActD/NA or puromycin/NA for 6 h. * p < 0.05, ** p < 0.01 versus untreated cells.

nM) or the cyclic AMP antagonists forskolin (5 μ M) and dibutyryl cyclic AMP (1 mM) for 6 h. The combination of lactacystin with NA, forskolin, or dibutyryl cyclic AMP did not change the effects on AANAT protein levels and activity that were elicited by lactacystin alone (Fig. 5g and h).

To determine whether ongoing protein synthesis is required for the lactacystin-induced increases in AANAT protein levels and activity, as is the case with NA, cells were preincubated with 50 μ g/ml puromycin for 30 min followed by treatment with a combination of 50 μ g/ml puromycin and 25 μ M lactacystin for 6 h. Under these conditions, puromycin totally blocked the effects of lactacystin and reduced AANAT protein levels and activity to nearly undetectable levels (Fig. 5g and h).

DISCUSSION

The results of this study are consistent with the conclusion that AANAT is continually synthesized in cultured bovine pinealocytes and that the abundance and activity of AANAT are controlled primarily by NA acting through the cyclic AMP pathway to prevent destruction of AANAT by proteasomal proteolysis. These findings are of general importance for neuroendocrine and neuronal signal transduction because they show for the first time that a neurotransmitter activates its target cells by inhibiting proteasomal proteolysis and thus attribute a

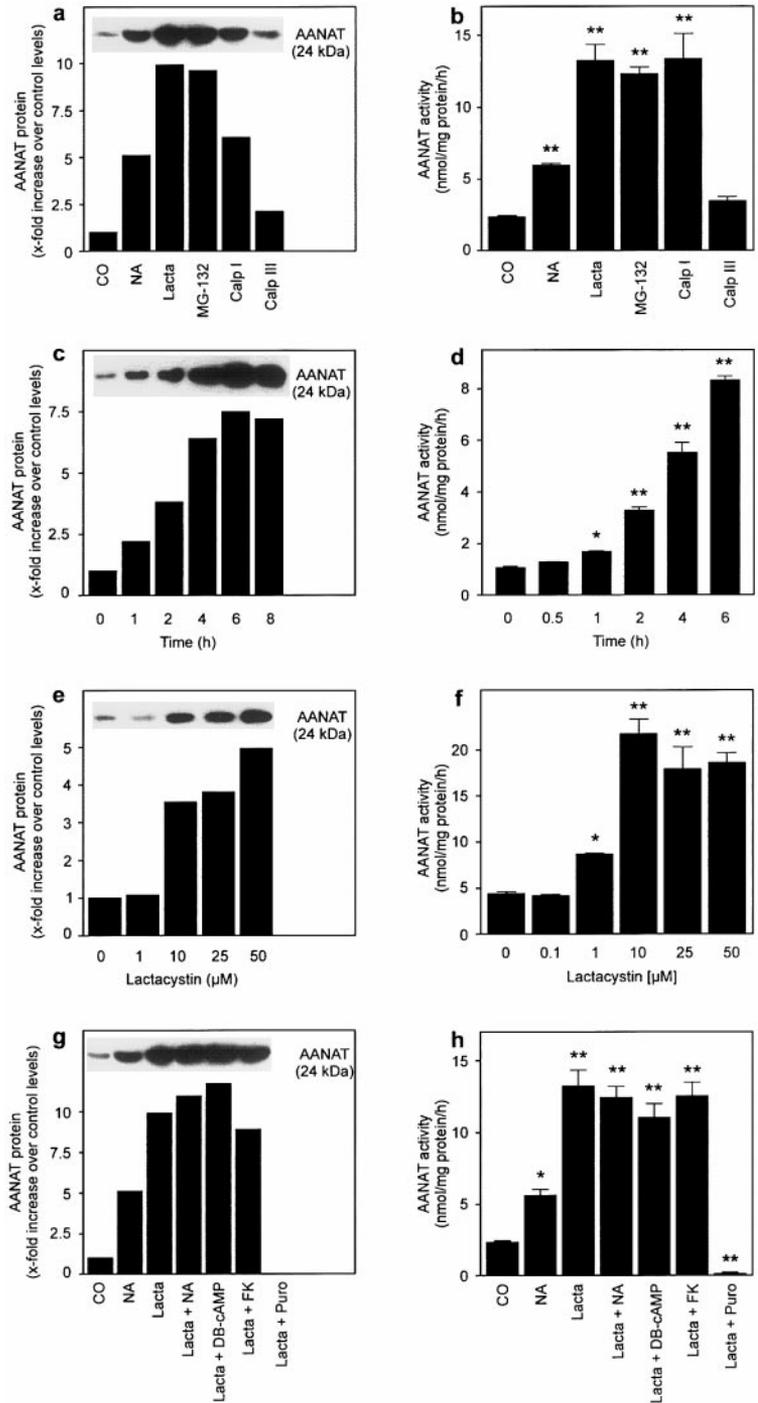
novel neuroregulatory role to proteasomal proteolysis. Our investigations are also of special interest in understanding pineal function because they clearly document a previously unknown, neurotransmitter-regulated switch-on mechanism of AANAT. It is reasonable to consider that this mechanism is widely distributed among mammals.

