

Interactive report

Natural melatonin ‘knockdown’ in C57BL/6J mice: rare mechanism truncates serotonin *N*-acetyltransferase¹

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Abstract

Pineal melatonin synthesis (serotonin → *N*-acetylserotonin → melatonin) is severely compromised in most inbred strains of mice, in many cases because serotonin is not acetylated by serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT). We have found that in the C57BL/6J strain, AANAT mRNA encodes a severely truncated AANAT protein, because a pseudo-exon containing a stop codon is spliced in. This is the first identification of a natural mutation which knocks down melatonin synthesis. The decrease in melatonin signaling may have been a selective factor in the development of laboratory strains of mice because melatonin can inhibit reproduction and modify circadian rhythmicity. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Circulating melatonin levels increase at night in essentially all vertebrates. This is due to an elevation in melatonin synthesis in the pineal gland, which reflects a large increase in the activity of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT, E.C. 2.3.1.87) [26]. AANAT is the penultimate enzyme in the melatonin synthesis pathway (serotonin → *N*-acetylserotonin → melatonin) [2,23]. The last enzyme in this pathway is hydroxyindole-*O*-methyltransferase (HIOMT), the activity of which does not change significantly on a day/night basis [23]. Melatonin mediates photoperiodic effects on reproduction [22] and may influence a variety of circadian activities including sleep [1,15].

In contrast to most vertebrates, many strains of inbred

mice appear to make very little or no melatonin [21,42] because one or both of the enzymes in the serotonin → melatonin pathway are severely compromised or undetectable [16,17]. Until now it has not been clear whether this reflects technical limitations, a developmental defect or a defect in one or both of the two melatonin synthesis enzymes. Here we report that the AANAT gene has been naturally knocked down in the C57BL/6J strain of mice as a result of a point mutation in the gene. This alters RNA splicing and results in the inclusion of a 102 base pair pseudo-exon in the mature AANAT mRNA which truncates the encoded protein because it contains a stop codon.

2. Materials and methods

2.1. Mice

C3H/HeJ and C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a control lighting environment (LD 14:10) with lights on at 0300. Mice were sacrificed by CO₂ asphyxiation, tissues were removed immediately, placed on solid CO₂, and stored at –80°C until use.

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2.2. Mouse AANAT gene isolation

A 129SV mouse genomic library constructed in Lambda FIX® II was obtained from Stratagene (La Jolla, CA). The library was constructed from a partial Sau3A I digest of liver DNA obtained from female mice (4–8 weeks old). The library was screened with the random-primed ³²P-labeled probe generated from the previously described rat AA-NAT clone (rLL13; Genbank acc. no. U38306) [33]. The four positive clones that resulted were plaque purified and screened by hybridization with a radiolabeled PCR product corresponding to bases 1–1392 of the rat AANAT promoter (Genbank acc. no. U77455) [3]. This probe detected one of the four clones (msg3), and this clone was used for sequence analysis and chromosomal localization.

2.3. Chromosomal localization

Fluorescent in situ hybridization (FISH) using the msg3 clone was done to determine the location of the mouse AANAT gene. Chromosomes obtained from mouse spleen cultures were used following the standard FISH protocol with modifications as described [31,45]. A symmetrical fluorescent signal was detected on sister chromatids at the distal segment of a medium-sized chromosome, and was localized on chromosome 11 at region E1.3–2.3. All chromosomes with a specific hybridization signal were positive for a chromosome 11-specific probe.

2.4. Northern blot analysis

Total RNA was extracted as previously described [6,34]. RNA was transferred to Hybond-N (Amersham, Arlington Heights, IL) by passive capillary transfer and probed using a ³²P-labeled random-primed probe (1207 base pairs) generated from a C3H/HeJ mouse retinal AANAT cDNA clone using primers from exons 2 and 4 (Genbank acc. no. AF004109). The sequence of this clone was similar that of the pineal cDNA clone described below except that it has a

single base pair difference in the 3' untranslated region and a 77 base pair insert in the coding region that corresponds to sequence in intron 2 of the mouse gene. Blots were hybridized at 68°C in QuikHyb (Stratagene, La Jolla, CA) and the final wash was at 60°C in 0.1 × SSC containing 0.1% SDS for 15 min. Hybridized blots were imaged and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

To normalize data for variations in RNA loading the Northern blots were also probed with a 983 base pair PCR product corresponding to human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) that had been amplified using primers specific to the coding sequence as previously described for rat G3PDH [33]. Transcript sizes were estimated by comparison with RNA markers (RNA Molecular Weight Marker I; Boehringer-Mannheim, Indianapolis, IN).

