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# Mitogen-activated protein kinase phosphatase-1 (MKP-1): >100-fold nocturnal and norepinephrine-induced changes in the rat pineal gland

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**Abstract The norepinephrine-driven increase in mitogen-activated protein kinase (MAPK) activity is part of the mechanism that regulates arylalkylamine *N*-acetyltransferase (AA-NAT) activity in the rat pineal gland. We now report a marked nocturnal increase in the expression of a MAPK phosphatase, MAP kinase phosphatase-1 (MKP-1), that was blocked by maintaining animals in constant light or treatment with propranolol. MKP-1 expression was regulated by norepinephrine acting through both α- and β-adrenergic receptors. These results establish a nocturnal increase in pineal MKP-1 expression that is under the control of a photoneural system. Because substrates of MKP-1 can influence AA-NAT activity, our findings suggest the involvement of MKP-1 in the regulation of the nocturnal AA-NAT signal.**


*Keywords:* MAP kinase phosphatase-1; Pineal; Mitogen-activated protein kinase; Arylalkylamine-*N*-acetyltransferase; Circadian; Adrenergic; Photoneuroendocrinology

## 1. Introduction

The rat pineal gland is stimulated by the nightly release of norepinephrine (NE) from the sympathetic nerve terminals, resulting in the activation of both α- and β-adrenergic receptors [1]. Stimulation of β-adrenergic receptors produces a 4- to 10-fold increase in cAMP and cGMP accumulation [1]. Stimulation of α-adrenergic receptors, which has no apparent independent effect on cAMP or cGMP accumulation, potentiates the β-adrenergic response resulting in a 50- to 100-fold increase in cAMP and cGMP accumulation [1–5]. cAMP acts through multiple mechanisms to increase arylalkylamine-*N*-acetyltransferase (AA-NAT) activity, the rate-controlling enzyme in melatonin biosynthesis [6–9].

In contrast to the established role of cAMP in pineal physiology, the importance of NE-stimulated cGMP accumulation in this tissue is only now beginning to become clear. The nocturnal activation of p42/44 mitogen-activated protein kinase ($p42/44^{MAPK}$) and its downstream kinase, the 90 kDa ribosomal S6 kinase, is primarily regulated by the NE → cGMP pathway [10–13]. Moreover, the NE activation of $p42/44^{MAPK}$ is modulated by the state of $p38^{MAPK}$ activation [14] and activation of AA-NAT activity by NE is modulated by MAPKs, specifically $p42/44^{MAPK}$ and $p38^{MAPK}$ [13,15]. Likewise, MAPK phosphatases are likely to play an important role in the regulation of pineal function, because the magnitude and duration of signaling through MAPKs reflects a balance between the upstream activators and a complex regulatory network of protein phosphatases [16–19].

One of these phosphatases, a dual specificity phosphatase, MAPK phosphatase-1 (MKP-1), is of special interest; MKP-1 is an important regulator of MAPK activity in a wide array of physiological processes [16–20]. Results from microarray analysis of pineal glands suggest that *MKP-1* mRNA increases when AA-NAT activity increases [21,22]; this is of interest because it points to the possibility that MKP-1 may play a role in controlling melatonin production through control of MAPKs. Accordingly, in the studies presented here, the physiological and neural regulation of *MKP-1* mRNA and protein were investigated.

## 2. Materials and methods

### 2.1. Materials

NE, isoproterenol, phenylephrine, prazosin and propranolol were obtained from Sigma Aldrich Co. (St. Louis, MO). Polyclonal anti-MKP-1 serum was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) serum was obtained from Ambion Inc. (Austin, TX). Polyclonal anti-$AA\text{-}NAT_{25–200}$ (AB3314) was raised in rabbit against a rat peptide $rAA\text{-}NAT_{25–200}$ [15]. All other chemicals were of the purest grades available commercially.

