Hydroxyindole-O-methyltransferase (HIOMT) activity in the retina of melatonin-proficient mice

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ABSTRACT

Numerous pieces of evidence support the expression by the mammalian retina of Hydroxyindole-O-methyltransferase (HIOMT, EC 2.1.1.4), the enzyme directly responsible for the biosynthesis of the pineal chronobiotic hormone melatonin (MLT). However, conflicting results obtained so far by enzyme-kinetic and immune-detection techniques still make HIOMT presence and relevance in the eye a matter of debate. This work aimed at evaluating unambiguously HIOMT activity in the mouse retina, a valuable model for studying the effects of MLT variations on ocular pathophysiology. Since laboratory mouse strains can bear genetic polymorphisms yielding defective enzymes of MLT biosynthesis, retinas and control pineal glands used in this study were obtained in a MLT-proficient crossing of A/J mice, the A/J/C57BL/10 strain. To improve the radiochemical reference assay, we tested different homogenization procedures coupled with HPLC detection. Concomitantly, we quantified MLT, and its precursor N-acetyl-serotonin (NAS) by HPLC coupled to electrochemical detection in retinas isolated from either light- or dark-adapted mice.

Results showed that the standard radio-chemical assay was successful for pineal HIOMT only, whereas specific homogenization buffers and HPLC were required to detect retinal activity, presumably due to interfering methyl-transferases inhibited by NAS. Under present conditions, retinal HIOMT Vmax accounted for by 3.5 fmol/h/mg protein, 2.6 hundreds-fold lower than the pineal counterpart, displaying equivalent KM(s) (~10 μM). Moreover, NAS and MLT rapidly decreased in light-exposed isolated retinas, corroborating light-sensitive in-situ MLT formation. Conclusively, we measured mouse retinal HIOMT kinetics under basal conditions, a useful result to elucidate the regulatory patterns, the possible impact on eye health, and therapeutic approaches related to this enzyme.

1. Introduction

Melatonin (N-acetyl-5-methoxy-tryptamine, MLT) is a methoxyindole endowed with a variety of biological activities in almost all living organisms (Tan et al., 2003; Macchi and Bruce, 2004; Reiter et al., 2014). In most vertebrates, MLT is an endogenous molecule, rhythmically synthesized and released at nighttime by the pineal gland, a neuroendocrine pacemaker that modulates the response of the whole organism to circadian light-dark cycles and photoperiod variations (Clastrat et al., 2005). Mammals, unlike lower vertebrates (Macchi and Bruce, 2004), control the biosynthesis of MLT through a neuronal circuit involving the retinal-hypothalamic tract, the Suprachiasmatic Nucleus (SCN), superior cervical ganglia and the pineal gland (Brzezinski, 1997; Arendt, 1998). This circuit converts environmental photic inputs into either the increase or inhibition of MLT synthesis by pinealocytes, being part of the body’s timekeeping system, such as sleep-time programming or photoperiod-related breeding, depending on the mammalian species (Pandi-Perumal et al., 2008).

Two main enzymatic steps catalyze MLT formation by the pineal gland: a first acetylation reaction, which transforms the tryptophan-derived neurotransmitter serotonin (5-HT) to N-acetylserotonin (NAS) by Arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87); a methyl-ination step then converts NAS and the co-substrate S-adenosyl-L-methionine (SAM) (Sugden et al., 1986) to MLT and S-adenosylhomocysteine by hydroxyindole-O-methyltransferase (HIOMT, EC 2.1.1.4), also known as

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Acetylsalicylic acid (ASA) or aspirin, a pain medication with anti-inflammatory and antiplatelet properties, also plays a role in modulating the circadian rhythm. It has been shown to inhibit the production of prostaglandins, which are involved in the inflammatory response, and it may also affect the expression of certain genes involved in the circadian clock, such as the PER2 gene.

The study by Hickey et al. (2016) found that ASA administration during the dark phase of the circadian cycle produced a significant increase in the expression of PER2 in the suprachiasmatic nucleus (SCN), which is the central pacemaker for the circadian rhythm. This suggests a potential role for ASA in resetting the circadian rhythm, which could have implications for the treatment of circadian disorders such as shift work sleep disorder.