2.5. cDNA sequence analysis

To obtain the AANAT sequences from the C3H/HeJ and C57BL/6J strains of mice, PCR was performed on mouse pineal cDNA synthesized as previously described [32]. The sequences of the PCR primers were derived from exons 1 and 4 of the msg3 genomic clone and corresponded to bases 1249–1273 and 5107–5082 (Genbank acc. no. U83462). The resultant PCR products were then gel purified and subcloned using the pGEM-T Easy Vector System (Promega, Madison, WI) and sequenced in both directions [35].

2.6. Recombinant AANAT protein expression and analysis

The expression of mouse AANAT protein was done essentially as previously described for the rat AANAT [12] with minor modifications. For each strain, cDNA sequences were subcloned in-frame into the glutathione-S-transferase fusion protein expression vector pGEX-4T-3 (Pharmacia Biotech, Piscataway, NJ). For the C3H/HeJ AANAT, the entire open reading frame was used. For the

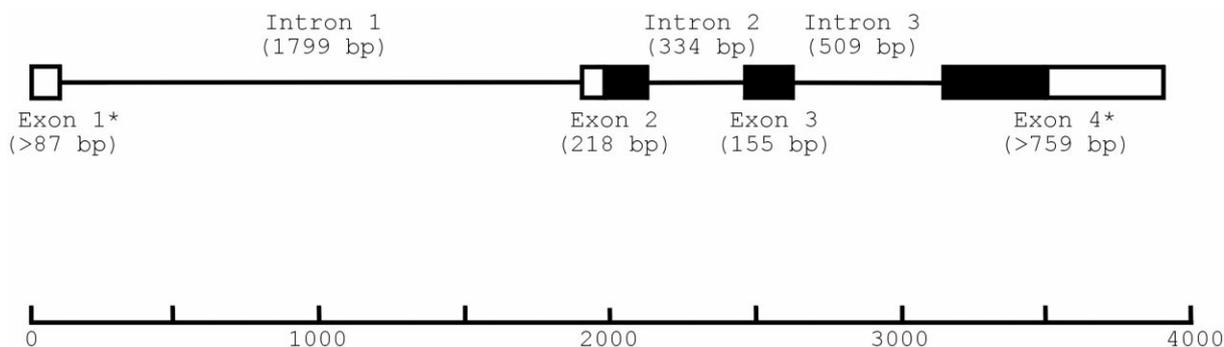


Fig. 1. Partial structure of the mouse AANAT gene. The structure was obtained by comparison of the sequence of the C3H/HeJ mouse pineal AANAT PCR product to the AANAT genomic sequence obtained from 129SV mouse DNA. Filled rectangles represent the open reading frame, and the numbers in parentheses are the size of the indicated segments. * Because the PCR primers that amplified the cDNA sequence were located in Exons 1 and 4, only partial sequence is known and the length given is a minimum for these two segments.

C57BL/6J AANAT cDNA, the two regions that correspond to the open reading frame of the C3H/HeJ cDNA along with the intervening 102 base pair pseudo-exon sequence were used. The constructs were transformed into

the *Escherichia coli* strain BL21(DE3)pLysS (Stratagene), and protein expression was induced with 0.2 mM isopropyl- β -thiogalactopyranoside (United States Biochemicals, Cleveland, OH) for 6 h at room temperature.