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*Abbreviations:* MKP-1, MAP kinase phosphatase-1; NE, norepinephrine; AA-NAT, arylalkylamine-*N*-acetyltransferase; MAPK, mitogen-activated protein kinase; ZT, zeitgeber time; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; SCN, suprachiasmatic nucleus

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*2.2. Animal handling and pineal gland isolation*

All procedures were reviewed and approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Sprague Dawley rats (male; weighing 150 g) were obtained from the University of Alberta animal unit. Animals were housed under a lighting regimen providing 12 h of light every 24 h with lights on at 06:00 h [zeitgeber time zero (ZT0)]. For pinealocyte cell culture, animals were killed 3 h after the onset of light by decapitation, and pineal glands were removed and stored in ice-cold PBS until trypsinization. For analysis of the pineal glands, groups of animals ($n = 3$–6 per groups) were killed at various time points as indicated, with one group kept in a lighted environment for an additional 3 h after the onset of darkness, one group kept in constant darkness for 48 h, one group exposed to light at ZT16, and another group injected intraperitoneally with aqueous propranolol solution (1 mg/kg body weight) 1 h prior to the onset of darkness. Pineal glands were cleansed in ice cold PBS, frozen on dry ice and stored at −75 °C until preparation for Western blot analysis or RNA extraction. When animals were killed during the dark period, illumination was provided by a dim red light (Bright Lab Jr Safelight, CPM Inc, Dallas, TX).

*2.3. Preparation of cultured pinealocytes and drug treatment*

Pinealocytes were prepared by trypsinization of freshly dissected rat pineal glands as described previously [23]. Cells were suspended in DMEM containing 10% fetal calf serum and maintained before the experiments at 37 °C for 24 h in a mixture of 95% air and 5% $CO_2$. Cells ($0.8 \times 10^5$ cells/0.3 ml DMEM) were treated with different drugs for the time periods indicated. Treated cells were collected by centrifugation (2 min, $12\,000 \times g$). Samples for Western blot analysis were boiled for 5 min in 1x sample buffer and stored at −20 °C until analysis.

*2.4. Preparation of pineal glands for western blot analysis*

Frozen pineal glands were homogenized in buffer (20 mM Tris–HCl; 2 mM EDTA; 0.5 mM EGTA; 2 mM phenylmethylsulfonylfluoride; 1 μg/ml each of aprotinin, leupeptin, and pepstatin (Sigma Aldrich Co.); 1 mM sodium vanadate; and 1 mM sodium fluoride, pH 7.5) by sonication. An aliquot of the homogenate was used for protein determination [24]. After adjusting for protein content, samples were mixed with 2× sample buffer, boiled for 5 min, and stored at −20 °C until electrophoresis. SDS–PAGE was performed according to the procedure of Laemmli [25] and described in our previous studies [10–15] using 10% acrylamide and 1 mg/ml sodium dodecyl sulfate (Mini-Protein II gel system, Bio-Rad Laboratories, Inc., Hercules, CA).

*2.5. RT-PCR*

Total RNA was isolated from both cultured pineal cells and intact frozen glands using RNeasy RNA extraction kits (Qiagen Inc., Valencia, CA). Frozen pineal glands were thawed in the supplied lysis buffer and homogenized with a plastic homogenizer before proceeding with RNA extraction. First strand cDNA was synthesized from the isolated RNA using an Omniscript reverse-transcriptase kit (Qiagen Inc., Valencia, CA) with an oligo-dT primer. For gland extracts, 1 μl of a 1:10 dilution of cDNA in PCR-grade water was used as template for PCRs. For cell extracts, 3 μl of a 1:10 dilution of cDNA was used. PCR was performed in a 29.3 μl reaction mixture containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.75 U Taq polymerase (Perkin–Elmer Cetus, Emeryville, CA) and 1 μM each of the two primers. PCRs were as follows: denaturing for 1 min at 94 °C, annealing for 1 min at 63 °C, and extension for 1 min at 72 °C. Initial denaturing and final extension were both 5 min in duration. Cycle numbers varied slightly between cell preparations, but in general, 22 cycles were used to amplify *GAPDH*, and 25 cycles to amplify both *MKP-1* and *AA-NAT* mRNAs. All reaction sets included water blanks as negative controls. Amplified products were separated on ethidium bromide-stained 1.5% agarose gels. PCR products were confirmed by sequencing. All primer sequences were designed using rat gene cDNA sequences obtained from BLAST [26] and primers were selected from 3′ regions as outlined in [27]. Primers used are as follows: *MKP-1*: left primer: 5′-CTG CTT TGA TCA ACG TCT CG-3′; right primer: 5′-AAG CTG AAG TTG GGG GAG AT-3′; *AA-NAT*: left primer: 5′-GGT TCA CTT TGG GAC AAG GA-3′; right primer: 5′-GTG GCA CCG TAA GGA ACA TT-3′. Sequences of the GAPDH primers used were previously described [28].