As indicated in the introductory section, pineal function has been studied extensively in the rat. In this species, adrenergic stimulation causes a >100-fold increase in AANAT mRNA content followed by increases in AANAT protein levels and activity with a temporal delay. The NA-induced increases in AANAT protein levels and activity are totally blocked by inhibiting transcription. This emphasizes the dominant role of transcriptional mechanisms for switching on AANAT in rodents. Switching-off rat AANAT at the end of the night involves transcriptional mechanisms (Borjigin et al., 1995; Roseboom et al., 1996) and the inhibitory transcription factor ICER (Stehle et al., 1993; Maronde et al., 1999a). Moreover, posttranscriptional mechanisms involving cyclic AMP-dependent proteasomal proteolysis are important for the rapid decrease of AANAT protein levels and activity that is induced by noradrenergic blockade or acute light pulses given during nighttime (Gastel et al., 1998). A dual transcriptional/proteolysis mechanism also regulates AANAT in the chicken (Bernard et al., 1997a,b; Klein et al., 1997; Zatz et al., 2000). However, it is difficult to determine the relative importance of proteolysis and translational influences for switching-on AANAT in these species because, in both the rat and the chicken, the AANAT mRNA level is regulated and varies significantly in conjunction with changes in AANAT activity and melatonin production. As shown in the present study, the bovine pinealocyte system makes it possible to examine this without potentially confounding transcriptional effects.

AANAT mRNA was readily detectable in untreated cultured bovine pinealocytes, and treatment with NA did not change AANAT mRNA levels but increased AANAT protein levels in parallel with AANAT activity. This indicates that transcriptional mechanisms are not involved in the switch-on of AANAT in cultured bovine pinealocytes, consistent with the results of *in vivo* studies (Coon et al., 1995; Craft et al., 1999); rather, it appears that downstream posttranscriptional mechanisms play a central role. This view was supported by the findings that the NA-induced increases in AANAT protein levels and activity were not blocked by the transcriptional inhibitor actinomycin D. In contrast to actinomycin D, puromycin totally blocked the NA-induced increases in AANAT protein levels and activity, indicating that NA actions require ongoing protein synthesis.

The effects of NA on AANAT protein levels and activity were mimicked by the cyclic AMP antagonists forskolin and dibutyryl cyclic AMP. Thus, the second messenger involved in increasing AANAT protein levels in bovine pinealocytes appears to be cyclic AMP, as has been proposed in a previous study by R uppel and Olcese (1991). The close correlation between AANAT activity

FIG. 5. Effects of inhibitors of proteasomal proteolysis on AANAT protein levels and activity. AANAT protein levels were analyzed semiquantitatively as described (Wicht et al., 1999) and expressed as the fold increase over the AANAT protein level in unstimulated cells (CO). **a** and **b**: Effects of treatment with different proteasomal inhibitors [lactacystin (Lacta), MG-132, and calpain inhibitor I (Calp I); 25 μ M each], a nonproteasomal protease inhibitor [calpain inhibitor III (Calp III) 25 μ M], or NA (100 nM) for 6 h on AANAT protein levels and activity. **c** and **d**: Time-dependent effects of Lacta (25 μ M) on AANAT protein levels and activity. **e** and **f**: Dose-dependent effects of Lacta treatment for 6 h on AANAT protein levels and activity. **g** and **h**: Effects of combinations of Lacta (25 μ M) with different cyclic AMP protag- onists or puromycin on AANAT protein levels and activity. Treatment of cells with combinations of Lacta/NA (100 nM), Lacta/dibutyl cyclic AMP (DB- cAMP; 1 mM), or Lacta/forskolin (FK; 5 μ M) for 6 h did not significantly change the levels of AANAT protein and activity compared with the effect elicited by Lacta (25 μ M) alone. Lacta effects were not apparent when used in combination with puromycin (Puro; 50 μ g/ml). * p < 0.05, ** p < 0.01 versus un- treated cells.



