pCUB-6 (*23*) was inserted between the Bcl I sites within the Bgl II–Sal I fragment of *INT1* cloned into the Bam HI–Sal I sites of pUC18 to yield the *int1*-disruption plasmid pCG05. pCG05 digested with Nsp V and Hind III was used to transform the Ura⁻ strain CAI4 (*23*) using a modified spheroplast transformation method [M. B. Kurtz, M. W. Cortelyou, D. R. Kirsch, *Mol. Cell. Biol.* **6**, 142 (1986)]. Ura⁺ transformants were screened by polymerase chain reaction (PCR) and Southern (DNA) blot analysis for integration of the disrupted *INT1* fragment. Southern blot analysis of transformant CAG1 confirmed that a single *hisG-CaURA3-hisG* cassette had integrated into the *INT1* locus. A Ura⁻ segregant (CAG2) was selected on 5-fluoroorotic acid (5-FOA); PCR and Southern blot analysis confirmed that CAG2 had lost *CaURA3* by recombination between the tandem *hisG* repeats. CAG2 was used in an identical round of transformation and 5-FOA selection; PCR and Southern blot analysis confirmed disruption of the second copy of *INT1* in the Ura⁺ *int1/int1* homozygote (CAG3) and Ura⁻ *int1/int1* homozygote (CAG4). Northern (RNA) blot analysis confirmed that *INT1* transcript was expressed in CAI4 and CAG1 and was absent in CAG3.

14. *INT1* was reintegrated into the genome of CAG4 by transforming with a Bgl II restriction digest mixture of pDF118, which contains the *INT1* Bgl II–Sal I fragment inserted between the Bam HI and Sal I sites of pVEC (provided by P. Magee and B. Magee, University of Minnesota). The presence of *INT1* in Ura⁺ transformants was confirmed by PCR. Conventional Southern blots as well as Southern blots of chromosomes separated by pulsed-field gel electrophoresis indicated that *INT1-CaURA3* reintegrated within the *int1* locus on chromosome 5.
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25. Protease-deficient *S. cerevisiae* strains YMH6 [BJ3501 (*MATα pep4::HIS3 prb1 his3-ura3 can1 gal2*) transformed with pCG01 (*3*)] and YMH7 [BJ3501 transformed with the *GAL1-10* vector pBM272 (*24*)] were grown for 8 hours at 30°C in SD minus uracil and containing 2% raffinose and 2% galactose. Cells were washed three times with 10 ml of phosphate-buffered saline (PBS) and resuspended in 400 μl of 50 mM $NaHCO_3/Na_2CO_3$ (pH 8.5) containing sulfo-NHS-LC-biotin (1 mg/ml). Cells were resuspended by vortexing for 1 s and were incubated on ice for 120 min, with periodic mixing. Cells were then incubated on ice for 1 hour with 1 ml of 100 mM tris-HCl (pH 8) to inactivate unreacted biotin. Cells were washed three times with PBS, pelleted, and resuspended in 500 μl of RIPA buffer [50 mM tris (pH 8.0), 300 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS] containing protease inhibitors [1 mM benzamidine, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.1 mM TLCK, 0.1 mM TPCK, leupeptin (3 μg/ml), and pepstatin A (3 μg/ml)] and disrupted by vortexing for 10 min at 4°C with an equal volume of 500-μm glass beads. The resulting lysate was cleared by centrifugation at 10,000*g*, 4°C, for 30 min. Protein (9 mg) from each lysate was incubated at 0°C with 8 μg of a mixture of affinity-purified antibodies (raised in rabbits against four peptides from the translated Int1p sequence) for 60 min in a total of 800 μl of 0.33× RIPA buffer (final concentration of antibodies, 10 μg/ml). An equal amount of lysate was incubated with a 1:850 dilution of Rap1p antiserum containing ~8 μg of Rap1p antibody [S. Enomoto, P. D. McCune-Zierath, M. Gerami-Nejad, J. Berman, *Genes Dev.