Real time RT-PCR based on SYBR green fluorescence (Molecular Probes, Inc., Eugene, OR) was used to provide a relative quantification of *MKP-1* and *AA-NAT* [29]. This was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Template cDNA samples were diluted 10-fold and serial 4-fold dilutions (from 1:4 to 1:65 536) were analyzed on a 384 well plate. cDNA template samples were standardized by their $OD_{260}$ readings and incorporation curves for *MKP-1* and *AA-NAT* were normalized to a standard curve constructed for amplification of *GAPDH* for each sample.

*2.6. Results and statistical analysis*

For quantitation of RT-PCR analyses, gel images were acquired with Kodak 1-D software on a Kodak 2000R imaging station (Eastman Kodak Co., Rochester, NY). For analyses of Western blots, exposed films were scanned and band densitometry of acquired images was performed with Kodak 1-D software. For comparison, densitometric and real time RT-PCR values were normalized so that peak values equal 100%. Data are presented as means ± S.E.M. from at least three independent experiments. Statistical analysis involved either a paired $t$ test or ANOVA with the Newman–Keuls test. Statistical significance was set at $P < 0.05$.

## 3. Results

*3.1. MKP-1 expression is induced at night in the rat pineal gland (Fig. 1)*

A rapid nocturnal increase in pineal *MKP-1* mRNA levels occurred with peak values observed at ZT15. The increase in *MKP-1* mRNA occurred more rapidly than that of *AA-NAT* mRNA. Between ZT15 and ZT19, *MKP-1* mRNA abundance rapidly declined; this differs from the nocturnal increase in *AA-NAT* mRNA, which exhibited a sustained elevation between ZT17 and ZT22. Real time RT-PCR analysis showed an ∼100-fold increase in *MKP-1* mRNA level at ZT15. At ZT18, *MKP-1* mRNA level was only 35% of the level at ZT15 ($P < 0.05$), while *AA-NAT* mRNA level was 3-fold higher ($P < 0.05$) (Fig. 1C). In the same experiment, MKP-1 protein increased with a peak at ZT15 followed by a rapid decline and the peak AA-NAT protein level occurred between ZT19 and ZT22 (Fig. 1B) as in previous studies [7,9].

*3.2. MKP-1 expression is reduced by injection of a β-blocker, is blocked by constant light and persists in constant darkness*

The role of adrenergic stimulation in the nocturnal induction of MKP-1 expression was investigated using treatments that reduce or block adrenergic stimulation of *AA-NAT* mRNA in the pineal gland [7], i.e., β-adrenergic blockade and constant light at night. Treatment with propranolol (1 mg/kg intraperitoneally) 1 h before onset of darkness reduced the nocturnal induction of *MKP-1* mRNA by ∼60% at ZT15; in the same experiment, this treatment reduced *AA-NAT* mRNA by ∼85% (Fig. 2A). Analysis of protein levels indicated that propranolol treatment had a small effect on pineal MKP-1 protein, but reduced AA-NAT protein by 75% (Fig. 2B). Whereas propranolol treatment produced partial inhibition of the increases in mRNA and protein, constant light treatment completely abolished the nocturnal induction of *MKP-1* and *AA-NAT* mRNA and protein. The nocturnal induction of MKP-1 expression in subjective night (ZT15) persisted in animals exposed to constant darkness for 48 h (Fig. 3).

*3.3. Neural stimulation of the pineal gland at night is blocked by exposure to light (Fig. 4)*

To investigate whether exposure to light in the middle of the night could alter *MKP-1* mRNA and protein expression,