and AANAT protein levels in cultured bovine pinealocytes in response to the activation of the NA/cyclic AMP pathway allows the conclusions that (a) AANAT activity is regulated by controlling the abundance of AANAT protein and (b) AANAT protein in the bovine pinealocyte is “active” following synthesis—as measured in homogenates—and does not appear to be subject to reversible posttranslational “on/off” regulation.

Considering the above-mentioned results with the rat and the chicken, we tested the hypothesis whether NA-induced inhibition of AANAT proteolysis might be the dominant mechanism involved in switching-on AANAT in those species in which the AANAT mRNA level does not change on a day/night basis. Our finding that inhibition of AANAT proteolysis with selective proteasomal inhibitors alone can switch-on AANAT enzyme activity

by increasing AANAT protein levels—in the absence of changes in AANAT mRNA levels—provides compelling evidence that this mechanism alone regulates AANAT protein. The effects of inhibition of AANAT proteolysis appear to require continual protein synthesis because puromycin totally blocked the lactacystin-induced increases in AANAT protein levels and activity. This finding is of special interest because it suggests that AANAT protein is continually being synthesized in bovine pinealocytes but only accumulates when proteolysis is blocked. The finding that combinations of lactacystin with NA or cyclic AMP antagonists did not increase the effects of lactacystin is consistent with the interpretation that NA and lactacystin act through a common mechanism, i.e., inhibition of AANAT proteolysis.

Accordingly, our results indicate that, in bovine pinealocytes, the selective inhibition of AANAT proteolysis by the proteasome is required for AANAT protein levels and activity to increase in response to adrenergic/cyclic AMP stimulation. This is the first system where an activating role of proteasomal proteolysis for neuroendocrine signaling has been clearly established because this occurs independently of changes in AANAT mRNA levels.

Our results allow us to construct a simple hypothetical model of adrenergic regulation of AANAT in bovine pinealocytes. The AANAT mRNA level is continually elevated, and it appears that AANAT is continually synthesized. Under unstimulated conditions, newly synthesized AANAT protein is immediately destroyed by proteasomal proteolysis. Under adrenergic stimulation, AANAT is protected from proteasomal proteolysis, and AANAT protein accumulates. We suspect that NA may protect AANAT from degradation via cyclic AMP-dependent phosphorylation of two highly conserved AANAT protein kinase A sites. This assumption is reasonable because the N-terminal protein kinase A site of bovine AANAT is located in the vicinity of a proline-rich region that, in other proteins, has been proposed as a motif destined for degradation via proteasomal proteolysis (Hershko and Ciechanover, 1998). Moreover, this N-terminal region of bovine AANAT contains a lysine residue that is conserved in the deduced AANAT amino acid sequences from sheep and humans and may function as a ubiquitination site targeting proteins for degradation by the proteasome (Hershko and Ciechanover, 1998). Alternatively, NA may protect AANAT from degradation via cyclic AMP-dependent phosphorylation of other proteins involved in targeting AANAT for degradation. A combination of these proposed mechanisms may also occur. In addition, a decrease in cyclic AMP content serves as a signal for rapid switch-off by allowing destruction of AANAT protein and suppression of AANAT activity (Gastel et al., 1998).

The evidence that AANAT is destroyed by proteasomal proteolysis raises the issue of whether a ubiquitinated form of AANAT exists. We have attempted to obtain convincing proof of this in several experimental systems but have not been successful using various treat-

ments and combinations of drugs (authors' unpublished data). There are at least three possible explanations for this. One is that AANAT is destroyed by the proteasome by conjugation to a ubiquitin-like protein—not to ubiquitin (Murakami et al., 1992; Yeh et al., 2000). The second is that the ubiquitinated form of AANAT is a highly transient molecule owing to rapid deubiquitination (D'Andrea and Pellman, 1998; Chung and Baek, 1999) and therefore does not accumulate. The third is that the tools we have used to study this issue are inadequate. Accordingly, the molecular steps leading to destruction of AANAT have not been fully described; however, this issue remains a focus of ongoing research.