In conclusion, the role of aspirin in the circadian rhythm is multifaceted and involves multiple pathways. Further research is needed to fully understand the mechanisms by which ASA affects the circadian system and how it may be used therapeutically.
All experiments complied with institutional guidelines in agreement with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and update, Directive 2010/63/EU.

2.3. Isolation of mouse retinas and pineal glands

All experiments used 4–8 weeks old mice. At this age stage (P30), the retina is fully developed, showing no age-dependent loss of photoreceptors. For HIOMT assay, mice were dark-adapted overnight (light off at ZT12, 7 pm, the day before the enzyme dosage) and kept in darkness till sacrifice at ZT2 (9 am) by a lethal dose of anesthetic (20% urethane in 0.9% saline i.p.). This timing was chosen to avoid possible interferences due to maximal levels of pineal activity at nighttime. Retinas were quickly isolated under dim red light, through a corneal slit made by an ophthalmic blade. Retinas were flash frozen in liquid nitrogen and then stored at -25°C until HIOMT assay. After upper skull bone removal under dim red light, the pineal was dissected from the bone under microscopic control using infrared light with the help of an infrared converter fitted on the dissecting microscope. The overall surgical procedure, from anesthetic administration to tissues freezing in liquid nitrogen, took less than 5 min, a timing that minimizes unspecific consequences on both retinal and pineal biochemistry.

In experiments conducted in support of MLT production by the retina and its physiological modulation by light, isolated retinas, obtained from dark-adapted mice sacrificed at ZT23, were immediately bathed in Locke’s solution, which has been shown to keep mouse retinas viable and light responsive up to 8 h (Demontis et al., 2009, 2012). The two retinas from each mouse, either dark- or light-adapted, were assigned to different samples to reduce between-mice variability.

2.4. HIOMT radio-enzymatic assay

For HIOMT assay, frozen retinas or pineal glands from 6 mice were suspended in ice-cold homogenization buffer (100μl retina or pineal), and sonicated three times for 3 s each, using the ultrasonic unit set at 0.3mW (Transsonic 420, ELMO). Three different homogenization buffers, buffers a, b, and c, were compared, as specified in Table 1, depending on either the presence or the absence of Dithiothreitol (DTT) or Triton-X100, respectively. After sonication, homogenates were centrifuged for 10' at 14,300g at 4°C, and supernatants used for HIOMT assay. Different sub-cellular fractions were also tested.

HIOMT activity was first assayed by measuring NAS conversion to MLT using [3H]-SAM and NAS as substrates, according to the reference method (Axelrod and Weissbach, 1968; Sugden et al., 1986). Unless specified, each tube contained 100μl of the sample (0.6–0.9 mg protein), either 50 or 0 μM NAS, 1–25μM cold SAM, and 0.1μM [3H]-SAM (corresponding to 379,922 dpm) in a final volume of 200μl of phosphate-buffered saline (PBS) 50mM pH 7.9. First, as a control, we carried out experiments on pineal glands of A/J mice. For pineal HIOMT, time-course pilot experiments were preliminary carried out to set assay duration (incubation time), up to 45 min, to prevent the deviation from linearity for times >1h, due to substrate depletion by the high enzymatic activity (data not shown). The incubation temperature was 30–32°C, and the reaction stopped by adding 200μl of Borate buffer 0.45M (pH 10) and 1 ml water-saturated chloroform (CHCl3). The chloroform phase was then washed twice with 200 μl borate buffer and the upper aqueous phase aspirated after careful mixing. The chloroform phase (800 μl) was then transferred into a pony vial, oven dried and counted by a β-counter Packard 1600, after the addition of 5 ml scintillation liquid, according to the reference method described by Sugden et al. (1986).