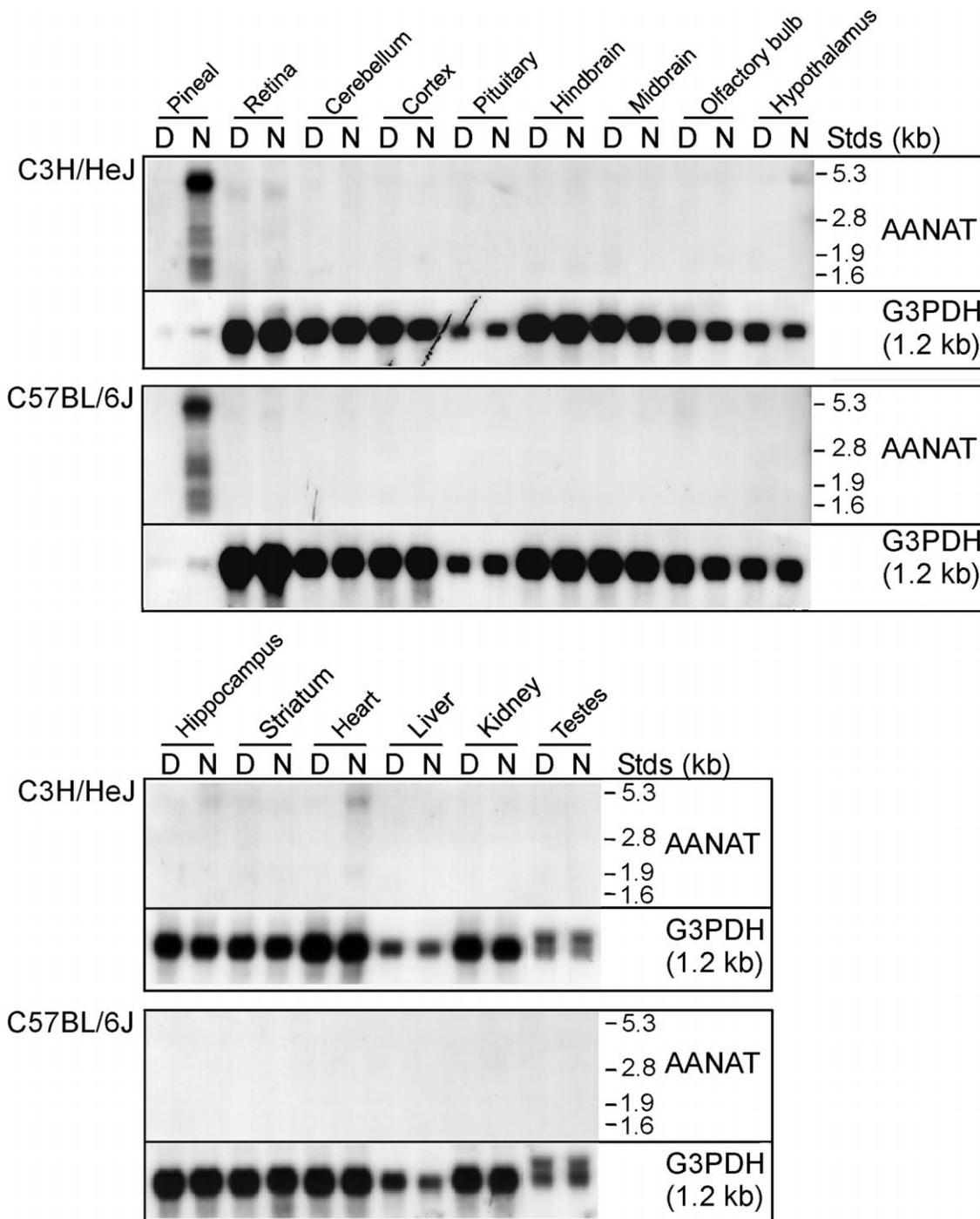


Fig. 2. Tissue distribution and day/night regulation of mouse AANAT mRNA expression in C3H/HeJ and C57BL/6J mice. Northern blot analysis was performed on total RNA obtained from day tissues (D) removed from 1000–1200 h and night tissues (N) removed from 2200–2400 h. Blots were probed with a C3H/HeJ mouse retinal AANAT PCR product (1207 base pairs). Each lane contains 15 μ g of total RNA except for the pineal lanes which contained only 2 μ g. To monitor for variations in RNA loading and degradation, the blots were stripped and probed for G3PDH. A similar night- and pineal-specific increase in AANAT mRNA in both strains of mice was seen in an independent study. Additionally, a nighttime increase in pineal AANAT mRNA has previously been detected in both C3H/HeJ mice, which have detectable pineal melatonin, and 129SV mice, which do not have detectable pineal melatonin [18].

