

A novel H28Y mutation in LEC rats leads to decreased NAT protein stability in vivo and in vitro

Abstract: Nocturnal melatonin production is reportedly controlled by the rhythms of serotonin N-acetyltransferase (NAT, or arylalkylamine N-acetyltransferase). While analyzing the melatonin synthetic pathways of Long Evans cinnamon (LEC) rats mutant for PINA, a pineal night-specific ATPase defective in Wilson disease, we discovered that NAT activity and protein levels are greatly reduced in LEC rats, and that the highly conserved histidine 28 is mutated to tyrosine. To study the effect of H28Y, we isolated a new strain of rat termed LPN that is mutant for NAT but wildtype for both PINA and coat color. Compared with control rats, the LPN rats displayed low NAT protein levels and enzyme activities. These results suggest that the H28Y mutation in NAT is the cause of reduced NAT levels in vivo. The identical H28Y mutation was also found in Sprague–Dawley rats from Zivic–Miller, suggesting it may be a common mutation in rodents. When analyzed in bacterial cells and HEK293 cells, the mutation resulted in reduction of both NAT protein stability and catalytic activity, confirming that the in vivo NAT phenotype in LPN rats was due to the H28Y mutation. Further analysis of the NAT-H28Y will focus on the mechanisms of the increased degradation both in vitro and in vivo, which will facilitate our understanding of how melatonin synthesis is controlled at the molecular level.

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Introduction

Melatonin is the major secretory product of the pineal gland [1] and is important in seasonal rhythms of reproduction and modulation of circadian functions such as sleep [2]. In all vertebrates examined, melatonin production displays dramatic circadian rhythms with night levels much higher than those of the daytime [3–5]. Melatonin is synthesized from tryptophan by four enzymes, which include tryptophan hydroxylase 1, aromatic amino acid decarboxylase, serotonin N-acetyltransferase (NAT, or arylalkylamine N-acetyltransferase), and hydroxyindole-O-methyltransferase [4]. Of the four enzymes involved, NAT is generally considered to play the most important role in controlling the diurnal rhythm of melatonin production, as its activity is much higher at night than during the day [3, 6]. We demonstrated that in rat the NAT transcriptional regulation dictates the diurnal rhythmicity of melatonin production [7]. In addition, post-transcriptional regulation of NAT protein also plays an important role in melatonin synthesis [3, 8].

To improve our understanding of melatonin production, we performed subtractive hybridization analysis of pineal day and night RNA and identified many transcripts that are enriched in the night pineal gland [7, 9, 10] including NAT and a few enriched in the day pineal gland such as 3OST2 [11]. One of the night-specific transcripts is pineal night-specific ATPase (PINA) [9], an alternative form of Wilson disease (WD) gene ATP7B [12], generated by an alternative promoter in ATP7B intron 8 [13]. In order to understand

the role of PINA in pineal physiology, we analyzed the pineal gland of the Long Evans cinnamon (LEC) rats [14], the animal model for WD [15] in which PINA is also deleted. This paper describes the identification of a mutant NAT phenotype in LEC pineal that has abnormally low NAT activity and protein content, and demonstrates that the aberrant NAT phenotype results from a single nucleotide change of C to T in NAT coding region that leads to H28Y mutation.

Materials and methods

Animals

Adult LEC rats were purchased from Charles River Japan, Long Evans (LE) rats from Charles River (Wilmington, MA, USA), PVG rats from Harlan (Indianapolis, IN, USA), Sprague–Dawley (SD) rats from Zivic–Miller (Pittsburgh, PA, USA). Rats were housed at 20–25°C with lights off at 19:00 hr [light:dark (LD) 12:12 hr]. Food and water were available ad libitum throughout the experiment. Illumination was supplied by white fluorescent lamps (600 lux at cage level). Rats were placed in the LD cycles for at least 2 wk before experiments.

Northern and Western blots

RNA samples subjected to Northern analysis were prepared using the RNeasy kit (Qiagen, Hilden, Germany) and

rat pineals isolated at the indicated time points. Northern blots were hybridized to ³²P-labeled full-length rat NAT cDNA and GAPDH cDNA. Total protein extracts from one-tenth of a single rat pineal gland were loaded in each lane, blotted onto a nitrocellulose membrane which was sequentially probed with a NAT polyclonal antiserum developed in our lab (against N-terminal 15 amino acids) and a monoclonal GAPDH antibody.