* **11**, 358 (1997)] under the identical conditions. An equal volume of a 10% slurry of protein A–Sepharose Fast Flow in 0.33× RIPA buffer with protease inhibitors was added to each sample and rotated for 60 min at 4°C. Samples were pelleted, and the bead pellet was washed three times with 500 μl of 0.33× RIPA buffer containing protease inhibitors. SDS-PAGE reducing buffer [20 mM tris (pH 6.8), 10% glycerol, 0.005% bromphenol blue, 2% SDS, and 5% β-mercaptoethanol; 200 μl] was added to the pellets and the samples were boiled for 5 min at 100°C. Samples were frozen at −80°C, thawed, and separated on a 7.5% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane that was incubated either with horseradish peroxidase (HRP)–avidin (1:50,000 dilution) or with antibodies (500 ng/ml), washed, and incubated with HRP-conjugated goat antibody to rabbit IgG. Blots were developed with the Supersignal CL-HRP Substrate System.
26. [^{35}S]Methionine (specific activity, 800 Ci/mmol) was diluted to a final concentration of 8.7 μM methionine and added to exponentially growing yeast cells. Unlabeled yeast cells used to calculate nonspecific adhesion were grown identically. Yeast cells were harvested in midexponential phase, incubated for 1 hour at 37°C with monolayers of human cervical carcinoma epithelial (HeLa) cells, and washed to remove nonadherent cells before release of the monolayer for scintillation counting. Specific adhesion was calculated as the difference between total adhesion [(cpm adherent cells/cpm total cells) × 100] and nonspecific adhesion, the latter measured in the presence of a 100-fold excess of unlabeled yeast cells as described [K. S. Gustafson, G. M. Vercellotti, C. M. Bendel, M. K. Hostetter, *J. Clin. Invest.* **87**, 1896 (1991)]. For antibody blockade, yeast cells were preincubated with nonimmune rabbit IgG (1 mg/ml) or Int1p antibody UMN13 (1 mg/ml). IgG fractions of rabbit antisera or nonimmune serum were prepared by elution at pH 3.0 from a protein A matrix. Statistical significance was determined by analysis of variance followed by Fisher's protected least significant difference (StatView, version 4.51). Significance for all statistical comparisons was set at $P < 0.05$. Results are reported as specific adhesion, expressed as means ± SE ($n \geq 3$).
27. To induce hyphal growth, we grew *C. albicans* strains to stationary phase in SD minus uracil at 30°C and then inoculated them on milk-Tween agar [S. Jitsurong, S. Kiamsin, N. Pattararangrong, *Mycopathologia* **123**, 95 (1993)] or on Spider medium (*16*) with 1.35% agar, followed by incubation for 5 days at 30° and 37°C, respectively, to yield approximately 100 colonies per plate.
28. *Candida albicans* strains were cultured in SD minus uracil at 30°C. Midexponential-phase cultures were harvested, washed twice with distilled water, and diluted to a final concentration of 10^7 cells/ml. The virulence of the strains was tested in a normal mouse model system as described [J. M. Becker, L. K. Henry, W. Jiang, Y. Koltin, *Infect. Immun.* **63**, 4515 (1995)]. Male ICR mice (22 to 25 g, Harlan Sprague-Dawley) were housed five per cage; food and water were supplied ad libitum, according to NIH guidelines for the ethical treatment of animals. Mice were inoculated with 10^6 cells in a final volume of 100 μl through the lateral tail vein. The genotype of recovered isolates was verified by PCR, using primers specific for the *INT1* locus.
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Melatonin Production: Proteasomal Proteolysis in Serotonin *N*-Acetyltransferase Regulation