Cells were pelleted and lysed by sonication in 2 × phosphate-buffered saline containing 10 mM DTT and a cocktail of protease inhibitors (1 tablet/50 ml, Complete™, Boehringer-Mannheim). Cellular debris was removed by centrifugation (4°C, 20 000 × g, 20 min) and AANAT enzyme activity was measured in the supernatant in the presence of 10 mM tryptamine and 0.5 mM [³H]AcCoA (4

μCi/μmole; New England Nuclear, Boston, MA) as previously described [44].

To determine the size of the expressed protein, proteins in bacterial extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [29] and transferred to Immobilon P (Amersham) [40]. AANAT proteins were detected immunochemically using an AANAT-

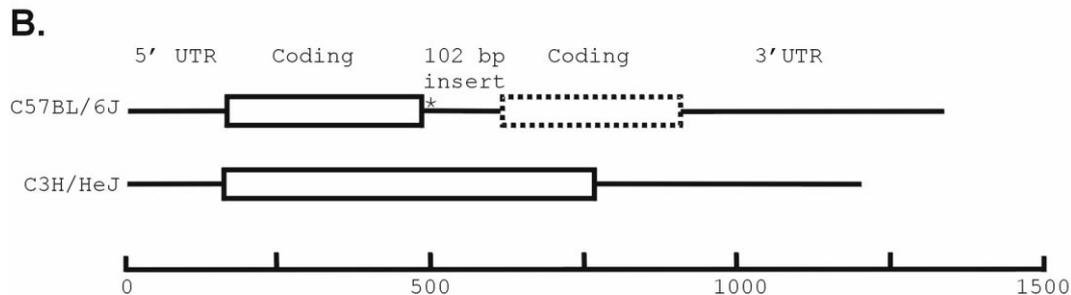
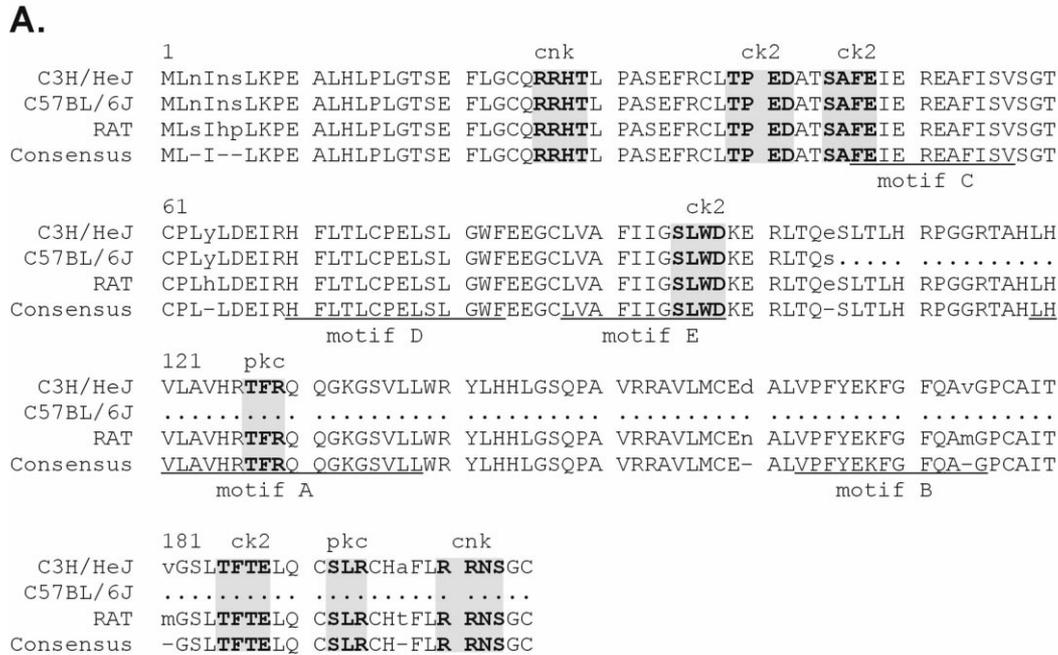