NAT enzyme assay

The NAT activity assay was performed according to the published methods [16]. Briefly, pineal glands isolated from night (02:00 hr) rats or rats killed at the indicated hours were homogenized in 100 μ L of 50 mM phosphate buffer (pH 6.8) on ice. One-tenth of the protein extracts was mixed with tryptamine, acetyl CoA, and acetyl ¹⁴C-CoA, and incubated at 37°C for 20 min. The reaction was stopped by addition of 0.2 M borate buffer, pH 10, and the labeled product extracted with 1 mL of chloroform. A portion (500 μ L) of the lower organic phase was transferred to new scintillation vial after two extractions with 0.2 M borate buffer, evaporated to dryness, and radioactivity measured in 10 mL of scintillation fluid after vigorous mixing.

NAT genomic DNA and cDNA sequence analysis

To obtain the NAT genomic sequence, a fragment containing exons 2 to 4 of NAT gene was isolated by PCR amplification of genomic DNA preparation from LEC and SD rats with primers corresponding to bases 7996315–7996335 (forward primer) and 7998317–7998296 (reverse primer) (Genbank accession no. NW_047343.1). The coding region of NAT was isolated from LEC night pineals by RNA preparation, 1st strand cDNA synthesis, and PCR amplification using the same primers. The PCR products were purified and sequenced directly on both strands.

Generation of LPN rats

To segregate the three independent mutations identified, LEC rats were mated to PVG rats, which are normal for PINA (and ATP7B), NAT, and coat color (black hooded). F1 LP (LEC \times PVG) rats thus produced were heterozygote for all three mutations. The segregation of coat color mutation was judged by visual inspection. PINA/ATP7B mutation was identified by genomic Southern blotting using a labeled PINA cDNA probe near the deletion breakpoint [15]. Genotyping of NAT was accomplished by genomic PCR and direct sequencing of NAT gene.

The F2 rats and rats of subsequent generations were produced by backcrossing to PVG rats (F2 = F1 \times PVG, F3 = F2 \times PVG, etc.). Rats at each generation were analyzed individually for PINA, NAT, and coat color mutations as stated above. Animals homozygous for each of the three traits were produced by crossing the male and female rats that are heterozygous for the same genes. Rats defective in NAT gene were designated as LPN. Rats who are wildtype in all three genes were designated as LPW. The LPN rats thus generated from the fifth generation

onward contain no mutations in PINA/ATP7B, and coat colors.

Recombinant NAT protein production and analysis in bacteria

Full-length NAT cDNA fragments from LEC and LE rats were subcloned in-frame into the maltose binding protein (MBP) fusion protein expression vector pMAL-c2 (New England Biolabs, Beverly, MA, USA). The plasmids were transformed into the BL21 (DE3) cells (Novagen, Madison, WI, USA) and protein expression was induced with IPTG (isopropyl-B-thiogalactopyranoside) at room temperature for 4 hr. Cells were collected by centrifugation and lysed by sonication in sodium phosphate buffer (50 mM, pH 6.8) in the presence of a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). The supernatant was assayed for NAT activity as described above. The expressed protein samples were analyzed by Western blotting using anti-NAT (generated in our lab) and anti-MBP (New England Biolabs) antibodies.

Recombinant NAT protein expression and analysis in HEK293 cells

Full-length NAT cDNA fragments from LEC and LE rats were engineered to contain a Kozak consensus sequence at the N-terminal and a C-terminal myc epitope [17]. The inserted myc epitope is preceded by five glycines to increase the flexibility of the epitope. The fragment was cloned into the pCISII expression vector [17]. HEK293 cells were seeded in 24-well plates at 70% confluency. Plasmid constructs expressing NAT-wt or NAT-H28Y, or empty vector were transfected the next day each in triplicate using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. Transfected cells were harvested 48 hr later, sonicated in chilled 50 mM sodium phosphate buffer, pH 6.8, and the total protein extracts analyzed by NAT enzyme assay (see above) and by Western blotting using anti-myc antibody (to probe NAT protein levels) and anti-14-3-3 (to control for protein loading).

Results

PINA is a pineal- and night-specific product generated from ATP7B gene locus [9, 13], and is absent in LEC rats, an animal model of WD, due to a large deletion in ATP7B gene [15] and PINA. Moreover, PINA displays an identical tissue distribution and night-specific expression with that of NAT. We sought to explore the functional consequences of PINA mutation in LEC pineals. Pineal glands of adult male LE (the parental strain of LEC rats) and LEC rats isolated at indicated times (Fig. 1) were analyzed for NAT enzyme activity (upper panel) and NAT protein content (lower panels). In comparison with LE rat, the LEC pineals contained much lower NAT activity, and low NAT protein content.