Several observations strongly suggest that the bovine model that emphasizes proteasomal proteolysis as the dominant switch-on mechanism of AANAT activity might also apply to primates and other ungulates. First, preliminary studies have provided an indication that the monkey pineal gland AANAT mRNA level is relatively high during the day (Klein et al., 1997), as is the case with sheep (Coon et al., 1995) and cattle. Second, in both primates (Reppert et al., 1979; Arendt, 1995) and ungulates (Hedlund et al., 1977; Namboodiri et al., 1985), there is an immediate increase in circulating melatonin levels at the start of the night period, which is made possible because AANAT mRNA is continually available. In contrast, in rodents there is a delay in the increase in melatonin production, which is imposed by the time required for AANAT mRNA to accumulate from nearly undetectable daytime levels to nighttime levels (Borjigin et al., 1995; Roseboom et al., 1996; Maronde et al., 1999a).

AANAT is the primary regulator of the melatonin signal that is used to coordinate optimally seasonal changes in environmental lighting cycles to the internal circadian and circannual rhythms in physiology, including reproduction. The differences in regulatory mechanisms of melatonin synthesis seen between rodents versus ungulates and primates might be linked to differences in the length of the gestation period requiring stimulation of reproductive activity during different seasons of the year. Rodents have a short gestation period, and spring birth requires breeding shortly before spring, whereas ungulates have a long gestation period, and spring birth requires fall breeding. Perhaps longer nocturnal periods of melatonin production in larger animals favor fall breeding, whereas short periods of melatonin production in rodents favor spring breeding.

The findings of our studies are of special interest because they represent the first evidence that neurotransmitter-regulated inhibition of AANAT proteolysis plays a dominant role in switching-on the day/night rhythm in AANAT activity in many mammals, including humans. These results have broad significance because they show that regulation of proteolysis of critical proteins, which is important for cell cycling (Hershko, 1997; Singer et al., 1999), immune response (Reits et al., 2000; Schubert et al., 2000), apoptosis (Canu et al., 2000; Qiu et al., 2000), and neurodegeneration (Alves-Rodrigues et al.,

1998), also plays a pivotal role in neuroendocrine and neural regulation.

Acknowledgment: We thank J. H. Stehle, F. Dehghani, C. Heck, and M. Pfeffer for helpful discussions, the Chemical Synthesis Program of the National Institute of Mental Health for providing coA-S-acetyltryptamine (under contract NO-IMH30003), and Dr. Igal Gery, National Eye Institute, National Institutes of Health, for a gift of anti-S-antigen serum. This study was supported in part by grant SFB 269 from the Deutsche Forschungsgemeinschaft and a Cooperative Research and Development Agreement between the National Institute of Child Health and Human Development and the Institut de Recherches Servier.

