Cellular/Molecular

Day–Night Changes in Downstream Regulatory Element Antagonist Modulator/Potassium Channel Interacting Protein Activity Contribute to Circadian Gene Expression in Pineal Gland

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The molecular mechanisms controlling the oscillatory synthesis of melatonin in rat pineal gland involve the rhythmic expression of several genes including arylalkylamine *N*-acetyltransferase (*AA-NAT*), inducible cAMP early repressor (*ICER*), and Fos-related antigen-2 (*fra-2*). Here we show that the calcium sensors downstream regulatory element antagonist modulator/potassium channel interacting protein (DREAM/KChIP)-3 and KChIP-1, -2 and -4 bind to downstream regulatory element (DRE) sites located in the regulatory regions of these genes and repress basal and induced transcription from *ICER*, *fra-2* or *AA-NAT* promoters. Importantly, we demonstrate that the endogenous binding activity to DRE sites shows day–night oscillations in rat pineal gland and retina but not in the cerebellum. The peak of DRE binding activity occurs during the day period of the circadian cycle, coinciding with the lowest levels of *fra-2*, *ICER*, and *AA-NAT* transcripts. We show that a rapid clearance of DRE binding activity during the entry in the night period is related to changes at the posttranscriptional level of DREAM/KChIP. The circadian pattern of DREAM/KChIP activity is maintained under constant darkness, indicating that an endogenous clock controls DREAM/KChIP function. Our data suggest involvement of the family of DREAM repressors in the regulation of rhythmically expressed genes engaged in circadian rhythms.

Key words: **calcium; repressor DREAM; cAMP; proteolysis; pineal gland; circadian rhythms**

Introduction

Circadian clocks are cellular oscillators that generate daily rhythms also in the absence of external timing cues (Pittendrigh, 1993; Dunlap, 1999). For vertebrates, the pineal gland is an important component of the circadian timing system that translates a clock-derived neuronal signal into rhythmic production and secretion of the hormone melatonin (Foulkes et al., 1997; Li et al., 1998). Melatonin is produced in the pineal gland and the retina from serotonin in a two-step process in which the arylalkylamine *N*-acetyltransferase (AA-NAT) is the rate-limiting enzyme (Borjigin et al., 1995; Coon et al., 1995). Melatonin synthesis is controlled by both cAMP and the intracellular concentration of Ca^{2+} ions, which act synergistically during the subjective night (Sugden et al., 1987; Nikaido and Takahashi, 1996). The mechanism involves the oscillatory expression of the immediate early genes Fos-related antigen-2 (*fra-2*) (Baler and Klein, 1995) and the inducible cAMP early repressor (*ICER*) (Molina et al., 1993; Stehle et al., 1993) which in turn regulate the expression of the gene coding for AA-NAT (Borjigin et al., 1995; Coon et al., 1995). Transcriptional regulation of the *fra*-2, *ICER*, and *AA-NAT* genes is remarkably similar, with a peak in mRNA levels at night and very low expression during the day, indicating a common regulatory mechanism (Stehle et al., 1993; Baler and Klein, 1995; Borjigin et al., 1995).

DREAM is a Ca^{2+}-binding protein of the neuronal calcium sensors family that specifically binds DRE sites as a tetramer and represses transcription in a Ca^{2+}- and cAMP-dependent manner (Carrion et al., 1999; Ledo et al., 2000). Other mechanisms also control the biological activity of DREAM; these include (1) differential splicing (Spreafico et al., 2001), (2) cleavage of the N-terminal part of DREAM by caspase-3 (Choi et al., 2001) that may affect the subcellular distribution of truncated DREAM, (3) palmitoylation (Takimoto et al., 2002) that directs DREAM toward a membrane localization, (4) phosphorylation by so far unidentified downstream kinases of the PI3 kinase pathway (Sanz et al., 2001) that alter the binding of DREAM to DRE sites, and (5) protein–protein interactions, i.e., a Ca^{2+}-dependent DREAM/cAMP response element-binding protein (CREB) interaction

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that displaces CREB from CRE sites and prevents the recruitment of CREB-binding protein to phospho-CREB. In this way, DREAM downregulates CRE-dependent transcription without direct binding of DREAM to CRE sites (Ledo et al., 2002).

DREAM belongs to a group of structurally and functionally related Ca^{2+}-binding proteins (KChIP-1 to -4) that interact with voltage-dependent K^+ channels of the Kv4 class modulating potassium currents in a Ca^{2+}-dependent manner in the plasma membrane (An et al., 2000; Holmqvist et al., 2002). Importantly, KChIP-3 corresponds to DREAM, suggesting that the transcriptional repressor activity of DREAM may be shared by these closely related KChIP proteins.

Here, we have investigated whether the presence of functional DRE sites within the *fra-2*, *ICER*, and *AA-NAT* genes indicate a role for transcriptional repressor DREAM in the transcriptional control of pineal-specific genes and hence a role in circadian gene expression.

Materials and Methods

Animals and sample preparation. Adult male Wistar rats (200–300 gm) were kept under a standard 12 hr light/dark cycle (L/D) (lights on from 7:00 A.M.–7:00 P.M.) or under constant darkness (D/D) with food and water available *ad libitum*. For D/D experiments, animals were adapted to this condition for at least 10 d before any manipulation. Rats were killed at the indicated time points under ambient light conditions for L/D animals during daytime or dim red light for D/D animals or L/D animals during nighttime. Pineal glands and retinas were removed, and whole-cell extracts were prepared as described by Konradi et al. (1994). Isoproterenol (15 mg/kg in saline) was injected intraperitoneally 1 hr before tissue harvesting.

Electrophoretic mobility shift analysis. Double–stranded oligonucleotides corresponding to the mouse *fra-2* DRE site ($DRE_{fra\text{-}2}$) 5′-TCGAGCTTGTCCTAAGTCAAGTGTCCGAGTC-3′, mouse *ICER* DRE sites (DRE_{ICER}) 5′-GGTGACGTC ACTGTGATGTCAGTG-3′, and rat *AA-NAT* DRE_1 site ($DRE_{2\ AA\text{-}NAT}$) 5′-GATCACATGCTGTCAGGGGGAATGC-3′ were labeled with [γ-^{32}P]ATP and T4 kinase and used as probes. In addition, the human prodynorphin DRE site (DRE_{DYN}) 5′-GAAGCCGGAGTCAAGGAGGCCCCTG-3′, the mutated prodynorphin DRE site ($mutDRE_{DYN}$) 5′-GAAGCCGGAGTCTAGGAGGCCCCTG-3′, and the Sp1 site 5′-ATTCGATCGGGGCGGGGCGAGC-3′ were used as controls. Protein extracts (5–10 μg) were incubated with the radioactive oligonucleotide probe for 20 min at room temperature in reaction buffer [10 mM HEPES, pH 7.9, 10% glycerol, 0.1 mM EDTA, 8 mM $MgCl_2$, 1 mM dithiothreitol, 0.15 μg/ml poly(dI-dC)]. Protein–DNA complexes were resolved in 5% nondenaturing polyacrylamide gels and visualized by autoradiography. DNA or antibody competition experiments, respectively, were performed as described (Carrion et al., 1998; Ledo et al., 2002).