To ascertain whether the lower NAT protein and enzyme levels were caused by lower NAT gene expression in the LEC rats, we performed Northern blot analysis of rat

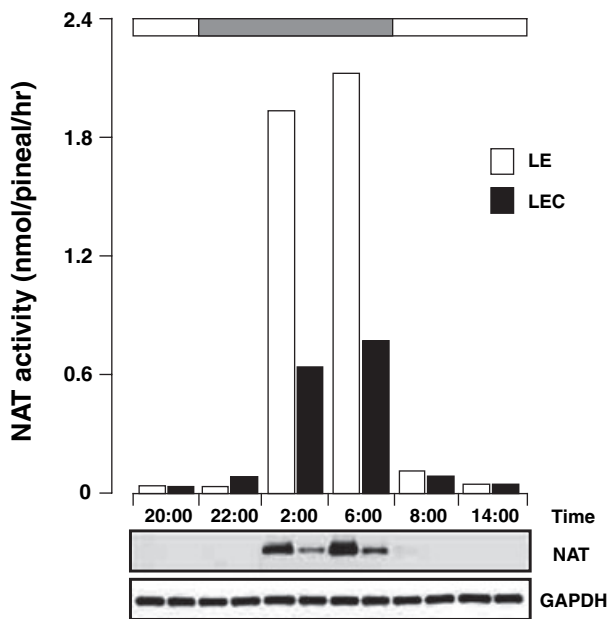


Fig. 1. Long Evans cinnamon (LEC) rats have reduced NAT activity and protein content at night. NAT activity (upper panel) and protein levels (lower panel) were assayed using pineal glands of LEC (black bars in upper panel and the corresponding lanes in protein blots below) and LE rats (open bars) isolated at indicated hours. Total protein extracts from one-tenth of single gland were loaded in each lane and probed with anti-NAT and anti-GAPDH antibodies. The shaded bar on the top indicates the dark period (19:00–07:00 hr).

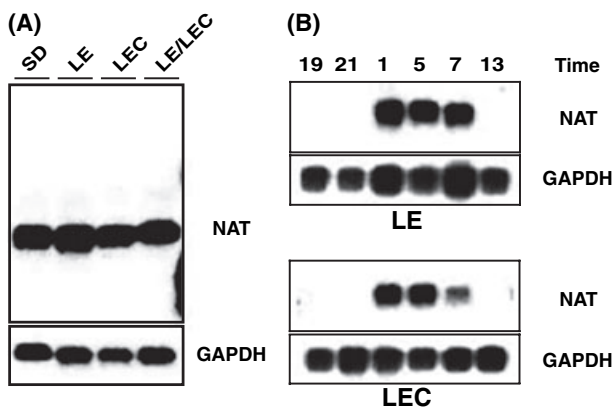


Fig. 2. N-acetyltransferase (NAT) RNA levels are unaffected in LEC pineals. (A) Northern blot analysis of pineal total RNA from Sprague–Dawley (SD), LE, LEC, and the F1 crosses of LE and LEC (LE/LEC) rats killed at night (02:00 hr). The RNA blot was first hybridized with a full-length rat NAT (clone 12.7) probe (upper panel), stripped, and reprobed with rat GAPDH probe (lower panel). (B) Northern blot analysis of pineal total RNA isolated at indicated hours of adult LE (upper panel) and LEC (lower panel) rats, probed with NAT and GAPDH. In both cases, the blots were analyzed by PhosphorImager to quantitate the signals. No statistical differences were found between LEC and LE (or SD or LE/LEC) rats.

pineal glands from both LE and LEC rats. As shown in Fig. 2A, the LEC pineals contained normal levels of NAT message compared with the SD, LE and LE/LEC (F1 rats

heterozygous for PINA mutation) at night. A circadian profiling of NAT expression in the pineals of LE and LEC rats shown in Fig. 2B demonstrates that LEC rats (lower panel) contain normal levels of NAT at all times of night compared with that of LE rats (upper panel). The higher level of NAT at 07:00 hr in LE rats was due to higher amount of RNA sample loaded.