Fig. 3. Strain and species differences in the AANAT protein and cDNA sequences. (A) Comparison of the deduced AANAT amino acid sequences encoded by the mouse C3H/HeJ clone (Genbank acc. no. AF004108), mouse C57BL/6J clone (Genbank acc. no. AF004110) and the rat clone (rLL13) (Genbank acc. no. U38306). The consensus sequence identifies sequences that are identical in both strains of mice and the rat. The conserved putative phosphorylation sites are shaded: cnk = cyclic nucleotide dependent protein kinase; pkc = protein kinase C; ck2 = casein kinase II. In addition, motifs weakly conserved in a wide variety of acetyltransferase (motifs A and B) [11],[39] and strongly conserved in AANATs (motifs C, D and E) are underlined [24]. (B) Comparison of the structures of the pineal AANAT cDNAs from C57BL/6J and C3H/HeJ mice. PCR was performed on cDNA prepared from mouse pineal total RNA using primers derived from exons 1 and 4 of the mouse AANAT gene. The asterisk indicates the location of the premature stop codon that interrupts the open reading frame. The dashed box represents the region in the C57BL/6J cDNA that corresponds to the open reading frame in the C3H/HeJ cDNA but which is not expressed due to the premature stop codon. A PCR product with the same 102 base pair insert was also obtained with C57BL/6J mouse retinal cDNA (Genbank acc. no. AF004111) using primers from exons 2 and 4 and corresponding to bases 3136–3155 and 5107–5082 in the msg3 clone.

specific antiserum (As2821) [19] that was raised against rat AANAT_{50–150} and affinity purified against recombinant rat AANAT protein as previously described [38].

2.7. Intron 3 sequence analysis

PCR was performed on 1 μ g of mouse liver genomic DNA with primers from exons 3 and 4 and corresponding to bases 3784–3803 and 4401–4382 of the msg3 clone. The resultant PCR products were subcloned without gel purification into pGEM-T Easy and sequenced in both directions with a Perkin-Elmer Applied Biosystems 370A DNA sequencer using *Taq* dideoxy termination sequencing kit (Perkin-Elmer, Norwalk, CT). To confirm sequencing results, 4 independent clones of the C3H/HeJ AANAT intron 3 PCR product, and 7 independent clones of the C57BL/6J AANAT intron 3 PCR product were sequenced.

3. Results

3.1. Isolation of the mouse AANAT gene

The mouse AA-NAT gene was cloned from a 129SV mouse genomic library using the rat AA-NAT full-length cDNA as a probe [33]. Determination of intron/exon junctions was made by comparison of the genomic sequence to that of C3H/HeJ cDNA described below. This revealed 4 exons and 3 introns (Fig. 1), which is the same genomic organization reported previously for the human [10], sheep and chicken AANAT genes [24].

Using the AANAT gene as a probe, we confirmed a recent report that located the mouse AANAT gene on chromosome 11 in region E1.3–2.3 [43], which is syntenic with the location of the human AANAT gene [10,13]. This is also consistent with the location of a mutation that knocks down AANAT activity as determined by analysis of recombinant inbred strains of mice obtained from an initial cross of C3H/He and C57BL/6 mice [20]. This suggests that a mutation in the AANAT gene itself, and not in an AANAT regulatory gene, is responsible for the decreased AANAT activity in C57BL/6 mice.

3.2. Northern blot analysis

To determine if AANAT mRNA is expressed normally in C57BL/6J mice, Northern blot analysis was done on RNA obtained from the C57BL/6J strain and the C3H/HeJ strain, which has functional AANAT activity. Total RNA was prepared from various tissues of mice sacrificed during day or in the evening. The results show that the night/day and tissue specific patterns of expression of AANAT mRNA are similar in both strains of mice (Fig. 2).