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The nocturnal increase in circulating melatonin in vertebrates is regulated by 10- to 100-fold increases in pineal serotonin *N*-acetyltransferase (AA-NAT) activity. Changes in the amount of AA-NAT protein were shown to parallel changes in AA-NAT activity. When neural stimulation was switched off by either light exposure or L-propranolol–induced β-adrenergic blockade, both AA-NAT activity and protein decreased rapidly. Effects of L-propranolol were blocked in vitro by dibutyryl adenosine 3′,5′-monophosphate (cAMP) or inhibitors of proteasomal proteolysis. This result indicates that adrenergic-cAMP regulation of AA-NAT is mediated by rapid reversible control of selective proteasomal proteolysis. Similar proteasome-based mechanisms may function widely as selective molecular switches in vertebrate neural systems.

An important component of vertebrate circadian and seasonal physiology is a large nocturnal increase in circulating melatonin (*1*), which results from an increase in pineal serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase) (AA-NAT) activity. High nocturnal values decrease rapidly (half-life ~3.5 min) after light exposure in the middle of the night (*2*). These changes are regulated by an adrenergic-cAMP mechanism (*3*), but are otherwise poorly understood.

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Analysis of pineal immunoreactive AA-NAT protein (irAA-NAT) (*4*) indicated a parallel change with AA-NAT activity (*5*) over a 24-hour period (Fig. 1). Exposure to constant light blocked the nocturnal increase (Fig. 1), and light exposure or β-adrenergic blockade in the middle of the night rapidly reduced AA-NAT activity and irAA-NAT (Figs. 1 and 2). This decrease was not due to inhibition of translation, which had only a minor effect (6) (Fig. 2). β-Adrenergic stimulation with isoproterenol blocked the rapid light-induced reduction in AA-NAT activity and irAA-NAT (Fig. 2); this treatment also rapidly reversed the effects of acute light exposure on irAA-NAT (Fig. 2), through a mechanism that required protein synthesis (*7*) (Fig. 2). These changes were not due to changes in mRNA levels (*8*) nor were they due to a reversible posttranslational process, because large changes in enzyme activity were always associated with parallel changes in enzyme protein (Figs. 1 to 3). Therefore, changes in AA-NAT activity appeared to result from changes in AA-NAT protein levels.

In vitro studies confirmed that occupancy of the pinealocyte β-adrenergic receptor maintained elevated levels of irAA-NAT because β-adrenergic agonists increased irAA-NAT, and subsequent adrenergic blockade with L-propranolol reversed these effects (Fig. 3). Cyclic AMP appears to be involved because dibutyryl cAMP increased irAA-NAT (*9*) and prevented L-propranolol–induced reduction in irAA-NAT (Fig. 3) (*10*).

The L-propranolol–induced reductions in AA-NAT activity and irAA-NAT were prevented by each of six proteasomal protease inhibitors, including lactacystin, calpain inhibitor I, and analogs of these compounds (Fig. 3) (*11*, *12*). This demonstrates that AA-NAT protein is destroyed by the proteasome and suggests that an adrenergic-cAMP mechanism stabilizes AA-NAT ac-

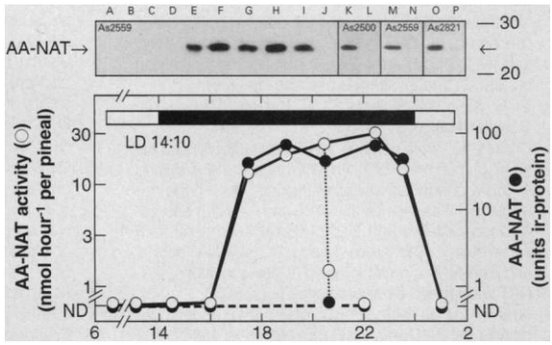
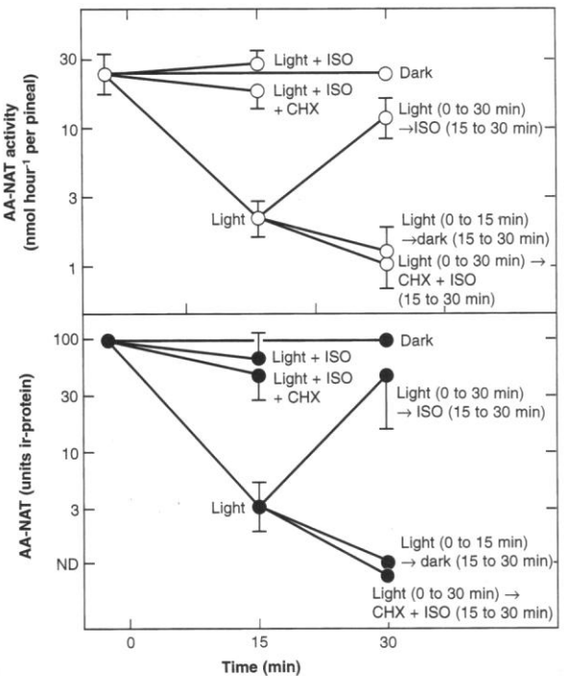


Fig. 1. Rat pineal AA-NAT protein and activity change in parallel. (Bottom) Rats were entrained to a light: dark (LD) 14:10 lighting cycle (light or dark bars) and pineal glands were obtained as indicated. One group of animals was not exposed to darkness on the day tissue was collected (dashed line), and another group was exposed to light for a 15-min period in the middle of the night (dotted line). Samples of pineal glands from two or three animals were analyzed for AA-NAT activity (*5*) (open circles, solid and dashed lines) or irAA-NAT (*4*) with As2559 (closed circles, solid and dashed lines); similar results were obtained with As2500 (not shown). (Top) Protein immunoblot analysis of irAA-NAT. Hour of harvest (Zeitgeber time, ZT): (A) 6, (B) 13, (C) 14.5, (D) 16, (E) 17.5, (F) 19, (G) 20.5, (H) 22, (I) 23.5, and (J) 1. Tissue obtained at ZT 21 with (+) or without (−) a 15-min light exposure: (K) −, (L) +; (M) −, (N) +; (O) −, (P) +; the antiserum used for each analysis is indicated. In three independent experiments, AA-NAT activity and irAA-NAT were either undetectable or less than twice background in samples obtained during the day or after several hours of light [$P < 0.005$, $n = 10$, Mann-Whitney U test (*21*)]. Exposure to light (15 min) at night significantly suppressed AA-NAT activity and protein values ($P < 0.005$, $n = 5$, Mann-Whitney U test). The SEM of the data presented is less than 30%.