Antibody preparation and Western blot analysis. Peptide-specific antibodies were prepared by serial injection of KLH-coupled peptides, residues 73–87 [antibody (Ab) 671] or residues 242–256 (Ab 668). A polyclonal antibody Ab 1013 was prepared using full-length recombinant DREAM. Antisera were affinity purified using the immunogenic peptides. All three antibodies recognized purified recombinant DREAM and monomer DREAM in nuclear extracts from NB69 cells as well as in nuclear extracts from human embryonic kidney (HEK) 293 cells overexpressing DREAM (data not shown). In addition, Ab 668 and Ab 1013 were able to react with all four KChIP proteins overexpressed in HEK293 cells, whereas Ab 671 showed partial cross-reactivity with KChIPs other than DREAM.

Cell culture, transfection, and chloramphenicol acetyl transferase analysis. Rat C6 glioma or human carcinoma HEK293 and HeLa cells were grown in DMEM–Ham F-12–Glutamax-I medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. Transfections and chloramphenicol acetyl transferase (CAT) activity assays were performed as described (Ledo et al., 2000). Eight micrograms of DNA containing 5 μg of reporter plasmid and 3 μg of expression vector were coprecipitated with calcium phosphate and added to the HEK293 or C6 cultures. Treatment with 50 mM KCl or 100 μM isoproterenol was performed 8 hr before cells were harvested. The expression vectors for the different human KChIP variants used in this study were KChIP-1.1, KChIP-2.6, full-length DREAM/KChIP-3, and KChIP-4. Site-directed mutagenesis at the DRE sites in the AA-NAT reporter plasmid was performed using the QuickChange method (Stratagene, La Jolla, CA). Mutated AA-NAT reporters include a GT to CA substitution at the GTCA DRE core at position −20 from the transcription start site (DRE_1) or at position +65 in the complementary strand in the 5′ untranslated region (DRE_2) or at both positions. Transfection experiments were performed in triplicates and repeated a minimum of four times. Statistical analysis of the results was performed using the Student's *t* test.

Qualitative and quantitative real-time RT-PCR. Qualitative RT-PCR was performed using total RNA from rat pineal gland, retina, or brain and specific primers: KChIP-1, forward 5′-GACACCACCCAGACAGGCTCT-3′ and reverse 5′-CAGAATGGCCAGTGTCCTCAGT-3′; KchIP-2, forward 5′-CAAGTTCACACGCAGAGAGT-3′ and reverse 5′-CCGAAGAATCACTGACAAAC-3′; DREAM/KchIP-3, forward 5′-AGCAAGAGGGAAGGCA-3′ and reverse 5′-GAAGAACTGGGAATAAATGA-3′; KchIP-4, forward 5′-CGTGAGAAGGGTGGAAAG-3′ and reverse 5′-GCAGGAGACGACGTTTTG-3′. PCR amplification was performed for 40 cycles: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Quantitative real-time RT-PCR was performed using specific primers and TaqMan minor groove binder (MGB) probes for KChIP-1, -2, and -4 supplied by Applied Biosystems (Foster City, CA). For DREAM/KChIP-3, the primers used were forward 5′-CACCTATGCACACTTCCTCTTCA-3′ and reverse 5′-ACCACAAAGTCCTCAAAGTGGAT-3′ and the probe FAM-′-TGCCTTCGATGCTGAT-3′-MGB. TaqMan MGB probes for DREAM/KChIP-3 and -4 were useful for amplification of all splice variants of these two transcripts. TaqMan probes for KChIP-1 and -2 map at the 5′ end of the coding sequence and amplify KChIP-1.1, -1.2, -2a, and -2b. Thus, splice variants 1a and 2c were not detected with our conditions.

Chromatin immunoprecipitation. We performed chromatin immunoprecipitation using a previously published method (Takahashi et al., 2000). Briefly, tissues were mechanically dispersed and cross-linked, nuclei were collected, and the chromatin were sonicated to an average length of 700 bp. The sheared chromatin was precleared with blocked protein-A/G Sepharose and used for immunoprecipitation, with 4.5 μg of affinity-purified polyclonal antibody 1013 against DREAM. Immunoprecipitated DNA was subjected to semiquantitative PCR with specific primers (forward, 5′-GGCCAGGGGGATTAGAAT-3′; reverse, 5′-TGTAAGAAGGAGTCCAGGTGTC-3′) to amplify a 162 bp fragment of the rat *AA-NAT* promoter region using a trace amount of [^{32}P]dCTP and 28 cycles of amplification, well within the linear range.

Results

DREAM and KChIPs are expressed in the rat pineal gland and bind to DRE sites present in the *ICER*, *fra-2*, and *AA-NAT* genes

The search for downstream regulatory elements within genes induced by calcium stimulation identified, among others, putative DRE sites in the *ICER*, *fra-2,* and *AA-NAT* genes (Fig. 1*A*), all three of which are related to the circadian changes of melatonin synthesis in the pineal gland (Foulkes et al., 1997). A cluster of two direct DRE sites and an inverted DRE is present in the mouse *ICER* gene (accession number S67785) 40 nucleotides upstream from the transcription initiation site. Similarly, the rat and human *AA-NAT* genes (accession numbers U77455 and AF375991, respectively) contain a cluster with two direct DRE sites and two inverted DREs located in close proximity to the transcription initiation site. Finally, the mouse *fra-2* gene (accession number X83970) contains a DRE site in the 5′-untranslated region of the gene.