REFERENCES

- Alves-Rodrigues A., Gregori L., and Figueiredo-Pereira M. E. (1998) Ubiquitin, cellular inclusions and their role in neurodegeneration. *Trends Neurosci.* **21**, 516–520.
- Arendt J. (1995) *Melatonin and the Mammalian Pineal Gland*. Chapman and Hall, London.
- Baler R., Covington S., and Klein D. C. (1997) The rat arylalkylamine *N*-acetyltransferase gene promoter. *J. Biol. Chem.* **272**, 6979–6985.
- Bernard M., Iuvone P. M., Cassone V. M., Roseboom P. H., Coon S. L., and Klein D. C. (1997a) Avian melatonin synthesis: photic and circadian regulation of serotonin *N*-acetyltransferase mRNA in the chicken pineal gland and retina. *J. Neurochem.* **68**, 213–222.
- Bernard M., Klein D. C., and Zatz M. (1997b) Chick pineal clock regulates serotonin *N*-acetyltransferase mRNA rhythm in culture. *Proc. Natl. Acad. Sci. USA* **94**, 304–309.
- Borjigin J., Wang M. M., and Snyder S. H. (1995) Diurnal variation in mRNA encoding serotonin *N*-acetyltransferase in the pineal gland. *Nature* **378**, 783–785.
- Bradford M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
- Burke Z., Wells T., Carter D., Klein D. C., and Baler R. (1999) Serotonin *N*-acetyltransferase promoter: conditional pineal/retinal gene expression in the transgenic rat. *J. Neurochem.* **73**, 1343–1349.
- Canu N., Barbato C., Ciotti M. T., Serafino A., Dus L., and Calissano P. (2000) Proteasome involvement and accumulation of ubiquitinated proteins in cerebellar granule neurons undergoing apoptosis. *J. Neurosci.* **20**, 589–599.
- Chik C. L. and Ho A. K. (1989) Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. *Prog. Biophys. Mol. Biol.* **53**, 197–203.
- Chung C. H. and Baek S. H. (1999) Deubiquitinating enzymes: their diversity and emerging roles. *Biochem. Biophys. Res. Commun.* **266**, 633–640.
- Coon S. L., Roseboom P. H., Baler R., Weller J. L., Namboodiri M. A. A., Koonin E. V., and Klein D. C. (1995) Pineal serotonin *N*-acetyltransferase: expression cloning and molecular analysis. *Science* **270**, 1681–1683.
- Craft C. M., Murage J., Brown B., and Zhan-Poe X. (1999) Bovine arylalkylamine *N*-acetyltransferase activity correlated with mRNA expression in pineal and retina. *Mol. Brain Res.* **65**, 44–51.
- D'Andrea A. and Pellman D. (1998) Deubiquitinating enzymes: a new class of biological regulators. *Crit. Rev. Biochem. Mol. Biol.* **33**, 337–352.
- Drijfhout W. A., van der Linde S., Kooi C., Grol B., and Westerink B. H. C. (1996) Norepinephrine release in the rat pineal gland: the input from the biological clock measured by in vivo microdialysis. *J. Neurochem.* **66**, 748–755.
- Fenteany G. and Schreiber S. L. (1998) Lactacystin, proteasome function, and cell fate. *J. Biol. Chem.* **273**, 8545–8548.
- Fleming J. V., Barrett P., Coon S. L., Klein D. C., and Morgan P. J. (1999) Ovine arylalkylamine *N*-acetyltransferase in the pineal and pituitary glands: differences in function and regulation. *Endocrinology* **140**, 972–978.
- Gastel J. A., Roseboom P. H., Rinaldi P. A., Weller J. L., and Klein D. C. (1998) Melatonin production: proteasomal proteolysis in serotonin *N*-acetyltransferase regulation. *Science* **279**, 1358–1360.
- Hedlund L., Lischko M. M., Rollag M. D., and Niswender G. D. (1977) Melatonin: daily cycle in plasma and cerebrospinal fluid of calves. *Science* **195**, 686–687.
- Hershko A. (1997) Roles of ubiquitin-mediated proteolysis in cell cycle control. *Curr. Opin. Cell Biol.* **9**, 788–799.
- Hershko A. and Ciechanover A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–479.
- Khalil E. M. and Cole P. A. (1998) A potent inhibitor of the melatonin rhythm enzyme. *J. Am. Chem. Soc.* **120**, 6195–6196.
- Klein D. C. (1985) Photoneural regulation of the mammalian pineal gland, in *Ciba Foundation Symposium 117: Photoperiodism, Melatonin and the Pineal Gland* (Evered D. and Clark S., eds), pp. 38–56. Pitman, London.
- Klein D. C. and Moore R. Y. (1979) Pineal *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase: control by the retinohypothalamic tract and the suprachiasmatic nucleus. *Brain Res.* **174**, 245–262.
- Klein D. C., Coon S. L., Roseboom P. H., Weller J. L., Bernard M., Gastel J. A., Zatz M., Iuvone P. M., Rodriguez I. R., Bégay V., Falcón J., Cahill G. M., Cassone V. M., and Baler R. (1997) The melatonin rhythm-generating enzyme: molecular regulation of serotonin *N*-acetyltransferase in the pineal gland. *Recent Prog. Horm. Res.* **52**, 307–358.
- Korf H. W., Moller M., Gery I., Zigler J. S., and Klein D. C. (1985) Immunocytochemical demonstration of retinal S-antigen in the pineal organ of four mammalian species. *Cell Tissue Res.* **239**, 81–85.
- Korf H. W., Schomerus C., and Stehle J. H. (1998) The pineal organ, its hormone melatonin, and the photoneuroendocrine system. *Adv. Anat. Embryol. Cell Biol.* **146**, 1–100.
- Maronde E., Pfeffer M., Olcese J., Molina C. A., Schlotter F., Dehghani F., Korf H. W., and Stehle J. H. (1999a) Transcription factors in neuroendocrine regulation: rhythmic changes in pCREB and ICER levels frame melatonin synthesis. *J. Neurosci.* **19**, 3326–3336.
- Maronde E., Wicht H., Tasken K., Genieser H. G., Dehghani F., Olcese J., and Korf H. W. (1999b) PKA type II regulation of CREB phosphorylation and melatonin synthesis in the rat pineal gland. *J. Pineal Res.* **27**, 170–182.
- Murakami Y., Matsufuji S., Kameji T., Hayashi S., Igarashi K., Tamura T., Tanaka K., and Ichihara A. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* **360**, 597–599.
- Namboodiri M. A. A., Sugden D., Klein D. C., Grady R. Jr., and Mefford I. N. (1985) Rapid nocturnal increase in ovine pineal *N*-acetyltransferase activity and melatonin synthesis: effects of cycloheximide. *J. Neurochem.* **45**, 832–835.
- Qiu J. H., Asai A., Chi S., Saito N., Hamada H., and Kirino T. (2000) Proteasome inhibitors induce cytochrome c–caspase-3-like protease-mediated apoptosis in cultured cortical neurons. *J. Neurosci.* **20**, 259–265.
- Reits E. A. J., Vos J. C., Grommé M., and Neeffjes J. (2000) The major substrates for TAP *in vivo* are derived from newly synthesized proteins. *Nature* **404**, 774–778.
- Reppert S. M., Perlow M. J., Tamarkin L., and Klein D. C. (1979) A diurnal melatonin rhythm in primate cerebrospinal fluid. *Endocrinology* **104**, 295–301.
- Roseboom P. H. and Klein D. C. (1995) Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: involvement of a β -adrenergic/cyclic AMP mechanism. *Mol. Pharmacol.* **47**, 439–449.
- Roseboom P. H., Coon S. L., Baler R., McCune S. K., Weller J. L., and Klein D. C. (1996) Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase mes-