2.5. High sensitive and specific HIOMT radio-enzymatic assay

Both assay sensitivity and specificity of the reference method increased in response to the following changes: sensitivity improved by using a higher [3H]-SAM concentration, up to 0.5μM (corresponding to 23,563,635 dpm), as well as by adjusting specific activity for saturation analysis from 20.6 to 1.65 Ci/mmol by cold SAM (2.5-4-7.5-10-18.5-25μM). The assay specificity increased upon introducing an HPLC step, to identify and quantify [3H]-MLT generated by HIOMT from exogenous NAS and SAM/[3H]-SAM.

Briefly, for the improved assay, the first step was carried out as described in the previous paragraph, leaving unchanged the incubation temperature (30–32°C) and the CHCl3 extraction procedure. At the end of the reaction, dried chloroform extracts were either immediately analyzed or stored at -80°C for later separation. Chloroform extracts injected either soon after drying or after one week at -80°C showed comparable chromatographic results, indicating sample stability after freezing. Dried chloroform extracts were analyzed by HPLC, using reversed-phase liquid chromatography (RP-LC), and eluted fractions collected for radioactivity counting.

The HPLC chromatograph device consisted in a high pressure binary pump module (Ultimate 3000 Dionex, Thermofisher Scientific, Waltham, MS, USA) coupled to a Pulsed-Electrochemical Detector (Dionex, Thermofisher Scientific, Waltham, MS, USA) equipped with an electrochemical cell composed by a graphite working electrode, set at the fixed voltage of +0.9 V, and an AgCl/Ag reference electrode. These modules were all managed by the Chromelone HPLC software (Dionex, Thermofisher Scientific). The injection valve (Reodyne) connected to a 100μl injection loop. For the chromatographic analysis, we slightly modified conditions described by Harumi et al. (1996). The mobile phase for isocratic separation was: 25% methanol and 75% aqueous 0.1 M NaH2PO4 (v/v), containing 4 mM sodium decansulphonate as ion-pairing agent, 1 mM Na-EDTA, pH 5. The analytical column was a C18 Atlantis T3, 150 x 4.6 mm, packed with 5 μm particles (Waters SpA, Milford, MA, USA) assembled with a Nova-pack C18 20 x 3.9 mm guard column (Waters SpA). The flow rate was set at 1 ml min⁻¹ at 30°C and total run time 35 min. Before assaying HIOMT assay in tissue samples, the MLT peak was identified by chromatographic runs of MLT standard solutions at known amounts (0.5–5.0 pmol), accordingly to retention time, under afore-described HPLC conditions. Specifically, to warrant the identity of the HIOMT-derived [3H]-MLT, we compared retention times in chromatograms of retinas’ spiked with known amounts of an MLT standard solution (1–2 pmoles) extracted in chloroform and those reported in the same extracts after the enzyme reaction.

Sample carryover and interferences deriving from sequential injections of different extracts were circumvented by carefully washing the injection valve as well as by mobile phase injections between each chromatographic run of retinal preparations or standards. As for the pineal gland, we set the incubation time for retinal HIOMT up at 75 min (linear, data not shown) by time-dependence experiments. For saturation kinetic analyses, six cold SAM concentrations were used, and the assay carried out as reported above. For HIOMT assay, dried chloroform extracts were dissolved in 120 μl of HPLC mobile phase and injected (100 μl) into the chromatograph under the same conditions; during the chromatographic runs, elution fractions (1 ml) were collected into pony vials for 30 min, oven-dried and radioactivity counted by a β-counter Packard 1600, after adding 5 ml of scintillation liquid.

The enzyme activity was expressed as fomtomoles of the product formed/hour/mg protein in the assays. Protein concentration was determined by the Coomassie Brilliant Blue G-250 colorimetric method under acidic conditions, using γ-globulins as the standard (Bradford BioRad assay kit).