Pineal RNA from both strains of mice showed a night-specific AA-NAT signal which consisted of five bands of 1.6kb, 1.8kb, 2.3kb, 2.6kb, and 5.2kb (Fig. 2). The 1.6kb band is of approximately the same size as that previously

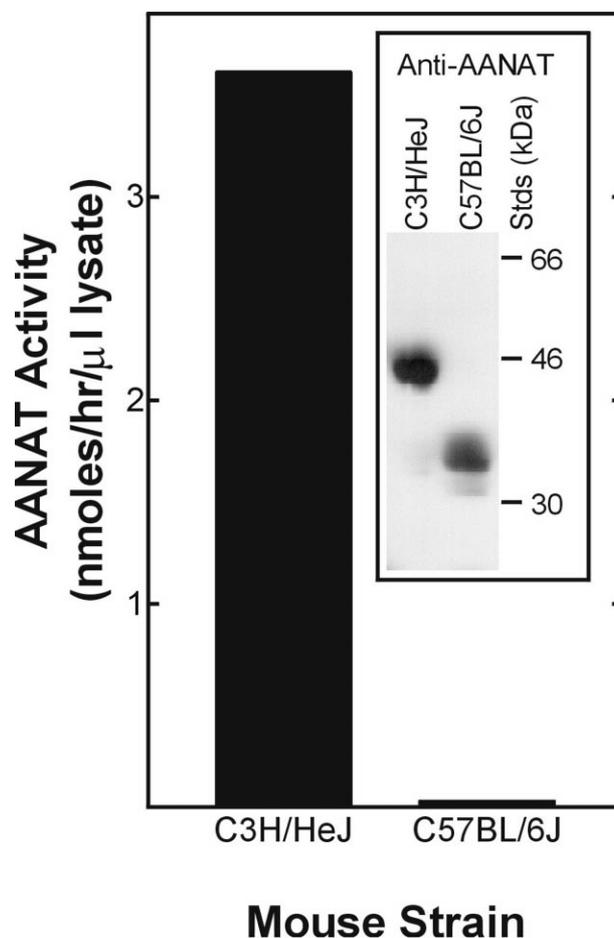


Fig. 4. Comparison of recombinant AANAT proteins expressed from the C57BL/6J and C3H/HeJ AANAT cDNAs. The cDNAs were subcloned into an expression vector and expressed as glutathione-S-transferase fusion proteins in bacterial cells. For the C3H/HeJ AANAT the subcloned insert corresponds to the open box in Fig. 3B and for the C57BL/6J AANAT the insert corresponds to the two open boxes in Fig. 3B including the intervening 102 base pair sequence. The bar graph presents AANAT activity measured in cell lysates. The boxed insert contains an immunoblot of the bacterial lysates generated using an affinity purified AANAT-specific antiserum (As2821). The estimated molecular weight of the C3H/HeJ AANAT fusion protein is 45.7 kDa and that of the C57BL/6J AANAT fusion protein is 36.3 kDa. The smaller C57BL/6J fusion protein is consistent with truncation of AANAT due to the premature stop codon described in Fig. 3B.

reported for rat pineal AANAT mRNA (1.7kb) [33]; the identity of the other four bands is unknown. In both strains of mice the relative abundance of the five bands was 5.2kb \gg 2.6kb \cong 2.3kb \cong 1.8kb \cong 1.6kb. Additionally, in neither strain was the AANAT mRNA signal detected by Northern blot analysis in the retina, cerebellum, cortex, pituitary, hindbrain, midbrain, olfactory bulb, hypothalamus, hippocampus, striatum, heart, liver, kidney, small intestine, or testes (Fig. 2)³.

³ A faint signal of approximately 4.5 kb was detected in day and night retina from both the C3H/HeJ and C57BL/6J mice, and in night heart, hypothalamus, and hippocampus from the C3H/HeJ mice. The identity of this signal is unknown.

3.3. cDNA sequence analysis and recombinant protein expression

To determine if there were sequence differences in the AANAT mRNA between the two strains of mice, the

pineal AANAT cDNAs were cloned from C3H/HeJ and C57BL/6J mice and the sequences compared. C3H/HeJ AANAT mRNA encodes a 205 amino acid protein (molecular weight = 23.1 kDa, pI = 7.4). The deduced protein sequence is 97% similar and 96% identical to the deduced

A.