The post-transcriptional defects in LEC NAT and the fact that PINA and NAT are both night-specific and pineal-specific led to our initial speculation that the NAT defect in LEC rats is caused by the PINA mutation. To ascertain the linkage between PINA mutation and the NAT defect, we crossed LEC rats to LE rats and generated F2 rats with three PINA genotypes: PINA^{-/-}, PINA^{-/+}, and PINA^{+/+}. Analysis of the pineal glands from rats with each of the three genotypes, however, demonstrated that NAT mutation is not associated with the PINA mutation (data not shown).

Sequence analysis of NAT genomic fragments and NAT cDNA from LEC rats demonstrates that LEC rats contain a C to T change in the NAT coding region (Fig. 3). This mutation converts a conserved histidine [3] to tyrosine at position 28. Interestingly, the histidine 28 is adjacent to the threonine residue at 29th position (T29) that is phosphorylated in vitro and in vivo by protein kinase A (PKA) [18–21] and protein kinase C (PKC) in vitro [22].

These results suggest that the LEC rats contain three mutations compared with the parental LE rats: PINA/ATP7B deletion, NAT-H28Y mutation, and cinnamon coat color mutation. To rule out potential contribution of PINA mutation and coat color mutation in the NAT phenotype expression in the LEC rats, we performed extensive crossbreeding studies with PVG rats to isolate the NAT mutation (Table 1). PVG rats were chosen as the mating

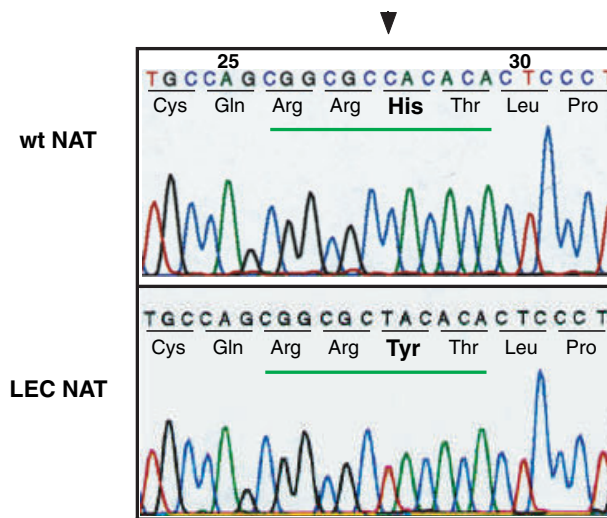


Fig. 3. Long Evans cinnamon (LEC) NAT contains a H28Y mutation in NAT gene. Sequence analysis of both NAT genomic DNA and NAT cDNA of LEC rats reveals a C to T change in NAT coding region (black arrow). The single nucleotide change leads to alteration of a conserved histidine residue at amino acid position 28 to tyrosine. Notice that the change is within the RRHT motif that is targeted by cAMP-dependent protein kinase (PKA).

Table 1. Isolation of LPN rats

Generations	Strains	Mutations		
		PINA/ATP7B	NAT	Coat color
F0	LEC (CrJ)	-/-	-/-	-/-
F0	PVG (CrUSA)	+/+	+/+	+/+
F1	LP (LEC × PVG)	+/-	+/-	+/-
F5 ^a	LPN (LP with NAT defect)	+/+	-/-	+/+
F5	LPW (LP with no known defect)	+/+	+/+	+/+

^aThe F5 rats were generated by backcrossing the F1 to PVG for additional 4 generations.

partner, because they are inbred, display similar coat color (black hooded) as the LE rats (the parental strain of LEC rats), and possess normal PINA/ATP7B and NAT genes (data not shown). As expected, the F1 LP (LEC × PVG) animals were heterozygous for all three alleles (Table 1). Subsequent crosses were performed between the LP rats and PVG rats. At each generation, the cinnamon coat color was confirmed visually, the PINA/ATP7B mutations were inspected by Southern blot analysis, and the NAT mutation validated by direct sequencing of genomic PCR products. By the fourth generation, we were able to obtain rats heterozygous for each of the three alleles. Rats homozygous for each allele were obtained at the fifth generation and maintained thereafter by sib mating. The rats that are homozygous for the cinnamon coat color were no longer maintained in our animal facility. The rats that are homozygous for ATP7B and PINA mutation, are functionally equivalent to the parental LEC rats in terms of liver cirrhosis, elevated copper concentration, and frequent fatality at 4 months of age associated with jaundice (data not shown), and therefore are a bonafide animal model for WD.