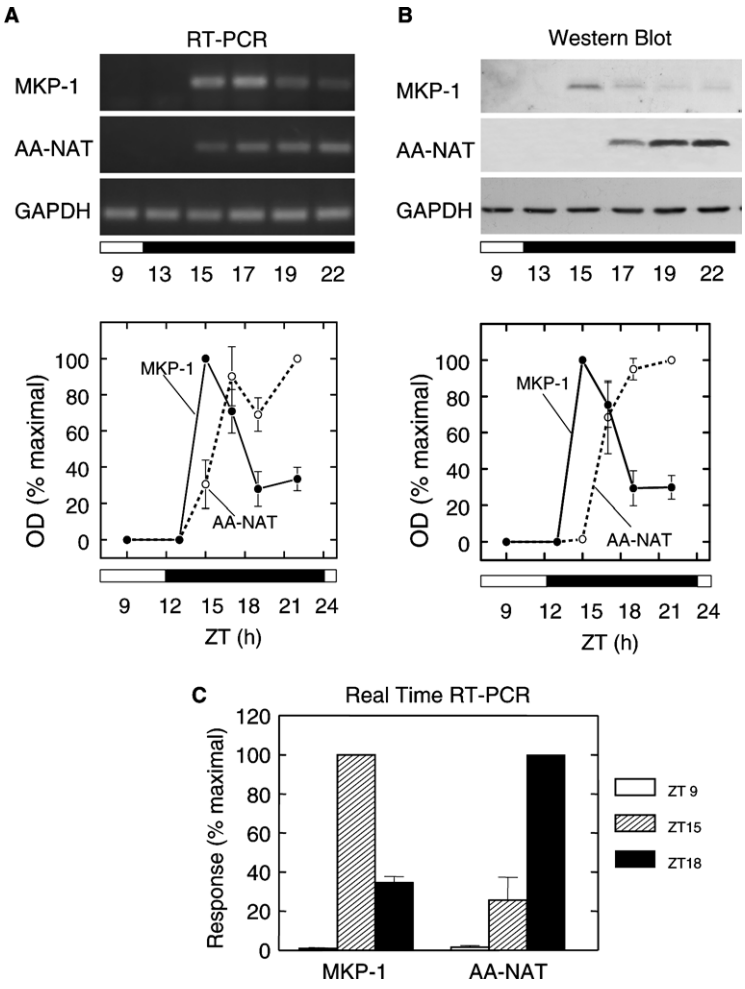


Fig. 1. MKP-1 is nocturnally expressed in the rat pineal. Pineal glands were collected from rats at the time points indicated. (A) Upper panel: representative ethidium bromide-stained agarose gels showing RT-PCR-amplified fragments of the *MKP-1* and *AA-NAT* mRNAs, *GAPDH* mRNA included to demonstrate loading consistency. Lower panel: densitometric measurements of *MKP-1* and *AA-NAT* mRNAs presented as % of peak value ($n = 3$–5 per group). (B) Upper panel: representative immunoblots of MKP-1 (40 kDa) and AA-NAT (23kDa) proteins, GAPDH (37 kDa) included to validate loading. Lower panel: densitometric measurements of MKP-1 and AA-NAT proteins presented as % of peak value ($n = 3$–5 per group). (C) Real time RT-PCR analysis of *MKP-1* and *AA-NAT* mRNAs. Histograms of real time RT-PCR *MKP-1* and *AA-NAT* mRNAs, normalized to *GAPDH* mRNA, expressed as % of the maximal increase ($n = 3$).

animals were maintained in a normal light/dark cycle and then exposed to light at ZT16; pineal glands were obtained at 30 min intervals for the next 90 min. This treatment caused *MKP-1* mRNA to decrease to day levels within 30 min and had a similar rapid impact on *AA-NAT* mRNA. In addition, this treatment caused MKP-1 and AA-NAT protein levels to return to day levels within 1 h. In contrast, in control animals in the dark, there was only a small decline in pineal *MKP-1* mRNA and protein levels between ZT16 and ZT17.5, while *AA-NAT* mRNA and protein levels were higher at ZT17.5 (see Figs. 4–6).

### 3.4. *MKP-1 expression is induced following NE stimulation of cultured pinealocytes (Fig. 5)*

To confirm that MKP-1 expression is under adrenergic control, isolated pinealocytes were treated with NE (3 μM) for 4 h; this caused a rapid increase in *MKP-1* mRNA levels that peaked at 1 h and declined gradually over the next 4 h. In the same experiment, this treatment caused a gradual increase in *AA-NAT* mRNA levels that peaked between 3 and 4 h. MKP-1 and AA-NAT proteins changed in parallel with mRNA levels following NE treatment.