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cDNA      CTTACTCAG..... 458
          |||||
Intron 3  CTTACTCAGGTGAGGGCAGGGGAGCTCCCGGGATGGAAGTCTCCTTCA 100
          Original Donor

cDNA      ..... ---
Intron 3  TTTTCATTGCTACTTCA†CAACTATAATCTTGTTACTGCTATAAATTGTAAT 300

cDNA      .....TCTTAGTCGACTCCTATGAAA 482
Intron 3  CTAAATCTCTGTGTTTTCTTTTTTTACAGT‡CTTAGTCGACTCCTATGAAA 350
          New Acceptor

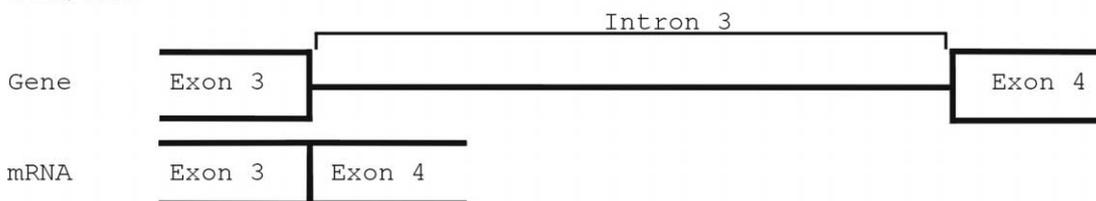
cDNA      CAGTCGTTTGACCTCCAAAGGGGTCACAACCCACAGGTTGAGAATTGCTG 532
          |||||
Intron 3  CAGTCGTTTGACCTCCAAAGGGGTCACAACCCACAGGTTGAGAATTGCTG 400

cDNA      CTTTAGAGTCCCGAACTGTAGGACTTTAGAT..... 563
          |||||
Intron 3  CTTTAGAGTCCCGAACTGTAGGACTTTAGATGTTAGGTAGCAGTGGGGTCC 450
          New Donor

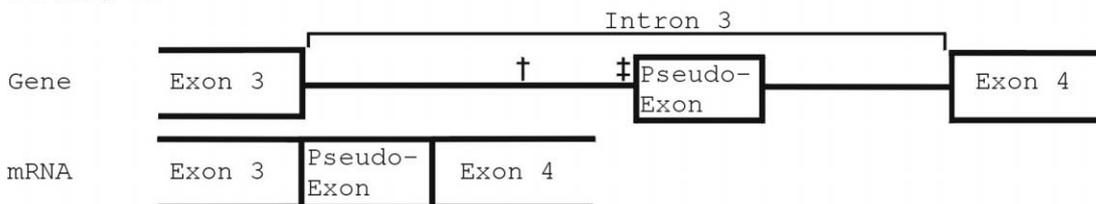
cDNA      .....GAGTCGCTGACACTACACAGGCCCGGAGGCCGC 596
          |||||
Intron 3  CCCTTGCCCATCCCCAGGAGTCGCTGACACTACACAGGCCCGGAGGCCGC 600
          Original Acceptor
    
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B.

C3H/HeJ



C57BL/6J



rat AANAT protein sequence [14,33] (Fig. 3A) and is generally similar to other AANAT sequences [25]. In contrast, the C57BL/6J pineal mRNA is 102 base pairs longer than the C3H/HeJ mRNA, yet encodes a protein that is 100 amino acids shorter. A premature stop codon is present in the 102 base pair insert (Fig. 3, A and B); based on this it is predicted that the encoded protein will be truncated.

This prediction was examined by expressing the C3H/HeJ AANAT coding region and the corresponding regions of the C57BL/6J AANAT cDNA, including the 102 base pair insert, as glutathione-S-transferase fusion proteins. It was found that the C57BL/6 AANAT protein is approximately 10 kDa smaller than C3H/HeJ AANAT protein (Fig. 4). The predicted protein would lack the putative catalytic and acetyl-CoA binding domains and therefore would be inactive [25]. This is consistent with the observation that the expressed C57BL/6J AANAT fusion protein has less than 1% of the activity of a similar amount of C3H/HeJ AANAT fusion protein (Fig. 4).

3.4. Intron 3 sequence analysis

Comparison of the 102 base pair sequence to the mouse AANAT gene revealed that the sequence was identical to a portion of intron 3. Analysis of intron 3 identified a G \leftrightarrow A conversion in the C57BL/6J sequence two bases upstream of the 102 base pair sequence (Fig. 5A). The conversion occurs in a cryptic splice acceptor site and increases the consensus score for the splice site from 76 to 92 (out of 100) as compared to a score of 88 for the C3H/HeJ site [37]. The activation of this cryptic acceptor splice site would result in the preferential use of a cryptic donor splice site to define the 102 base pair pseudo-exon in the mature C57BL/6J AANAT mRNA according to the 'exon definition' model of RNA splicing [5] (Fig. 5B). Such a mechanism is considered to be very rare [30].