- senger ribonucleic acid in the rat pineal gland. *Endocrinology* **137**, 3033–3044.
- Rüppel R. and Olcese J. (1991) Bovine pinealocytes in monolayer culture: studies on the adrenergic regulation of melatonin secretion. *Endocrinology* **129**, 2655–2662.
- Schaad N. C., Parfitt A., Russell J. T., Schaffner A. E., Korf H. W., and Klein D. C. (1993) Single-cell $[Ca^{2+}]_i$ analysis and biochemical characterization of pinealocytes immobilized with novel attachment peptide preparation. *Brain Res.* **614**, 251–256.
- Schomerus C., Laedtke E., and Korf H. W. (1999) Analyses of signal transduction cascades in rat pinealocytes reveal a switch in cholinergic signaling during postnatal development. *Brain Res.* **833**, 39–50.
- Schubert U., Antón L. C., Gibbs J., Norbury C. C., Yewdell J. W., and Bennink J. R. (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**, 770–774.
- Singer J. D., Gurian-West M., Clurman B. and Roberts J. M. (1999) Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. *Genes Dev.* **13**, 2375–2387.
- Stehle J. H., Foulkes N. S., Molina C. A., Simonneaux V., Pevét P., and Sassone-Corsi P. (1993) Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. *Nature* **356**, 314–320.
- Tamotsu S., Schomerus C., Stehle J. H., Roseboom P. H., and Korf H. W. (1995) Norepinephrine-induced phosphorylation of the transcription factor CREB in isolated rat pinealocytes: an immunocytochemical study. *Cell Tissue Res.* **282**, 219–226.
- Wicht H., Maronde E., Olcese J., and Korf H. W. (1999) A semiquantitative image-analytical method for the recording of dose–response curves in immunocytochemical preparations. *J. Histochem. Cytochem.* **47**, 411–417.
- Yeh E. T. H., Gong L. M., and Kamitani T. (2000) Ubiquitin-like proteins: new wines in new bottles. *Gene* **248**, 1–14.
- Zatz M., Gastel J., Heath J. R. III, and Klein D. C. (2000) Chicken pineal melatonin synthesis: light and cyclic AMP control abundance of serotonin *N*-acetyltransferase protein. *J. Neurochem.* **74**, 2315–2321.