### Table 1

| Experimental condition | PBS | pH | DTT | Triton-X100 |
|------------------------|-----|----|-----|-------------|
| Buffer a                | 50 mM | 7.9 | 1 mM | 0.00 %      |
| Buffer b                | 50 mM | 7.9 | 1 mM | 0.02 %      |
| Buffer c                | 50 mM | 7.9 | 0 mM | 0.02 %      |
2.6. HPLC assay of endogenous MLT and NAS using ElectroChemical detection (ECD)

For the measurement of endogenous MLT and NAS, freshly-isolated retinas from 9 dark-adapted mice were kept in saline for 90 min, either in darkness or in the presence of room light. After incubation, retinas were flash-frozen in liquid nitrogen and then stored at -25 °C. Frozen samples were homogenized in 0.1 M ice-cold trifluoroacetic acid (TFA) using a glass–glass potter (about 20 strokes). The homogenization ratio was: 6 retinas/500 μl TFA. The ensuing homogenate was collected, diluted with additional 500 μl of ice-cold TFA, and the final homogenate centrifuged at 10,000×g for 30 min at 4 °C. The supernatant was collected, pH adjusted to 4.5 with 1N KOH while adding EDTA to a final concentration of 1 mM. Buffered EDTA-containing supernatants were then slowly filtered through 0.2 μm filters (13 mm GHP, Waters). C18 sample extraction cartridges (Sep-Pak, Waters) were pre-conditioned with 2 ml methanol, and 2 ml distilled water, according to manufacturer instructions. After loading the filtered supernatants and removing water-soluble components by 2 ml of 10% v/v methanol in MilliQ water, compounds of interest were eluted with 1 ml of methanol, and the eluates subsequently dried using a high-vacuum device (Savant, SpeedVac concentrator SC100, ThermoFisher Scientific), attached to a refrigerated trap (Savant RT4104). Dried samples were stored at -25 °C until assay. The recovery of the procedure was about 98% for MLT and NAS, as assessed by the ratio between measured and added amounts of reference compounds, either for standard solutions or homogenates spiked with known quantities of MLT and NAS. For retinal analyses, dried samples were concentrated 3.5× in the mobile phase, sonicated and injected into the chromatograph under the same chromatographic conditions and procedures described above for HIOMT enzyme assay. Peaks of interests were identified by the retention time of known standards. Calibration lines of NAS and MLT were also carried out by spiking samples with known amounts (0, 1, 2 and 5 pmol) of standards to enable quantitative analysis, according to peak areas: the slope of the linear regression analysis between charge and pmoles of standards was used to convert sample areas into pmoles. Peak area integration was carried by fitting data with a Gaussian function implemented in Origin 6.0 Pro (MicroCal Software Inc., MA, USA). The same program was also used for identifying NAS and MLT peaks in spiked and non-spiked extracts.

2.7. Statistics

Data are expressed as the mean ± SEM. HIOMT Michaelis-Menten kinetic parameters (Km, μM, and Vmax, fmoles/h/mg protein) were determined by regression analysis using Graph Pad Prism software (version 5.0, S. Diego, CA USA). Student t-test was carried out for statistical comparisons, with the two-tailed statistical threshold set at P = 0.05.

3. Results

3.1. HIOMT radio-enzymatic assay

Table 2 reports radioactive counts measured in homogenates of either pineal glands or retinas isolated from A/J or C57BL/6 mice using the HIOMT reference radiochemical method described above (45 min incubation at 30–32 °C), under three different experimental conditions (Table 1). Using Buffer a, containing 1 mM DTT without Triton X-100 as reported in the reference method (Sugden et al., 1986), about 80% of radioactive counts in the chloroform phase were dependent on exogenous NAS, consistent with a specific activity in the supernatant fraction of pineal glands. Pineal crude homogenates and supernatants provided quite comparable results using Buffer a (data not shown) in A/J mice. Fig. 1 plots a typical HIOMT saturation kinetic curve obtained in the crude homogenates from pineal glands of A/J mice using Buffer a. In these conditions, we failed to detect a specific activity in retinal supernatant fractions: radioactive counts were higher in the absence (0 NAS or blank) than in the presence of exogenous NAS (Table 2).

By homogenizing both pineal and retinal samples in Buffer b, containing 1 mM DTT and 0.02% Triton-X100 to permeabilize membranes, we could observe an overall increase of non-specific counts in pineal gland supernatants, while reporting an about 10-fold increase in total counts in retinal supernatant fractions, still displaying higher radioactivity in the absence of NAS (Table 2). In retinal pellets, under these same experimental conditions, we found specific HIOMT activity of about 19% of total counts (Table 2).