To test the possibility that DREAM is related to pineal function regulating the expression of genes involved in circadian rhythms, we first checked for the expression of DREAM in the

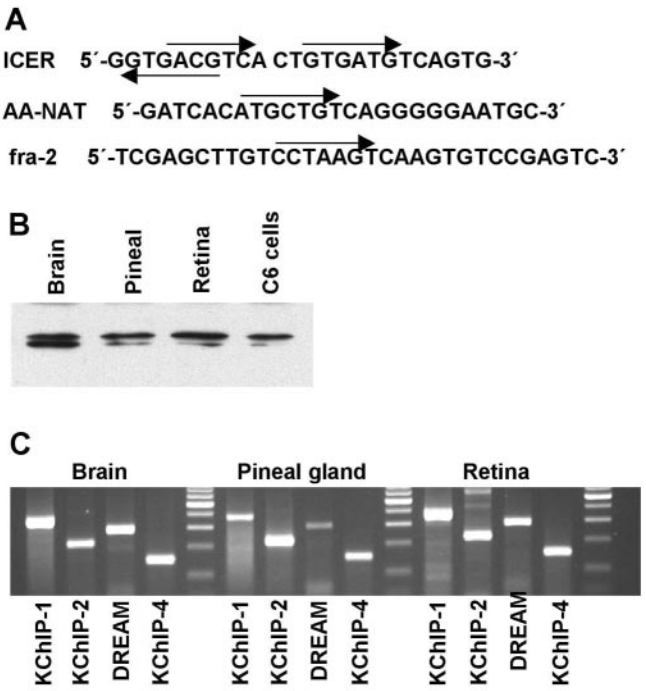

Figure 1. DREAM/KChIPs are expressed in the pineal gland and the retina. *A*, Putative DRE sites present in *ICER*, *AA-NAT*, and *fra-2* regulatory regions. Arrows indicate the orientation of the DRE sequence. *B*, Western blot analysis of whole-cell extracts from pineal gland and retina. For comparison, brain and C6 rat glioma cell extracts are shown. *C*, RT-PCR using total RNA from brain, pineal gland, and retina and specific primers for the different KChIPs.

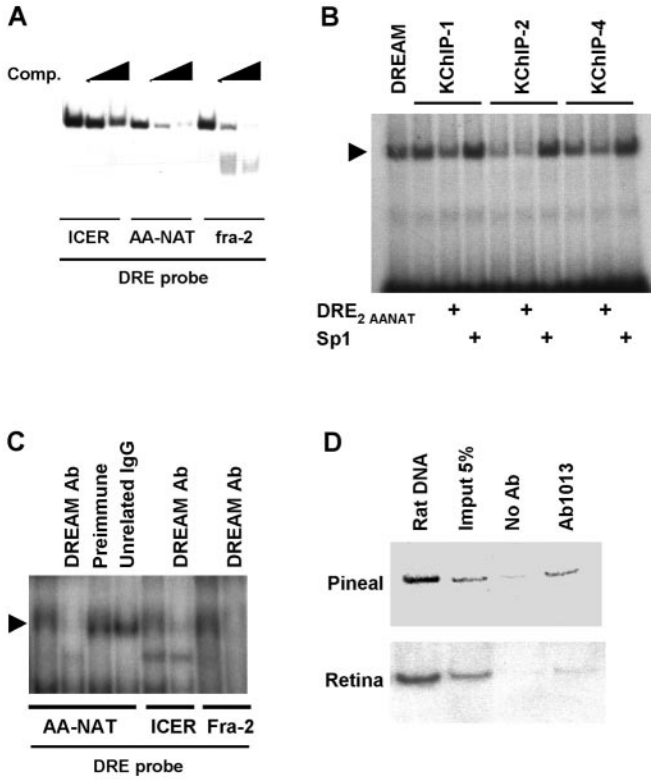

Figure 2. DREAM/KChIPs bind DRE sites in pineal-related genes. *A*, EMSA using recombinant DREAM protein and representative DREs from pineal-related genes as probes. Competitions with 5- to 50-fold DRE_{DYN} are shown for each probe. *B*, EMSA using whole-cell extracts from HeLa cells transfected with KChIP-1, -2, and -4 and the $DRE_{AA\text{-}NAT}$ probe. Competitions with fivefold cold $DRE_{2AA\text{-}NAT}$ or Sp1 oligonucleotides are shown. *C*, Supershift of specific DRE retarded bands (arrow) obtained with pineal gland whole-cell extracts, the indicated DRE probes, and the Ab1013 for DREAM/KChIPs. *D*, Chromatin immunoprecipitation using affinity-purified polyclonal Ab1013 and DNA samples from pineal gland and retina. PCR amplification of the AA-NAT promoter was not detected when no antibody was added.

pineal gland as well as in the retina, an area related to the central origin of circadian rhythms that drives oscillatory melatonin synthesis (Tosini, 2000). Western blot analysis revealed DREAM immunoreactivity in the pineal gland and in the retina as a doublet with a migration corresponding to ~29 kDa monomer DREAM (Fig. 1*B*). As a control, DREAM protein was detected in rat brain and in C6 glioma cells using this antibody (Fig. 1*B*). Posttranslational modifications of the DREAM protein may account for the appearance of the doublet, although its precise nature has not yet been investigated. Alternatively, the faster migrating immunoreactive band may correspond to other KChIP proteins closely related to DREAM (An et al., 2000). To assess this possibility, we used total RNA isolated from rat pineal gland or retina and specific primers for each one of the KChIP transcripts. RT-PCR amplified bands corresponding to DREAM and the other KChIP mRNAs are shown in Figure 1*C*. As a positive control, amplification of all KChIPs in rat brain total RNA is also shown. Direct PCR on rat genomic DNA did not amplify any of the DREAM/KChIP bands (data not shown). Taken together, these data indicate that DREAM as well as other KChIPs are expressed both in the pineal gland and in the retina. Noteworthy, expression of KChIP proteins in pineal gland parallels the expression levels of Kv4 potassium channels in this tissue (Isbrandt et al., 2000).

Next we analyzed the functionality of the DRE sites present in *ICER*, *fra*-2, and *AA-NAT* promoters. For this, we performed electrophoretic mobility shift analysis (EMSA) using recombinant DREAM and oligonucleotides containing a representative DRE site from each of these genes as a probe (Fig. 1*A*). In all cases, a major DRE retarded band was observed that were competed with a 5- to 50-fold excess of a nonlabeled oligonucleotide encompassing the DRE_{DYN} site (Fig. 2*A*). Because other KChIPs are also expressed in circadian-related tissues, share a high sequence similarity with DREAM, and show a common function regulating the conductance of Kv4 potassium channels, we analyzed the ability of KChIP-1, -2, and -4 to bind the DRE site in EMSA experiments. Not surprisingly, a DRE-retarded band was observed with nuclear extracts from HeLa cells overexpressing KChIP-1, -2, or -4 and the DRE_{DYN} probe (Fig. 2*B*). The retarded bands were competed with a fivefold excess of nonlabeled $DRE_{AA\text{-}NAT}$ but were not affected by a 50-fold excess of cold oligonucleotide containing a mutated DRE_{DYN} site or the nonrelated Sp1 site (Fig. 2*B*). Furthermore, to confirm Western blot and RT-PCR data and to probe the functionality of the DREAM/KChIP proteins present in pineal extracts, we analyzed the appearance of DRE-retarded bands using whole-cell extracts from rat pineal gland and the different DRE sites tested previously. Again, all three probes gave a specific retarded band comparable in migration with the retarded band obtained with DRE_{DYN} (Fig. 2*C*). Moreover, these retarded bands could be competed with cold DRE_{DYN} (data not shown) or after preincubation with polyclonal DREAM antibody Ab 1013 able to react with all KChIP proteins (Fig. 2*C*) (and data not shown). Taken together, these experiments demonstrate that DREAM and KChIP-1, -2, and -4 are expressed in the pineal gland and *in vitro* can bind DRE sites present in the regulatory regions of the *ICER*, *AA-NAT*, and *fra-2* genes.