### 3.5. *Dual α- and β-adrenergic control of MKP-1 expression in rat pinealocytes (Fig. 6)*

The subtype of adrenergic receptors involved in pineal MKP-1 expression was investigated in culture using selective adrenergic antagonists at doses which produced α- and β-adrenergic selective effects in this system [3–5]. NE-induced *MKP-1* mRNA expression was reduced by treatment with prazosin (3 μM) or propranolol (3 μM) and blocked by the combined treatment with prazosin and propranolol. In contrast, propranolol alone was effective in inhibiting the NE-induced *AA-NAT* mRNA expression. The decreases in NE-stimulated MKP-1 and AA-NAT protein levels paralleled the corresponding reduction in mRNA levels.

## 4. Discussion

In many physiological processes mediated by growth factors or stress stimuli, MKP-1 is an immediate early gene that is induced to target inactivation of MAPKs [18,19]. In rat pinealocytes, the activation states of $p42/44^{MAPK}$ and $p38^{MAPK}$

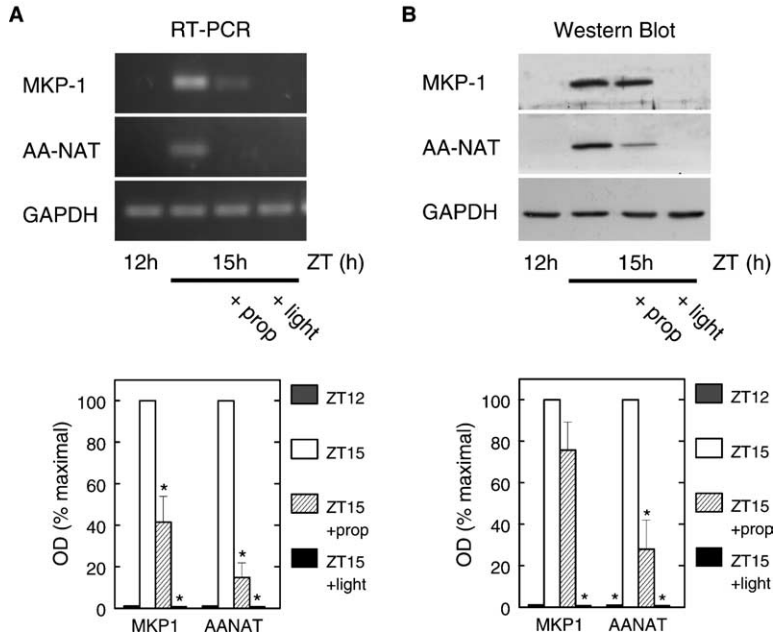


Fig. 2. MKP-1 induction is blocked by constant light and reduced by injection of a β-blocker. Pineal glands were collected from rats at ZT12, ZT15, ZT15 with propranolol treatment (1 mg/kg IP at ZT11) (+prop), and ZT15 with light remaining on from ZT12 (+light). (A) Upper panel: representative ethidium bromide-stained agarose gels showing *MKP-1*, *AA-NAT* and *GAPDH* mRNAs. Lower panel: densitometric measurements of *MKP-1* and *AA-NAT* mRNAs presented as % of peak value. (B) Upper panel: immunoblots of MKP-1, AA-NAT and GAPDH proteins. Lower panel: densitometric measurements of MKP-1 and AA-NAT proteins presented as % of peak value ($n = 3$ for ZT12 and ZT15+light, $n = 6$ for ZT15 and ZT15+prop). $P < 0.05$, compared with ZT15.