When using Buffer c conditions, containing 0.02% Triton-X100 only, we could observe specific counts in both supernatants and pellets from A/J mouse retinas. Under this condition, pineal homogenates showed again increased non-specific counts. In control experiments using the same homogenization buffer, we also evaluated HIOMT activity in crude pineal homogenates, containing both supernatant and pellet fractions, from the low-prolific MLT C57BL/6 mouse strain (Kashara et al., 2010). Data indicate a HIOMT specific activity (NAS-dependent) in C57BL/6 pineal glands (about 2,100 dpm) lower than in A/J ones (about 6,500 dpm) (Table 2). Assays carried out in retinal homogenates of C57BL/6 vs. A/J/C57BL/10 mice, also showed higher specific NAS-dependent counts, by considering the sum of supernatant and pellet specific dpm counts.

3.2. High specific and sensitive HIOMT radio-enzymatic assay

To address the puzzling results presented in Table 2, we resolved by HPLC the MLT, newly synthesized by HIOMT using NAS as a substrate, from unrelated labeled compounds in the total chloroform extracts.

Under the established chromatographic conditions, as indicated in the Material and Methods Section, MLT standards showed a retention time of 17.5 min. Using this approach, injections of resuspended dried chloroform extracts from both retinal pellets and supernatants into the chromatograph, and peak quantification by ECD revealed that labeled chloroform extracts, derived from two independent experiments. HIOMT activity was assayed as indicated in the Material and Method section, using blanks without NAS and without enzyme. For assays related to homogenizations in Buffer a and Buffer b, 2.5 μM SAM and 0.04 μM [3H] SAM were used; for Buffer c assays, 2.5 μM SAM and 0.1 μM [3H] SAM were used. Total protein amounts were 0.5 and 0.7 mg for pineal gland and retinal homogenates, respectively.

Sub-cellular compartment: Supernatant, P Pellet, H Homogenate.

Table 2

| Buffer    | a    | b    | c    |
|-----------|------|------|------|
| NAS (μM)  | 50   | 0    | 50   |
| Pineal A/J-C57BL/10 | 9.775–10,405$^{5}$, 1,673–1,815$^{5}$ | 10,000–10,336$^{5}$ | 4,014–4,014$^{5}$ | 1879–11089$^{91}$, 4930–5010$^{91}$ |
| Pineal C57BL/6 | NA   | NA   | NA   | 7804–7708$^{91}$ | 5543–5739$^{91}$ |
| Retina A/J-C57BL/10 | 691–923$^{5}$, 1,036–1,408$^{5}$ | 7,983–7,179$^{5}$ | 8,436–8,480$^{5}$ | 3,415–2,763$^{9}$, 1,855–2,016$^{9}$ |
| Retina C57BL/6 | NA   | NA   | NA   | 5,713–5,327$^{9}$ | 4,457–4,457$^{5}$, 2,175–2,090$^{9}$, 1,751–1,816$^{9}$ |

Values are [3H]-dpm counts of chloroform extracts, derived from two independent experiments.
products generated in the supernatant fractions of retinas homogenized in Buffer a and b were unrelated to authentic MLT. Indeed, Fig. 2 depicts superimposed experiments carried out in pineal and retinal supernatants using Buffer b: a prominent radioactive peak in the extracted pineal sample (open circles), eluted at about 17 min (MLT elution time). On the other hand, we did not observe a signal at the same elution time in the retinal chromatogram (filled circles), and the radioactivity eluted after about 6 min.

For retinal HIOMT assay by HPLC using Buffer c, we used crude homogenates to collect the MLT generated in both pellet and supernatants fractions (Table 2). For the A/J mouse retina homogenate under these experimental conditions, a radioactive peak elutes at the time of authentic MLT (Fig. 3, filled circles), increasing in proportion to unlabelled SAM concentrations. No [3H]-MLT was instead formed in the absence of exogenous NAS (Fig. 3, open squares), supporting the assay specificity. The chromatogram also indicates that a substantial part of chloroform-extracted tritium elutes either before or after the MLT peak, demonstrating that the resolution achieved by HPLC allows the separation of HIOMT-generated [3H]-MLT from other interfering radiolabeled compounds.