To assess whether DREAM/KChIP proteins bind *in vivo* to

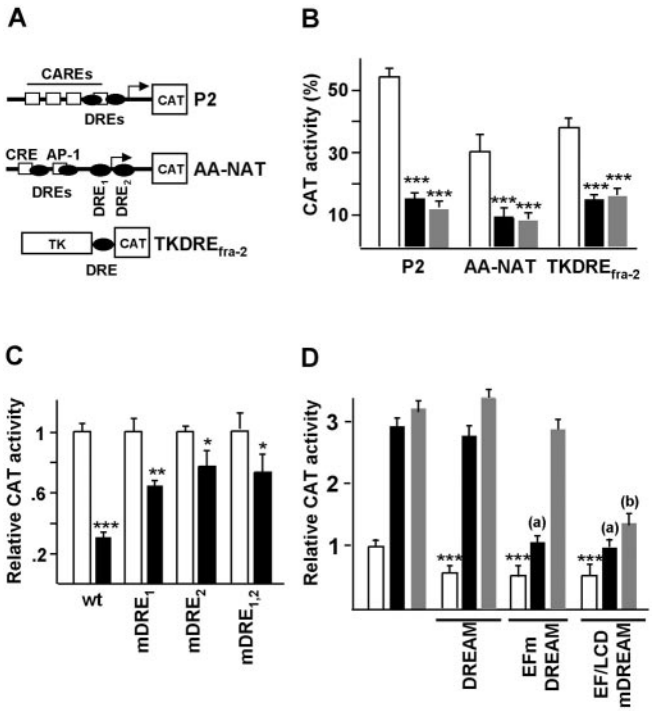

Figure 3. DREAM/KChIPs regulate *ICER*, *AA-NAT*, and *fra-2* promoters. *A*, Schematic representation of the reporter plasmids used in these experiments. Arrows indicate the transcription start site for *ICER* (P2) and the *AA-NAT* genes. Relevant regulatory elements are indicated. *B*, DREAM and KChIP-2 repress basal transcription from *ICER*, *AA-NAT*, and *fra-2* reporter plasmids. Shown is basal reporter activity (open bars), activity after DREAM (black bars), and KChIP-2 (gray bars) overexpression. *C*, Mutation of DRE1 or DRE2 sites, or both, flanking the transcription start site blocked DREAM-mediated repression of the AA-NAT reporters. *D*, Transactivation of the *AA-NAT* reporter in basal conditions (open bars) and after potassium (black bars) or cAMP (gray bars) stimulation. Effects of the Ca^{+2}–insensitive (EFmDREAM) and of the double Ca^{+2}- and cAMP-insensitive (EF/LCDmDREAM) dominant-negative mutants are shown. Asterisks represent statistically significant differences between the means relative to corresponding controls (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; Student's *t* test). Statistically significant blockage of EFmDREAM or EF/LCD mDREAM relative to potassium-induced ([a]$p < 0.001$; Student's *t* test) or cAMP-induced transactivation ([b]$p < 0.001$; Student's *t* test), respectively.

promoters of genes involved in circadian rhythms, we performed chromatin immunoprecipitation using affinity-purified polyclonal Ab 1013 and retina or pineal gland tissues. The paucity of available information about rat genomic sequences limited the analysis to the AA-NAT promoter. Nevertheless, after immunoprecipitation with Ab 1013 of chromatin prepared from either pineal or retina, a fragment of the *AA-NAT* promoter encompassing a cluster of DRE sites located around the transcription start site was amplified (Fig. 2*D*). Omission of the DREAM antibody or use of preimmune serum resulted in no amplification or background amplification (Fig. 2*D*) (and data not shown).

DREAM/KChIPs repress transcription from *ICER*, *AA-NAT*, and *fra-2* reporters

We next addressed whether DREAM and other KChIPs can repress basal transcription from reporters containing regulatory sequences from the *ICER*, *fra*-2, and *AA-NAT* genes (Fig. 3*A*). Transient transfection in HEK293 cells, a cell line without endogenous DREAM activity (Carrion et al., 1999), showed that basal expression from the *P2-ICER* and the *AA-NAT* promoters was equally reduced after cotransfection with DREAM or KChIP-1, -2, or -4 (Fig. 3*B*) (and data not shown). In parallel experiments, DREAM and other KChIPs also repressed transcription of reporter plasmids containing the DRE sites from *ICER*, *fra-2*, or *AA-NAT* genes cloned downstream from a heterologous promoter, the tk minimal promoter from the human herpes simple virus, (Fig. 3*B*) (and data not shown). Similar results for DREAM have been described previously for the prodynorphin DRE site (Carrion et al., 1998, 1999). Furthermore, to confirm that binding to DRE sites is responsible of the DREAM-mediated repression of these promoters, we performed parallel experiments using AA-NAT reporters bearing single or double point mutations at the DRE sites surrounding the transcription start site. Mutation of either DRE site equally blocked the repressor activity of DREAM (Fig. 3*C*), whereas the double mutation did not result in a stronger block of DREAM repressor activity (Fig. 3*C*). These results suggest that (1) both DRE sites flanking the transcription start site cooperatively participate in the repression of the AA-NAT reporter by DREAM, and (2) the two DREs located in the proximity of the CRE site could be responsible for the residual repressive effect of DREAM on the double mutant AA-NAT reporter. Taken together, these data indicate that DREAM and other KChIPs bind to DRE sites and are equally efficient in repressing basal expression of *ICER*, *fra-2*, and *AA-NAT* promoters.

The activity of the *AA-NAT* and *ICER* promoters is controlled primarily by cAMP and Ca^{2+} (Foulkes et al., 1997; Li et al., 1998). To confirm the regulation by DREAM, we aimed to block their inducibility using dominant-negative DREAM mutants unable to sense the rise in nuclear Ca^{2+} or the increase in cAMP (Ledo et al. 2000, 2002). For this study we choose C6 glioma cells, a cell line that has been used to study cAMP-dependent regulation of the *AA-NAT* promoter (Fitzgerald et al., 1996; Baler et al., 1997; Burke et al., 1999) and that expresses endogenous DREAM (Fig. 1*B*). Treatment of C6 cells with 100 μM isoproterenol, a selective agonist of β-adrenergic receptors that increases cAMP levels, or stimulation with depolarizing concentrations of K^+ dramatically increased the activity of wild-type or DRE-mutated AA-NATCAT reporters (Fig. 3*D*) (and data not shown). Isoproterenol treatment also strongly transactivated the P2CAT reporter, as described in a different cell system (Molina et al., 1993), but had no effect on the activity of empty reporter pBLCAT3 (data not shown). Overexpression of EFmDREAM reduced basal and K^+-induced reporter activity from AA-NATCAT (Fig. 3*C*), whereas the double mutant EFmDREAML47,52V, which is insensitive to both Ca^{2+} and cAMP stimulation (Ledo et al., 2000), blocked basal as well as potassium- and isoproterenol-induced AA-NATCAT transactivation (Fig. 3*C*). The double mutant EFmDREAML47,52V blocks DREAM–CREM interaction, preventing CREM-mediated derepression at the DRE site (Ledo et al., 2000). Importantly, this double mutant does not interact with CREB and does not affect CRE-dependent transcription (Ledo et al., 2002), suggesting that the block of AA-NAT transactivation is only related to a block of the derepression process. Similar results were obtained with the P2CAT reporter (data not shown), indicating that in transient transfection experiments DREAM regulates *ICER* and *AA-NAT* promoters. Furthermore, these data show that the *AA-NAT* promoter is regulated also by increasing concentrations of intracellular Ca^{2+} ions.