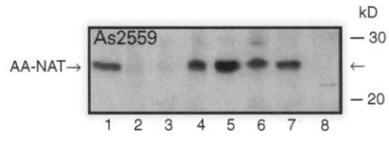


Fig. 2. In vivo β-adrenergic control of irAA-NAT. (Top) Animals were treated as indicated between 5 and 7 hours after lights were turned off and then killed under dim red light. AA-NAT activity (*5*) was measured and irAA-NAT (*4*) was determined with As2559 and As2500. Data shown are the pooled results of experiments performed on three sets of animals. (Bottom) Treatment groups for immunoblot (lane number): (1) dark, no treatment; (2) dark, then light for 15 min; (3) dark, L-propranolol [5 mg/kg, intraperitoneal (ip)] for 15 min; (4) dark, cycloheximide (CHX, 20 mg/kg ip) for 20 min; (5) dark, isoproterenol (ISO, 5 mg/kg, ip), then exposure to light for 15 min; (6) as in (5) except that cycloheximide was injected 5 min before isoproterenol; (7) dark, then 15 min of light, then isoproterenol (5 mg/kg) in the presence of light for an additional 15 min; (8) same as (7) except that during the initial 15-min period of light exposure, rats were injected with cycloheximide (20 mg/kg). When animals were first exposed to 15-min light at night and then placed in the dark for an additional 15 min, irAA-NAT was not detectable and AA-NAT was barely detectable—consistent with previous reports on AA-NAT activity (*7*). AA-NAT activity and irAA-NAT values in groups 2 and 3 are statistically lower than values at night [$P < 0.025$, $n = 4$, Mann-Whitney U test (*21*)], group 8 is not statistically different from group 2 ($P = 0.09$, $n = 3$, Student's t test), and groups 5, 6, and 7 are different from group 2 (AA-NAT activity: $P < 0.001$; and irAA-NAT: $P < 0.025$; $n = 3$, Student's t test). IrAA-NAT is either undetectable or barely detectable in three experiments in groups 2, 3, and 8, as determined with either As2500 or As2559. Bars indicate SEM.

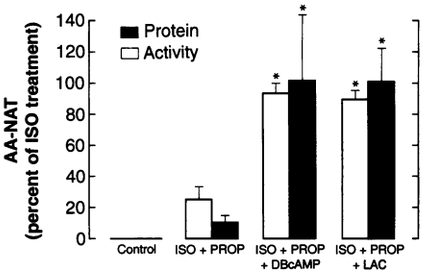


Fig. 3. L-Propranolol–induced decrease in irAA-NAT and AA-NAT activity is prevented by dibutyryl cAMP and lactacystin. Pinealocytes (*20*) were incubated for 24 hours and then treated with isoproterenol (ISO, 100 nM, 5 hours; not shown) or water (control). All treatment groups shown received L-propranolol (PROP, 10 μM, 4 to 5 hours); in addition, one group was also treated with dibutyryl cAMP (DBcAMP, 1 mM, 3.5 to 5.0 hours) and another with lactacystin (LAC, 50 μM, 2.0 to 5.0 hours). IrAA-NAT protein was detected as labeled immunoprecipitated protein (*11*). AA-NAT activity and irAA-NAT values after 4 and 5 hours of ISO treatment were similar (data not shown). Each determination of activity and protein was based on a pool of cells obtained from two wells; results were normalized to the 4-hour ISO treatment group value (100%) ($n = 4$). *$P \leq 0.001$ compared with ISO + PROP alone for AA-NAT activity; $P < 0.025$ for irAA-NAT (*21*).

tivity by preventing proteasomal proteolysis of AA-NAT protein. A reasonable hypothetical mechanism underlying the action of cAMP is inhibition of proteasomal targeting by ubiquitination (*13*).

Cyclic AMP appears to regulate mammalian AA-NAT activity through complementary stimulation of transcription and inhibition of proteasomal proteolysis of AA-NAT protein. Although transcriptional control is not important in all vertebrates (*14*), inhibition of AA-NAT proteasomal proteolysis may be conserved (*13*, *15*). β-Adrenergic agents may act in a similar manner to control degradation of proteins in other tissues (*13*, *16*).