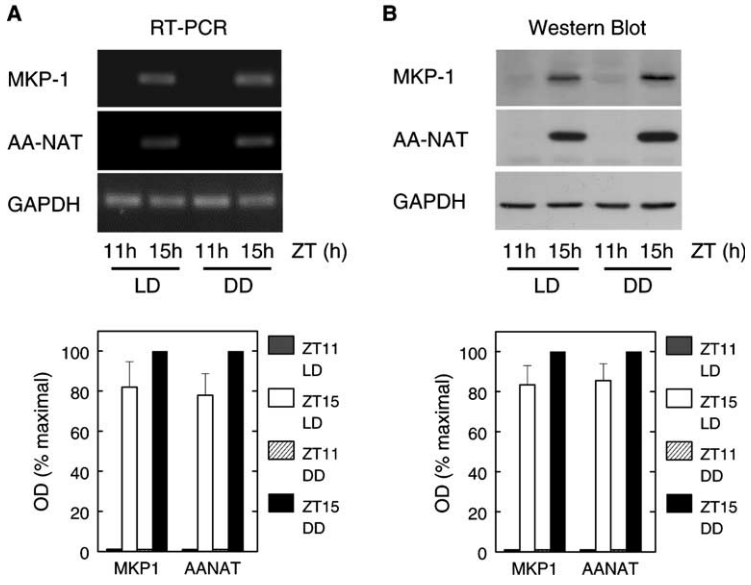


Fig. 3. MKP-1 induction persists in constant darkness. Pineal glands were collected at ZT11 and ZT15 from rats subjected to either 12 h of light every 24 h with lights on at 06:00 h (LD) or 48 h of constant darkness (DD). (A) Upper panel: representative ethidium bromide-stained agarose gels showing *MKP-1*, *AA-NAT* and *GAPDH* mRNAs. Lower panel: densitometric measurements of *MKP-1* and *AA-NAT* mRNAs presented as % of peak value ($n = 4$). (B) Upper panel: immunoblots of MKP-1, AA-NAT and GAPDH proteins. Lower panel: densitometric measurements of MKP-1 and AA-NAT proteins presented as % of peak value ($n = 4$).

can be increased by NE stimulation [11,15]; similarly, there is a nocturnal increase in $p42/44^{MAPK}$ and $p38^{MAPK}$ activity in the pineal glands [12,30]. In view of the evidence that NE control of AA-NAT activity is modulated by $p42/44^{MAPK}$ and $p38^{MAPK}$ [15,17], it appears that MKP-1 may shape the nocturnal MT signal in rats by regulating the activities of MAPKs.

Results from the present studies demonstrate that the marked nocturnal induction of MKP-1 expression is tightly regulated by environmental lighting and under adrenergic control. This is based on observations that pineal *MKP-1* mRNA and protein levels increase at night and that subjecting the animals to constant light from the onset of darkness is

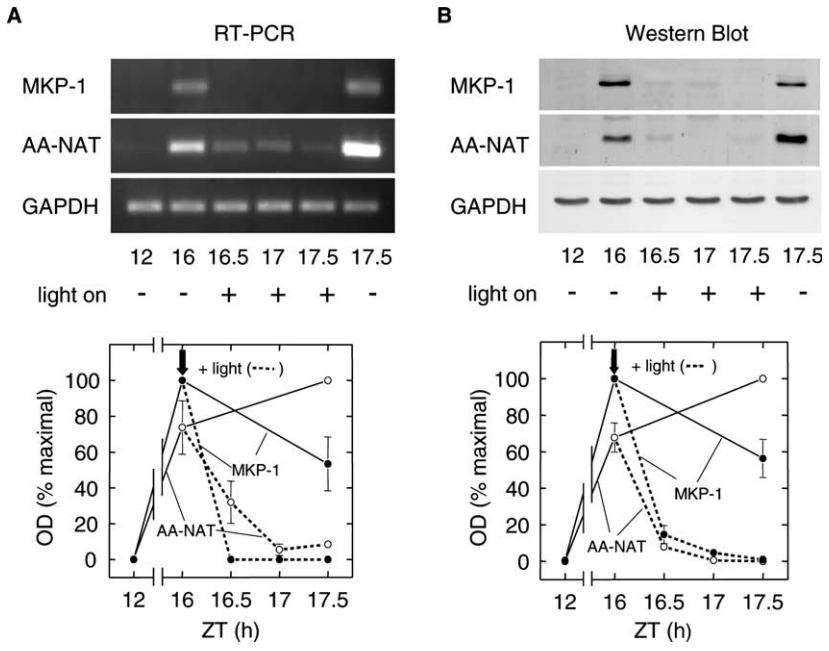


Fig. 4. Neural stimulation of the pineal gland at night is blocked by exposure to light. Pineal glands were collected at the time points indicated in the absence or presence of acute light exposure at ZT16. (A) Upper panel: representative ethidium bromide-stained agarose gels showing *MKP-1*, *AA-NAT* and *GAPDH* mRNAs. Lower panel: densitometric measurements of *MKP-1* and *AA-NAT* mRNAs presented as % of peak value ($n = 3$). (B) Upper panel: representative immunoblots of MKP-1, AA-NAT and GAPDH proteins. Lower panel: densitometric measurements of MKP-1 and AA-NAT proteins presented as % of peak value ($n = 3$).