A circadian clock controls DRE binding activity in the pineal gland

To test the hypothesis that DREAM/KChIPs participate in the circadian regulation of pineal gene expression by rhythmic repression and derepression of target genes, we analyzed the changes in the DRE binding activity in pineal extracts during a normal circadian cycle. Whole-cell extracts from rat pineal glands collected at different times of the circadian cycle were

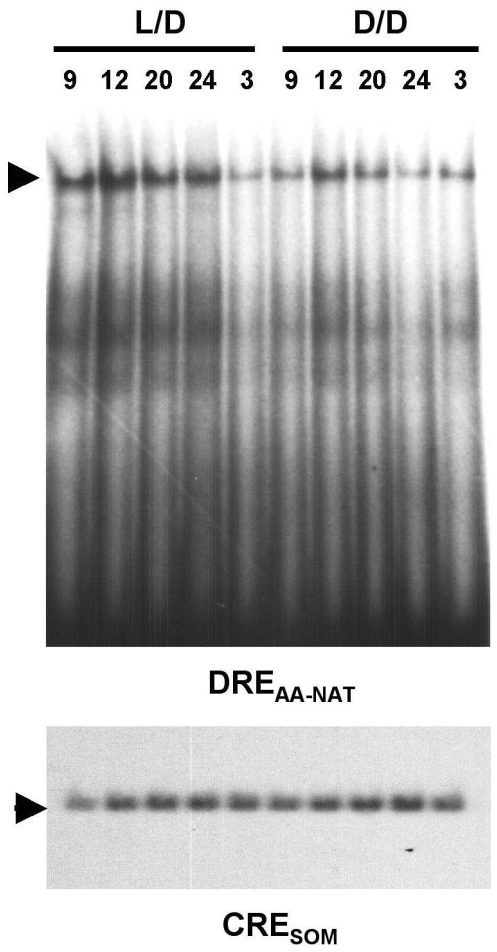

Figure 4. Circadian oscillations of DRE binding activity in the pineal gland: EMSA with the DRE_{AA-NAT} probe and whole-cell extracts from pineal gland collected at the indicated times. Animals were kept under normal light/dark (L/D) cycling conditions or under constant darkness (D/D). As control, EMSA using the same extracts and CRE_{SOM} as a probe is shown in the bottom panel. The L/D experiment was repeated three times, and the D/D experiment was repeated twice.

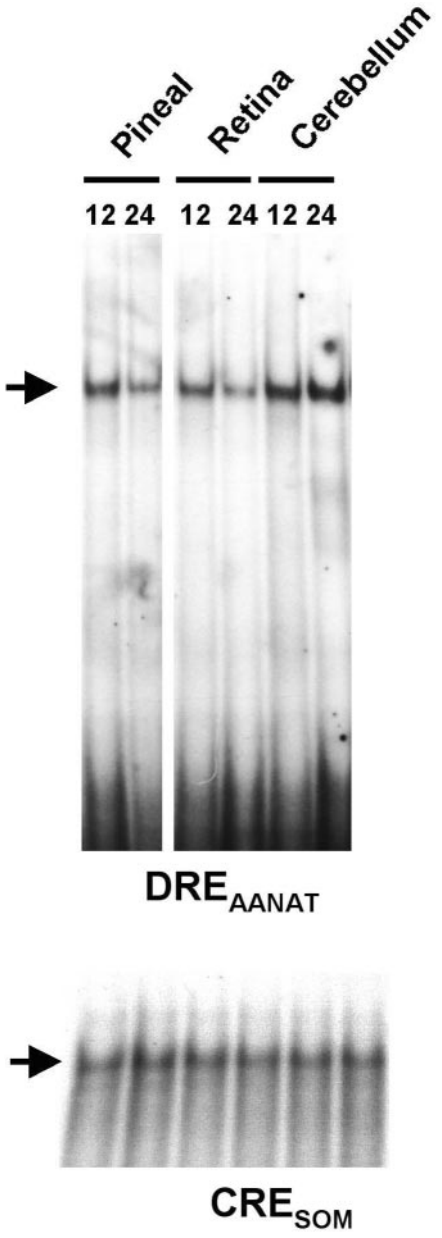

Figure 5. Circadian oscillations of DRE binding activity in the pineal gland and in the retina: EMSA with the DRE_{AA-NAT} probe and whole-cell extracts from the indicated tissues collected at noon (12) or midnight (24). Animals were kept under normal light/dark cycling conditions. As control, EMSA using the same extracts and CRE_{SOM} as a probe is shown in the bottom panel. Arrowheads show the DRE- or CRE-specific retarded bands, respectively.

subjected to EMSA using an oligonucleotide containing the DRE_{AA-NAT} site as a probe. EMSA analysis revealed a circadian fluctuation of DRE binding activity with the highest binding to DRE at 12:00 A.M. (Fig. 4, top panel). The intensity of the DRE-retarded bands decreased, reaching the lowest value at 3:00 A.M. Similar results were obtained with a DRE_{DYN} probe (data not shown), supporting the idea of a day–night change in the binding of pineal DREAM/KChIPs to DRE targets. To control for equal loading in all lanes, the same extracts were assayed using an oligonucleotide encompassing the CRE site of the somatostatin gene. No change in the CRE-retarded band was observed at any time (Fig. 4, bottom panel).

To examine whether DREAM/KChIPs rhythm is endogenously generated, rats were shifted to D/D 10 days before pineal glands were collected at the same time that intervals and extracts were subjected to EMSA. Importantly, DRE binding activity displayed an identical pattern throughout subjective day–night cycles under constant darkness (Fig. 4, top panel). Nevertheless, the intensity of the DRE-retarded bands in D/D extracts was reduced as compared with the intensity in L/D samples. EMSA using a CRE probe was used to control for equal loading (Fig. 4, bottom panel). Importantly, no difference in CRE band intensity was observed between L/D and D/D samples. These data indicate that a circadian clock controls the endogenous DRE binding activity and suggest that oscillatory repression and derepression of DRE sites may participate in the circadian expression of *ICER*, *fra*-2, and *AA-NAT* genes.