The LPN rats, which are devoid of PINA mutation and coat color mutation (Table 1), were analyzed for NAT enzyme activity and NAT protein content. As shown in Fig. 4, individual LPN rats all contained much lower levels of NAT activity (upper panel) and protein (lower panel),

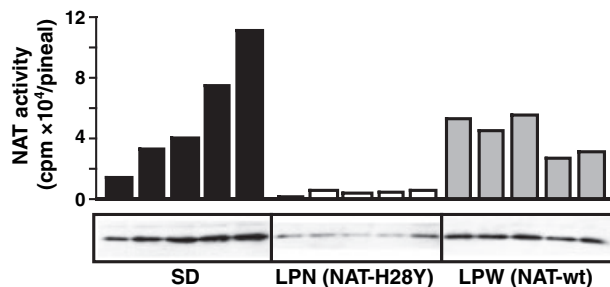


Fig. 4. The LEC NAT phenotype is caused by the H28Y mutation. LPN rats containing only the H28Y mutation and LPW rats (wildtype in all three genes PINA/NAT/coat color) were generated as described in the text. NAT activity (upper panel) and protein content (lower panel) were measured for five individual male rats of SD (from Zivic-Miller), LPN, and LPW strains. A portion (10%) of a single pineal extract is loaded in each lane and probed with anti-NAT antibody (lower panel).

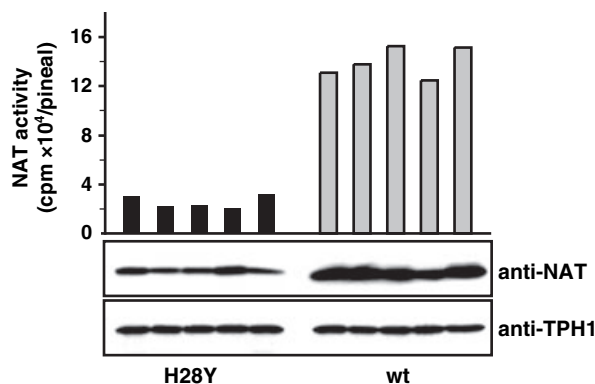


Fig. 5. The H28Y mutation in NAT is also found in SD rats. Five individual SD rats that are either wildtype (wt) or mutant for NAT gene (H28Y) were analyzed for NAT enzyme activity (upper panel) and protein contents (lower panel). The protein blots were hybridized with anti-NAT (upper) and anti-tryptophan hydroxylase 1 (TPH1) antibodies.

compared with the SD and the LPW wildtype rats. These results strongly suggest that the H28Y mutation in NAT is the cause for the low protein expression and enzyme activity in the LEC and LPN rats.

While analyzing each full-length NAT clones we isolated from SD rats [7], we came across one (clone 12.7) that contains the same H28Y mutation, which was initially attributed to error in our night pineal cDNA library construction. Identification of the H28Y mutation in LEC rats and confirmation of strong linkage of NAT phenotype with the mutation in vivo prompted us to analyze SD rats from two commercial sources: Zivic-Miller SD rats that were used for our night pineal library construction, and Harlan SD rats that have been used routinely in our laboratory since 1998. Genomic DNA was isolated from tail biopsy samples of 50 SD rats from Zivic-Miller and 50 SD rats from Harlan, and NAT genes were PCR amplified and analyzed by sequencing the entire coding regions. While there was no NAT mutation found in SD rats from Harlan, the H28Y mutation was frequently found in the SD rats from Zivic-Miller. Of the 50 analyzed, eight animals were homozygous, 20 heterozygous for H28Y mutation, and 22 were wildtype. Analysis of the night SD pineals revealed that NAT activity (upper panel) and protein content (lower panel) were lower in rats harboring the H28Y mutation (Fig. 5). These results confirm our observations made with LEC (Fig. 1) and LPN (Fig. 4) rats, and are consistent with the heterogeneous NAT expression levels seen in Fig. 4 for SD rats from Zivic-Miller. They also suggest that H28Y may be a hot spot for genomic mutations of NAT even in outbred rats.