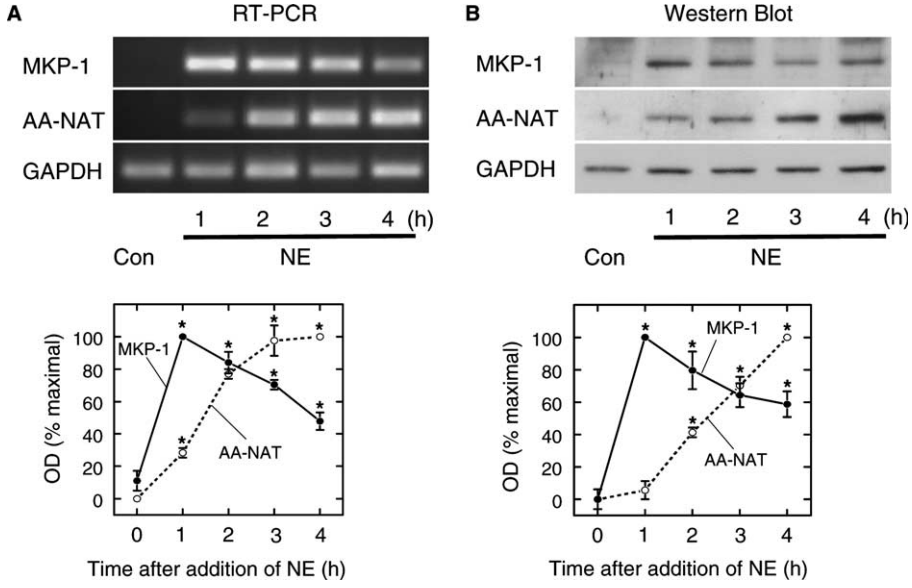


Fig. 5. MKP-1 is induced by NE treatment of rat pinealocytes. Pinealocytes ($0.8 \times 10^5$ cells/0.3 ml) were cultured for 24 h and treated with NE (3 μM) for different time periods. (A) Upper panel: representative ethidium bromide-stained agarose gels showing *MKP-1*, *AA-NAT* and *GAPDH* mRNAs. Lower panel: densitometric measurements of *MKP-1* and *AA-NAT* mRNAs presented as % of peak value ($n = 4$). (B) Upper panel: representative immunoblots of MKP-1, AA-NAT and GAPDH proteins. Lower panel: densitometric measurements of MKP-1 and AA-NAT proteins presented as % of peak value ($n = 4$). Con = control, $P < 0.05$, compared with time 0.

effective in blocking the nocturnal induction in *MKP-1* mRNA and protein levels. The nocturnal induction of pineal MKP-1 expression is also reduced by injection of the β-blocker propranolol. It is known that NE release at night in the pineal gland is driven by the endogenous circadian clock in the suprachiasmatic nucleus (SCN) [31,32]; accordingly, it would appear that the daily rhythm in MKP-1 expression in the rat pineal gland is driven by the SCN. This is supported by our finding that the nocturnal induction of MKP-1 expression persists in constant darkness.

The observation that the nocturnal induction of pineal MKP-1 expression is completely blocked by exposing animals to constant light, but reduced by injection of the β-blocker propranolol suggests the involvement of mechanisms not regulated