To further substantiate these results, we next wondered whether the cycling DRE binding activity is specific for the pineal gland, is a feature present in other central pacemakers, or is simply a general property of DREAM/KChIPs. For this, we performed EMSA using extracts from retina and cerebellum, a brain area not directly related to the generation of circadian rhythms. Tissues were harvested at noon (L) or at midnight (D) from rats kept under standard light/dark conditions. Importantly, extracts

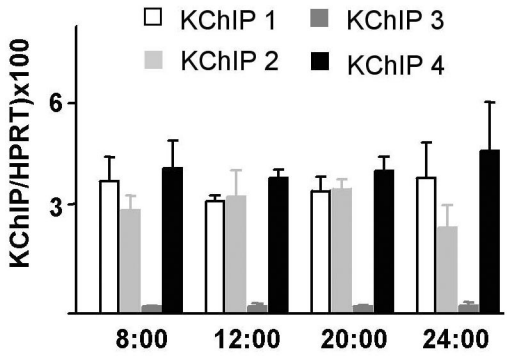

Figure 6. Expression of DREAM/KChIP mRNAs is not modified during the circadian cycle. Quantification was by real-time RT-PCR using specific primers and TaqMan MGB probes for the different KChIPs.

from retina obtained at night but not extracts from cerebellum displayed a significant lower binding to the $DRE_{AA\text{-}NAT}$ probe compared with extracts from tissue harvested during the midday (Fig. 5, top panel). As before, we controlled for equal loading in all lanes by using an oligonucleotide containing the CRE site of the somatostatin gene as a probe (Fig. 5, bottom panel).

Thus, an intrinsic circadian oscillation regulates the endogenous DRE binding activity in pineal gland and retina. Rhythmic changes in DRE binding activity could be a reflection of an oscillatory mechanism controlling the expression of the DREAM/KChIP genes or changes in the posttranslational processing of the DREAM/KChIP proteins, or both.

Expression of DREAM/KChIP mRNAs in pineal gland is constant through the circadian cycle

To investigate whether changes at the transcriptional level could account for the circadian oscillation of DREAM binding activity in pineal gland, we analyzed DREAM/KChIPs mRNA levels in total RNA from pineal samples by real-time quantitative RT-PCR using specific TaqMan MGB probes for DREAM and each of the other KChIPs. As shown in Figure 6, no significant change in DREAM/KChIPs mRNA content was observed in the pineal at any time of the photoperiod. The results are normalized by quantification of the housekeeping hypoxdutine phosphoribosyl transferase gene, which by itself did not show any variation at different times during the day (data not shown). Interestingly, relative values for DREAM/KChIPs confirmed the results after nonquantitative RT-PCR and showed that all four KChIPs are expressed and that the levels of DREAM mRNA were lower compared with any of the other KChIP mRNAs. These results indicate that transcription of DREAM/KChIPs in pineal gland is not subjected to circadian control and suggest that changes in DRE binding activity could be related to clock-regulated posttranslational modifications in DREAM/KChIP proteins.

Rhythmic changes in DREAM/KChIP immunoreactivity in the pineal gland

To check whether circadian changes in the DRE binding activity in pineal extracts are related to changes at the protein level, we analyzed rat pineal extracts by Western blot using the peptide specific antibody Ab 668. Importantly, the intensity of the DREAM/KChIPs doublet did not show any consistent variation at any circadian time in three independent experiments (Fig. 7*A*); however, a group of faster migrating immunoreactive bands was specifically observed at the time point corresponding to the beginning of the dark phase and persisted until midnight in some

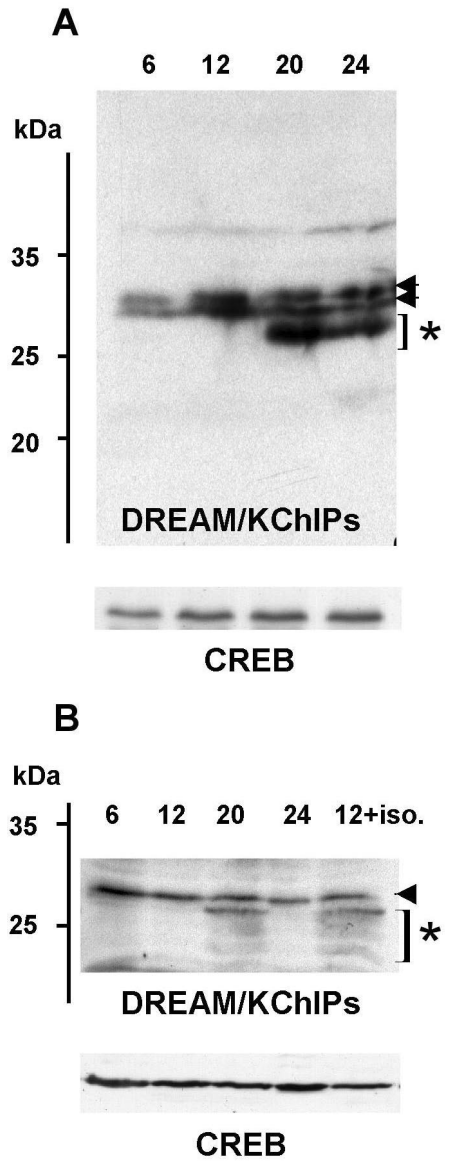

Figure 7. Oscillations of DREAM/KChIP protein levels in the pineal gland during the photoperiod: Western blot analysis with polyclonal antibody 668 (*A*) or polyclonal antibody 671 (*B*) of extracts from the pineal gland at indicated times. Bands corresponding to monomer DREAM/KChIP are indicated by arrows. Faster migrating immunoreactive bands are indicated by asterisks. For each case, control for loading is shown in the bottom panel using an antibody for CREB. Intraperitoneal injection of isoproterenol at noon mimics the entrance in the dark phase of the photoperiod.

experiments (Fig. 7*A*). Importantly, appearance of the low molecular weight immunoreactive bands coincided with the reduction in DRE binding activity at the entrance to the dark phase of the cycle, suggesting a link between these two events. Similar results were observed in extracts from retina, whereas in the cerebellum the low molecular weight bands were not observed at any time (data not shown). Use of a CREB polyclonal antibody demonstrated the integrity of the CREB protein and no variation in the levels of CREB protein in the different pineal samples (Fig. 7*A*). These results suggest that a clock-dependent posttranslational mechanism is operating on DREAM/KChIP proteins to regulate their binding to DRE sites in target genes at the entrance to the dark phase. Alternatively, an accumulation of the shorter splice variants of the different KchIPs, like KChIP-1a and -2c not

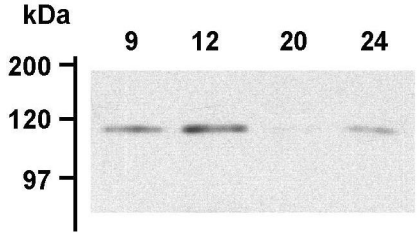

Figure 8. Oscillation of tetramer DREAM levels in the pineal gland during the photoperiod. Southwestern blot analysis of pineal extracts at indicated times using a $DREAM_{AA\text{-}NAT}$ oligonucleotide probe.

detected by the TaqMan probes used in this study or new short splice variants not yet described in rat pineal gland or retina, could also be responsible for the appearance of smaller KChIP isoforms coinciding with the reduction in light intensity. So far, this last possibility has not been investigated further.