In addition to the G \leftrightarrow A conversion described in the text, a second G \leftrightarrow A conversion in the C57BL/6J intron 3 sequence was located 64 base pairs upstream of the 102 base pair sequence. This single base difference is not contained within a consensus splice sequence and the functional significance, if any, is unknown at present.

4. Discussion

The present study indicates that the low level of AANAT activity in C57BL/6J mice results from a point mutation

in the AANAT gene, which in part can explain the decreased pineal melatonin production in this strain of mice. AANAT activity is also severely compromised in the pineal gland of several other strains of mice [16]. However, preliminary findings indicate that the critical G \leftrightarrow A conversion in the C57BL/6J strain is not seen in BALB/c and 129SV strains, suggesting that in these strains other mutations have knocked down melatonin signaling. Further evidence of multiple mutations in this system is supported by the finding that the last enzyme in melatonin synthesis, hydroxyindole-O-methyltransferase (HIOMT) is also undetectable in some strains [16].

These findings raise the question of the significance of the mutation identified in this study. Melatonin appears to play a role in the readjustment of rhythms in response to changes in photoperiod. Examination of circadian behavior in C3H/He and C57BL/6 strains reveals both have robust circadian locomotor rhythms that entrain to light-dark cycles and display light-induced phase shifts [4,36,41]. Direct comparison of these strains indicates that C57BL/6 mice adjust more rapidly to a reversal of the light/dark cycle as compared to C3H/He mice [27]. This suggests that melatonin modulates the effects of light on circadian rhythms in mice and that further analysis of strains that differ in their ability to produce melatonin will provide insight into the effects of melatonin on circadian rhythms.

These findings also lead to the question of what was the selective pressure to 'knockdown' melatonin signal transduction in laboratory mice. We suspect that this may involve the inhibitory effects melatonin has on reproduction in rodents and that the selective pressure for knocking down melatonin signal transduction was successful reproduction in a laboratory setting, in which lighting intensity and/or cycle might otherwise inhibit reproduction [8,9]. If this is correct, it is probable that those strains of mice that synthesize melatonin have a defect downstream in the signaling pathway which knocks out inhibitory effects of melatonin on reproduction.

It is clear that inbred mice are a source of natural mutations in the melatonin signal transduction pathway. Identification of the sites of these mutations in known components of the system will make it possible to knock in functional genes and determine the effect of such manipulations. For example, it will be of interest to determine if knocking in functional AANAT and HIOMT in C57BL/6J mice will suppress reproduction and increase the size of the pineal gland, which is smaller in this strain,

Fig. 5. Strain differences in AANAT gene sequence and RNA processing. (A) Sequence comparison between a fragment of the C57BL/6J mouse AANAT intron 3 PCR product and the corresponding portion of the C57BL/6J mouse pineal AANAT cDNA. The regions corresponding to the consensus sequences for donor and acceptor splice sites are underlined. The locations of the two single base differences between the C3H/HeJ and C57BL/6J AANAT intron 3 PCR products are denoted by † and ‡. The mutation denoted by ‡ results in the activation of a cryptic acceptor splice sequence and the inclusion of 102 base pairs of intron 3 in the mature mRNA for the C57BL/6J pineal AANAT. (B) Schematic representation of the structure of the C3H/HeJ and C57BL/6J mouse AANAT gene and mature mRNA sequences in the region of intron 3. Open boxes represent flanking partial exonic sequence, solid lines represent intron 3, and closed boxes represent that portion of intron 3 that is retained in the mature C57BL/6J pineal AANAT mRNA.

relative to the C3H/HeJ strain (Baler et al., unpublished observation, [7,28]). It will also be of interest to determine if this alters visual transduction in the retina, a common site of melatonin synthesis in vertebrates. Eventually, it should also be possible to use inbred mice to identify mutations in unknown components of melatonin signal transduction pathways using reverse genetics.

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