We next tested the mutant NAT protein in bacterial cells. Expression of wildtype NAT and NAT-H28Y mutant MBP fusion proteins in *Escherichia coli* cells demonstrates that the mutation leads to reduced fusion protein production (lower panels) and low NAT-H28Y enzyme activity (Fig. 6, upper panel). Expression of NAT wt and the H28Y mutant protein in HEK293 cells also demonstrate that H28Y mutation consistently leads to decreased protein expression and correspondingly reduced enzyme activity (Fig. 7). The

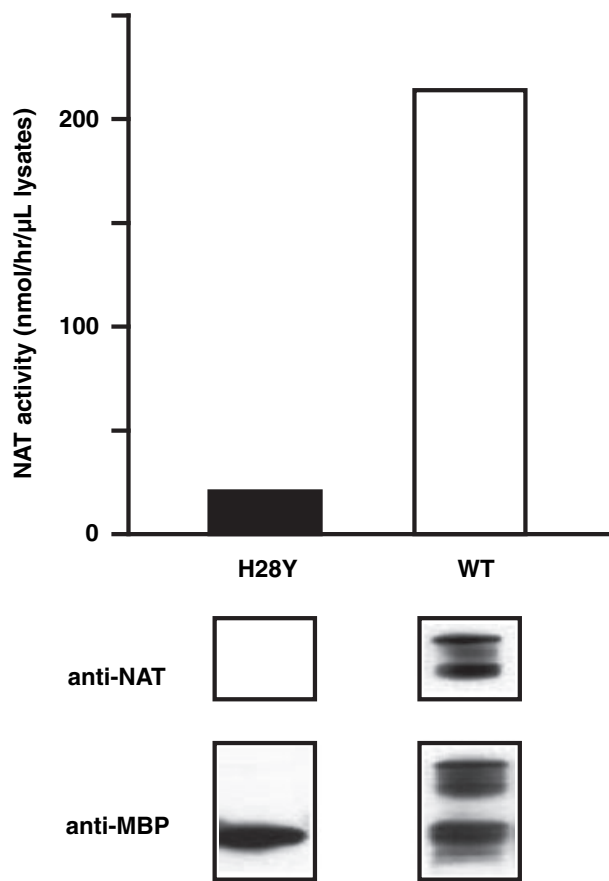


Fig. 6. Analysis of NAT-H28Y in bacterial cells. The coding regions of wildtype (wt) and H28Y mutant NAT (H28Y) cDNA were fused to the C-terminal of maltose binding protein (MBP) and expressed as fusion proteins. The bar graph represents NAT activity measured in total cell lysates (upper panel). The immunoblots of the lysates were reacted with anti-NAT antibody (upper) and anti-MBP antibody (lower).

data obtained in each well is normalized against the average value of the NAT wildtype samples for both activity (open bars) and protein contents (solid bars).

Discussion

In this paper, we report a novel H28Y mutation of NAT in rats. The H28Y mutation found initially in LEC rats was segregated into wildtype rat strains that do not have the PINA mutation (LPN rats), and later were also found in SD rats from Zivic-Miller. In vivo, the NAT-H28Y protein is expressed at lower levels in the pineal gland at night correlating with a proportional decrease in NAT enzyme activity. The same mutant, when expressed in vitro in bacterial cells or mammalian cells, also leads to lower levels of NAT protein production and a parallel decrease in activity. These data indicate that the H28 residue in NAT is crucial for NAT protein stability both in vitro and in vivo.

The defect in NAT protein expression and activity was initially attributed to the PINA defect in LEC rats, as NAT and PINA share identical expression patterns both temporally and spatially [8]. An extensive genotype-phenotype