Posttranslational processing of DREAM/KChIP proteins has been studied in more detail for DREAM/KChIP-3 (Choi et al., 2001; Sanz et al. 2001; Takimoto et al., 2002). Among posttranslational modifications, cleavage of DREAM by caspase 3 has been described as a Ca^{2+}-dependent process that occurs at position 63 in the DREAM protein (Choi et al., 2001) and could account for the fast migrating immunoreactive band. To investigate a potential clock-dependent proteolytic process, we performed Western blot analysis of pineal extracts using a different peptide-specific polyclonal antibody (Ab 671). Western blots with Ab 671 showed monomer DREAM as a unique band of ~29 kDa that was not modified at the different circadian time points (Fig. 7*B*). Importantly, a group of several immunoreactive bands of lower molecular weight were observed also with Ab 671 at the beginning of the dark phase. Thus, similar results were obtained with two peptide-specific antibodies targeting different epitopes in DREAM/KChIP proteins.

Because the levels of monomer DREAM/KChIP remained practically unchanged through the different time points, a proteolytic mechanism could only be understood if we assume a clock-dependent proteolytic process modifying the levels of multimeric DREAM protein. To check this hypothesis, we analyzed pineal extracts by Southwestern analysis, a technique that allows visualization of tetramer DREAM (Carrion et al., 1998, 1999). In Southwestern blots prepared with rat pineal extracts, DREAM/KChIP binds to the DRE probe as a tetramer with an apparent molecular mass of 110 kDa (Fig. 8) as reported previously for brain extracts (Carrion et al., 1998, 1999). Importantly, the intensity of the 110 kDa band showed a circadian pattern with a peak at noon and a drastic reduction at the beginning of the dark phase (Fig. 8). Whether other KChIPs participate in the 110 kDa band observed in Southwestern analysis is presently unknown, although formation of multimers has also been shown recently for KChIP-1 (Chang et al., 2003). Thus, a clock-dependent posttranslational modification of DREAM/KChIPs tetramers is responsible for the regulation of DRE binding activity. Whether this involves proteolysis of DREAM/KChIP proteins remains to be studied further.

cAMP-dependent posttranslational processing of DREAM/KChIP proteins

To further understand the molecular mechanisms controlling the rhythmic changes of DREAM/KChIP proteins in the pineal gland, we mimicked the cAMP stimulation that occurs in the pineal gland at the beginning of the dark phase of the photoperiod by intraperitoneal administration of the β-adrenergic receptor agonist isoproterenol (Drijfhout et al., 1996). Interestingly, administration of isoproterenol at 11:00 A.M. resulted in the appearance of the same group of fast-migrating DREAM/KChIP immunoreactive bands in pineal extracts prepared 1 hr after the injection (Fig. 7*B*). Detection of DREAM processed bands in pineal extracts 1 hr after isoproterenol was also observed with Ab 668 (data not shown). Thus, the clock-related posttranslational modification of DREAM/KChIP in pinealocytes appears to be cAMP-dependent and regulated by external inputs to the pineal gland.

Discussion

A highly dynamic network of transcription factors with activity that is regulated both at the transcriptional level and posttranslationally drives rhythmic gene expression in the pineal gland (Foulkes et al., 1997; Li et al., 1998; Hastings, 2000). Nocturnal norepinephrine release has been shown to trigger CREB phosphorylation (Roseboom and Klein, 1995; Tamotsu et al., 1995; Maronde et al., 1999; Shaywitz and Greenberg, 1999) and cAMP-dependent induction of AA-NAT activity, which is blocked by the PKA antagonist (R_p)-8-CPT-cAMP-S (Roseboom and Klein, 1995). Similarly, the cAMP-dependent regulation of the P2 *ICER* promoter has been demonstrated, and ICER is involved in turning off cAMP-dependent transcription at the end of subjective night (Stehle et al., 1993; Pfeffer and Stehle, 1998; Maronde et al., 1999). Nevertheless, disruption of a CRE site in the *AA-NAT* promoter by site-directed mutagenesis did not lead to a complete inhibition of responsiveness to cAMP stimulation (Baler et al., 1997). In addition, Fra-2 acts as a repressor of *AA-NAT* gene expression through binding to an AP-1 site present in its promoter (Baler et al., 1997). Importantly, in *CREM* knock-out mice lacking ICER proteins as well as in transgenic rats designed to suppress Fra-2 expression specifically in the pineal gland, the rhythmic expression of AA-NAT persists (Foulkes et al., 1996; Smith et al., 2001). Our study revealed that all four DREAM/KChIP proteins are expressed in pineal gland and retina and, indistinguishably, function as transcriptional repressors of the *ICER*, *AA-NAT*, and *fra-2* genes. Interestingly, site-directed mutagenesis identified the cooperative function of both DRE sites flanking the transcription start site in the repression by DREAM of the AA-NAT promoter. Moreover, supporting a role of DREAM/KChIP in pineal function, we found that DRE binding activity undergoes a circadian regulation with a peak during the light period of a typical light/dark cycle in pineal gland and retina. Interestingly, application of constant darkness for 10 d reduced DRE binding activity but did not alter its circadian pattern, indicating that DREAM/KChIP is under the control of an endogenous clock. The kinetics of DRE binding activity is highly suggestive of a physiological role in pineal function because the highest binding to DRE sites and hence DREAM/KChIP-mediated transcriptional repression coincides with the lowest level of *ICER*, *AA-NAT*, and *fra-2* expression (Foulkes et al., 1997; Li et al., 1998).