These findings indicate that proteasomal proteolysis has a role in neural regulation in vertebrates, as in invertebrates (*17*). Our results indicate that receptor-regulated proteasomal proteolysis can function as a precise, selective, and very rapid neural switch. In the pineal gland, this mechanism regulates the conversion of minute-to-minute changes in environmental input into profound global changes in physiology (*18*). Such neurally regulated and selective proteasomal proteolysis may play a similarly important role in other aspects of vertebrate physiology and behavior.

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9. Norepinepherine, isoproteronol, dibutyryl cAMP, and 8-bromocyclic AMP reproducibly increased AA-NAT activity (*2*) and irAA-NAT (*4*) in parallel in pineal organ culture (*2*) and in isolated pinealocytes (*20*).
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11. Cells (~10^6) were homogenized in 200 μl of 50 mM tris (pH 7.5), 500 mM NaCl, 1 mM dithiothreitol (DTT), and 100 μM phenylmethylsulfonyl fluoride (PMSF); the homogenate was then centrifuged, and AA-NAT was immunoprecipitated with 2 μl of As2821 (2 hours, 4°C) and protein A–Sepharose (1 hour, 4°C). AA-NAT activity and irAA-NAT are stable under these conditions. AA-NAT was labeled by incubation (1 hour, 30°C, final volume = 20 μl) with 10 μCi of [^{32}γ]ATP (adenosine triphosphate), 100 mM NaCl, 50 mM tris-HCl (pH 7.5), 1 mM DTT, 10 mM MgCl, and 33 U of protein kinase A (PKA, Promega); radioactive bands were analyzed and quantitated as described (*4*). This technique is quantitative within the range of AA-NAT values used in these experiments.
12. Rat pinealocytes or pineal glands were treated in experiments similar to those in Fig. 3. Unless otherwise indicated, the concentration of all protease inhibitors was 100 μM. The following protease inhibitors (targeted class) were ineffective: (serine) PMSF (Sigma), leupeptin (ICN), aprotinin (ICN), *N-p*-tosyl-L-phenylalanine chloromethyl ketone (Calbiochem); (lysosomal) chloroquine (0.2 mM, Sigma); (aspartic) pepstatin (ICN); (metallo) ethylenediamine tetraacetic acid (Sigma); (cysteine) *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)butane (Sigma), calpeptin (Calbiochem), and α-*N*-acetyl-leucine-leucine-methioninal (Calbiochem). The following protease or proteasome inhibitors preserved more than 50% of the activity or AA-NAT protein compared with drug alone: calpain inhibitor I and Mg115 [K. L. Rock *et al.*, *Cell* **78**, 761 (1994)], Mg132 (V. J. Palombella, O. J. Rando, A. L. Goldberg, T. Maniatis, *ibid.*, p. 773), Z-leucine-leucine-leucine-vinyl sulfone [M. Bogyo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6629 (1997)], lactacystin [G. Fenteany *et al.*, *Science* **268**, 726 (1995)], and β-clastolactacystin [L. R. Dick, L. Cruikshank, F. D. Grenier, S. L. Melandri, R. L. Stein, *J. Biol. Chem.* **271**, 7273 (1997)].
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Regulation of Flowering Time by *Arabidopsis* Photoreceptors

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The shift in plants from vegetative growth to floral development is regulated by red–far-red light receptors (phytochromes) and blue–ultraviolet A light receptors (cryptochromes). A mutation in the *Arabidopsis thaliana CRY2* gene encoding a blue-light receptor apoprotein (CRY2) is allelic to the late-flowering mutant, *fha*. Flowering in *cry2/fha* mutant plants is only incompletely responsive to photoperiod. Cryptochrome 2 (cry2) is a positive regulator of the flowering-time gene *CO*, the expression of which is regulated by photoperiod. Analysis of flowering in *cry2* and *phyB* mutants in response to different wavelengths of light indicated that flowering is regulated by the antagonistic actions of phyB and cry2.

The blu e–ultraviolet A (UV-A) light receptors, cryptochromes, and red–far-red light receptors, phytochromes, mediate light-regulated plant growth and development from seed germination to flower initiation. Phytochrome A (phyA), phytochrome B (phyB), and cryptochrome 1 (cry1) function in both early photomorphogenesis (*1*–*5*) and floral induction (*6*–*9*). We report that in *Arabidopsis thaliana*, the