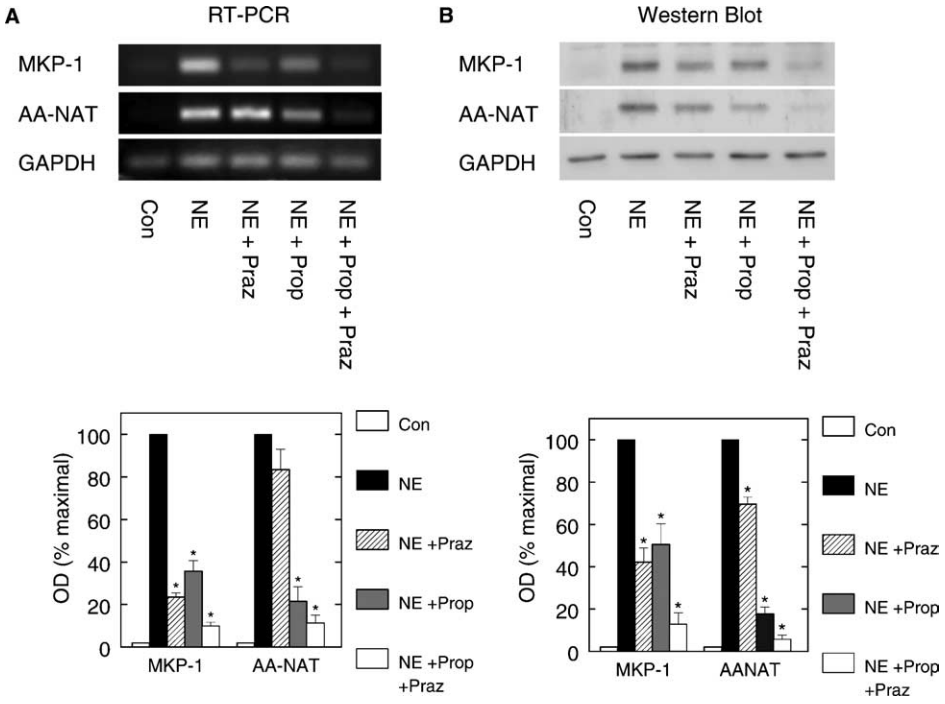


Fig. 6. NE-stimulated MKP-1 expression in rat pinealocytes is reduced by pre-treatment with both α- and β-blockers. Pinealocytes ($0.8 \times 10^5$ cells/0.3 ml) were cultured for 24 h and treated with NE (3 μM) in the absence or presence of prazosin (Praz, 3 μM), propranolol (Prop, 3 μM), or Praz (3 μM) + Prop (3 μM) for 3 h. (A) Upper panel: representative ethidium bromide-stained agarose gels showing *MKP-1*, *AA-NAT* and *GAPDH* mRNAs. Lower panel: densitometric measurements of *MKP-1* and *AA-NAT* mRNAs presented as % of peak value ($n = 4$). (B) Upper panel: representative immunoblots of MKP-1, AA-NAT and GADPH proteins. Lower panel: densitometric measurements of MKP-1 and AA-NAT proteins presented as % of peak value ($n = 4$). Con = control, $P < 0.05$, compared with NE.

by the β-adrenergic receptors. This is confirmed by pinealocyte cell culture studies that showed involvement of both α- and β-adrenergic receptor mechanisms in the NE-induced MKP-1 expression. Blockade of either α- or β-adrenergic receptors is effective in reducing NE-stimulated MKP-1 expression but blockade of both receptors are required to abolish the NE-stimulated response. Whereas our results support that MKP-1 is regulated by an adrenergic system, additional studies are required to fully describe the contributions of α- and β-adrenergic receptors to the in vivo regulation of this gene.

Comparisons of the temporal profiles of the nocturnal induction of pineal MKP-1 and AA-NAT expression reveal that pineal *MKP-1* mRNA and protein levels increase at night more rapidly than *AA-NAT* mRNA and protein and the induction of pineal MKP-1 expression is less sustained. This suggests possible differences in transcriptional and protein-level control between MKP-1 and AA-NAT. Because this difference in temporal profiles is replicated in pinealocyte cell culture, it will be possible to characterize in detail the molecular differences in the control mechanisms involved in MKP-1 and AA-NAT induction and degradation.

Our studies also raise the question of what MAPKs are substrates for MKP-1 in the pineal gland. Studies in other cell types have indicated that $p38^{MAPK}$ is the preferred substrate for MKP-1 [33] and $p42/44^{MAPK}$ activity is normal after deletion of *MKP-1* gene in mice [34]. In the case of the rat pineal gland, we have previously shown that NE treatment increases phosphorylated $p42/44^{MAPK}$ that peaks at 5 min and is back to basal levels at 1 h without having an effect on $p42/44^{MAPK}$ protein [11]. This precedes the NE induction of MKP-1 expression that peaks at 1 h and declines gradually over the next 4 h. In comparison, NE increases phosphorylated $p38^{MAPK}$ at 1 h and the increase is sustained for 4 h [15]. Together, these results suggest that MKP-1 may mediate the rapid decline in $p42/44^{MAPK}$ and the delayed activation of $p38^{MAPK}$.

The observations in this report of MKP-1 add to the growing body of the literature on a role of the MAPK pathway in circadian biology. The coupling of light to resetting of the SCN clock in rats [35], regulation of AA-NAT oscillation in the chick pineal gland [36], and regulation of AA-NAT by MAPKs in the pineal gland [13,15] point to the existence of a common conserved regulatory MAPK mechanism associated with the biological clock.

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