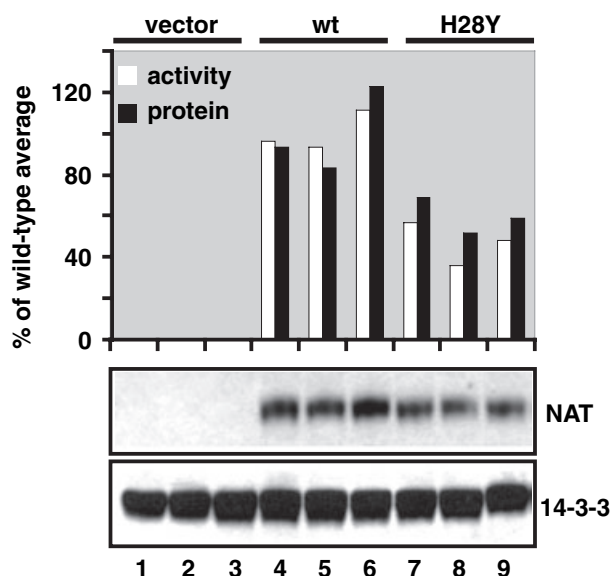


Fig. 7. Analysis of NAT-H28Y in mammalian cells. Myc-tagged full-length NAT wildtype (wt) and the H28Y mutant (H28Y) were transiently transfected in HEK293 cells in triplicate along with vector control. Portions of cell extracts were assayed for NAT enzyme activity (upper) and protein expression (lower panels). Wildtype average in upper panel is the average value of the triplicates from wildtype samples. Values for each sample were expressed as percentage of the wildtype average. Vector-alone samples contained no detectable NAT activity. The expressed NAT protein (NAT) was analyzed using anti-myc antibody, while total extracts loading was controlled with anti-14-3-3 antibody. Lanes 1–3 were from the wells transfected with vector alone, lanes 4–6 with wildtype NAT-myc, and lanes 7–9 with NAT-H28Y-myc constructs. The amount of plasmids transfected in each wells is identical (lanes 1–9).

analysis of LEC rats was performed, leading to the discovery of NAT-H28Y mutation in LEC rats, and suggesting that the H28Y mutation could be the cause of the LEC pineal phenotype. This study revealed the unusual existence of two independent mutational events in LEC rats that affect two genes whose expression is pineal- and night specific. The isolation of the H28Y mutation in a wildtype background (without PINA and coat color mutations) was a necessary step toward an accurate characterization of the consequences of H28Y mutation in vivo. The consistent correlation of low NAT expression with the H28Y mutation in LEC, LPN, and SD rats demonstrates that the in vivo phenotype of low NAT is due to the H28Y mutation. In vitro analysis of NAT-H28Y demonstrates that the H28Y mutation consistently leads to lower NAT protein expression in both bacterial cells and mammalian cells, suggesting that the H28 residue is important for maintaining NAT protein stability.

The H28 residue is located in the middle of a RRHT motif that is a target of phosphorylation site by both cAMP-dependent PKA [18, 19], and calcium-dependent PKC [22]. The RRHT motif is conserved in NAT from rat [7, 23], human [24], monkey [25], sheep [26], cow (Genbank accession no. O02785), hamster [27], mouse [28, 29], chicken [30], owls [31], frog (Genbank accession no.

AAP57668), rainbow trout (Genbank accession no. BAA34809), Gilthead sea bream [32], puffer fish (Genbank accession no. CAG10779), and zebrafish [33]. This striking conservation of the RRHT motif highlights the importance of the conserved H28 residue in NAT activity. It is tempting to speculate that H28 is important in phosphorylation of T29 by PKA and PKC; however, the third position in the consensus motif RRXT (X = H in NAT) is not predicted to influence phosphorylation [34]. It remains to be determined whether H28 functionally interacts with phosphorylated T29 to regulate protein stability in the pineal.

The phosphorylation of T29 in rat NAT and the subsequent interaction of phosphorylated NAT with 14-3-3 have been demonstrated independently by Li [18] and Ganguly et al. [19]. Formation of this NAT/14-3-3 complex is thought to enhance NAT activity by shielding NAT from proteolysis [18, 19]. In fact, NAT protein stability increases when phosphorylated at T29, the protein is less stable when T29 is mutated to valine when expressed in tissue culture cells [22]. These data suggest that stability of NAT protein is maintained in part by phosphorylation at T29 residue and by subsequent interaction with 14-3-3. As the H28Y is predicted not to affect phosphorylation of NAT at the T29 residue (see above) and NAT-H28Y is unstable compared with the wildtype NAT in vivo and in vitro (this study), the interaction of 14-3-3 with RRHT site of NAT may require the H28 residue. When T29 residue is mutated to alanine (T29A) and transfected side by side to HEK293 cells with H28Y mutant, the two mutants exhibited similar degree of protein stability (Z. Huang & J. Borjigin, unpublished data), suggesting that H28 and T29 residues are equally important for NAT stability. Future studies will be focused on testing the ability of NAT-H28Y mutant to be phosphorylated and to interact with 14-3-3.

As NAT was proposed to be the rate-limiting enzyme for melatonin formation [3, 6], we measured melatonin levels of LPN and SD rats that harbor H28Y mutation. Surprisingly, the melatonin levels are unaffected by the H28Y-NAT mutation that resulted in a two to 10 times reduction of NAT activity (data not shown). These data suggest that NAT may not be the rate-limiting enzyme at night in the pineal gland.

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