Levels of DREAM/KChIP mRNA appear to be constant during the circadian cycle, suggesting that their transcriptional impact is independent of *de novo* protein synthesis. Notably, it has been shown that the induction of the *AA-NAT* gene does not require ongoing synthesis of new proteins (Roseboom et al., 1996). Several mechanisms may contribute to the circadian rhythmicity of DRE binding activity in the pineal gland. First, clock-driven oscillations of cAMP levels in pinealocytes (Vanecek et al., 1985; Sugden et al., 1986; Nikaido and Takahashi 1989)

may directly account for the circadian changes in DREAM binding activity. During the day–night cycle, signals from the retina use a multisynaptic pathway to reach the pineal gland. The last step in this pathway is a rhythmic nocturnal norepinephrine input that is transduced by adrenergic receptors into intracellular increases of cAMP (Vanecek et al., 1985; Sugden et al., 1986; Sugden et al., 1987; Moore, 1996). cAMP-dependent phosphorylation of CREM repressor isoforms α or ϵ results in an increased interaction with DREAM, the block of DREAM–DRE binding, and the derepression of the DRE site (Ledo et al., 2000). Blockage of the CREM–DREAM interaction using mutant EFmDREAML47,52V notably reduced transactivation of the AA-NAT reporter in C6 glioma cells after isoproterenol exposure. Whether a similar protein–protein interaction occurs between CREM and KChIP-1, -2, or -4 is presently unknown. Second, circadian variations in Ca^{2+} levels in pinealocytes have been shown to be the result of increased expression of the nonselective cationic channel I_{LOT} in chick pineal cells during the night (D'Souza and Dryer, 1996). Increased activity of I_{LOT} channels would facilitate Ca^{2+} entry through voltage-dependent calcium channels and unbinding of DREAM/KChIP from DNA. Interestingly, increased Ca^{2+} entry would in turn activate Ca^{2+}/calmodulin-dependent adenylate cyclase, which increases cAMP levels (Choi et al., 1992). Thus, cyclic changes in intracellular Ca^{2+} and cAMP levels provoke a coordinated and rhythmic regulation of the affinity of DREAM/KChIPs for DRE sites in target genes (Carrion et al., 1999). Third, the change in DRE binding activity may reflect a change in the posttranslational processing of the DREAM/KChIP proteins. Interestingly, posttranslational modifications have been shown to regulate the activity of several clock-related proteins *in vivo* (Lee et al., 2001). Thus, phosphorylation of clock proteins modifies subcellular localization and the interactions between mammalian Per (Period), CLOCK (circadian locomotor output cycles Kaput), and BMAL (brain muscle ARNT-like) proteins, which are normally present in the nucleus as multimeric complexes containing also cryptochromes and casein kinase Iϵ (Lee et al., 2001). Interestingly, it has been proposed that DREAM binds DNA as a tetramer (Carrion et al., 1999), and phosphorylation by downstream kinases of the PI3 kinase pathway modifies DREAM binding activity (Sanz et al., 2001). Whether a clock-dependent change in the phosphorylation status of specific residues in DREAM is the signal that controls tetramer formation and function is presently unknown. Fourth and last, the circadian variation in DREAM activity may reflect a change in the stability of the DREAM/KChIP proteins. Indeed, the reduction in DRE binding activity at the beginning of the subjective night coincides with the appearance of a group of faster migrating DREAM/KChIP bands on Western blots. It is noteworthy that a proteolytic pathway could represent a rapid mechanism to eliminate DNA-bound DREAM/KChIP proteins necessary to derepress the target genes *ICER*, *AA-NAT*, and *fra-2* and to increase melatonin synthesis in the pineal gland as soon as the light photoperiod is finished. A light-dependent activation of the proteosomal degradation pathway has been involved in the rapid degradation of AA-NAT protein in the fish pineal organ (Falcon et al., 2001) as well as in the mammalian retina (Fukuhara et al., 2001). Moreover, clock-dependent degradation of Per proteins in the mammalian circadian clock by Slimb, an F-box/WD40-repeat protein of the ubiquitin ligase Skp1/cullin/F-box protein complex, is preceded by the hyperphosphorylation of Per by casein kinase Iϵ and δ, the mammalian functional homologs of the *Drosophila* Timeless protein (Akashi et al., 2002; Grima et al., 2002; Ko et al., 2002). Light-dependent degradation of Timeless follows after phosphorylation of tyrosine residues and ubiquitinization (Naidoo et al., 1999) and is regulated by the levels of the putative circadian cryptochrome cryptochrome (Lin et al., 2001). Importantly, a Ca^{2+}-dependent proteolytic processing of DREAM has been described at Ser63 by the action of caspase-3 (Choi et al., 2001) and requires phosphorylation of DREAM by casein kinase I at Ser63 (Choi et al., 2003). The appearance of several faster migrating DREAM/KChIP immunoreactive bands, as detected by either of the polyclonal peptide-specific antibodies 668 or 671, suggest that the fast migrating bands are antigenically related to DREAM/KChIP. Whether the circadian changes in DRE binding activity involve caspase 3 or other proteases is presently unknown. Taken together, these data suggest that the circadian fluctuation of DRE binding activity may be caused by oscillations in the intracellular availability of DREAM/KChIP, which seems to fulfill essential requirements for a circadian oscillator (Hardin et al., 1992; Pittendrigh, 1993; Sassone-Corsi, 1994).

Circadian fluctuations in DRE binding activity are also observed in the retina, whereas it is constant in the cerebellum. Changes in DRE binding activity in retinal ganglion cells could be related to a circadian modulation of intracellular Ca^{2+} levels. Several observations support this idea. (1) In retinal cells from *Bulla gouldiana* and *Aplysia californica*, clock-regulated K^+ currents control basal membrane potentials and impose larger rises in intracellular Ca^{2+} during the subjective night (Michel et al., 1993; Geusz et al., 1994). (2) Light pulses fail to produce phase shifts in ocular rhythms at low extracellular Ca^{2+} concentrations or in the presence of the Ca^{2+} channel antagonist, nickel chloride (Colwell et al., 1994). (3) Serotonin-mediated ocular phase shifts in *A. californica* involve an increase in ocular cAMP levels (Colwell et al., 1994). (4) It has been reported recently that a circadian oscillator in cultured chick cones modulates the affinity of cGMP-gated ion channels for cGMP and the nature of the signal transduction cascade after dopamine D2 receptor activation (Ko et al., 2003). Taken together, our results support a Ca^{2+}-mediated role for DREAM/KChIP in *AA-NAT* gene expression in retina.

A central role of changes in the intracellular Ca^{2+} concentration has also been reported for the oscillatory activity of neurons in the suprachiasmatic nucleus (Pennartz et al., 2002). In this case, a diurnal increase of L-type Ca^{2+} currents in suprachiasmatic neurons contributes to the generation of spontaneous oscillations in the membrane potential that are tightly coupled to spike generation only during daylight. Interestingly, a common mechanism of circadian regulation has been suggested for the *Per1* and *AA-NAT* genes in the retina (Fukuhara et al., 2000). Whether *per*, *clock*, *BMAL*, or additional genes are targets for DREAM/KChIP repression in suprachiasmatic neurons, retinal ganglion cells, or pinealocytes remains to be investigated.

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