3.3. Saturation kinetic analysis of HIOMT in the pineal and retinal tissues

By monitoring HPLC-resolved MLT, we performed a kinetic analysis using SAM, the second substrate of HIOMT, to estimate HIOMT parameters in mouse retinal homogenates in the presence of 50 μM NAS, as described by Abe et al. (1999). Fig. 4 shows a typical saturation experiment and Lineweaver-Burk linearization (insert) to estimate the Vmax and apparent KM in A/J mouse retinas.

Table 3 reports instead the comparison of HIOMT kinetic parameters obtained in homogenates from A/J pineal gland (Buffer a) and those reported from crude retinal homogenates (Buffer c): the pineal gland has Vmax values more than 2-hundreds-folds higher than the retinal extracts, without apparent differences of the Michaelis-Menten constants, KMs.

Fig. 1. HIOMT saturation and Lineweaver-Burk plot analyses in mouse pineal gland homogenates. Representative Michaelis-Menten curve of mouse pineal gland HIOMT, homogenized in Buffer a and assayed under radiochemical standard assay conditions (Sugden et al., 1986) using SAM concentrations ranging from 1 to 25 μM and NAS 50 μM (0.75 h incubation at 30–32 °C). The inset displays the Lineweaver-Burk plot. SAM specific activity ranged from 8.16 to 0.32 Ci/mmol, as described in the Materials and Methods section.

Fig. 2. Chromatograms of the extracted pineal and retinal homogenates. Data points plot radioactive counts of HPLC-eluted fractions, following injections of samples derived from the supernatants of either retina (filled circles; 5,933 dpm injected) or pineal glands (open circles; 23,000 dpm injected) homogenized in Buffer b. HIOMT assays carried out by incubating samples with SAM (2.5 μM) and NAS (50 μM), at 30–32 °C for 75 min (retina) and 45 min (pineal).

Fig. 3. Chromatograms of extracted retinal homogenates. Data points plot counts of HPLC-eluted fractions following injections of HIOMT assay products carried out in permeabilized membranes of retinas homogenized in Buffer c. The HIOMT assay was carried out at 30–32 °C for 75 min. Symbols plot assays carried out in SAM 2 and NAS 50 μM (filled black circles; 11,680 dpm injected); SAM 25 μM and NAS 50 μM (filled gray circles; 1,226 dpm injected); SAM 2 μM and NAS 0 μM (open squares; 10,793 dpm injected).
rapidly suppressed the retinal synthesis of these compounds. Comparing the activity of HIOMT in mouse retina vs. pineal gland, Student’s t-test, p < 0.05 (**): HIOMT Vmax means were significantly different between pineal glands and retinas, Student t-test, p > 0.05 (****): HIOMT Km means were not significantly different between pineal glands and retinas; Student’s t-test, p < 0.05 (**): HIOMT Vmax means were significantly lower in retinas vs. pineal glands, Student t-test, p < 0.0001.

3.4. HPLC assay of endogenous MLT and NAS using ElectroChemical detection (ECD)

To support the endogenous production of MLT by retinas, we evaluated whether light might suppress MLT production in dark-adapted mice. Using conditions as mentioned earlier, the retention time of MLT in TFA-extracted retinal homogenates was 17.5 min, as already found for the chromatographic separation of standards, and 4.3 min for the precursor NAS. The observation of the reduced peak’s area in retinal samples isolated at ZT 9 vs. ZT 23 (data not shown) provides further support for the identity of the tritium-labeled peak eluting at 17.5 min.

We thus measured both MLT and NAS levels in isolated retinas either exposed to room light for 90 min or kept dark-adapted for the same time after isolation. For the analysis of MLT peak, a double Gaussian was used, whereas retinas in buffer a, whereas retinas in buffer c, pineal and retinal HIOMT were appraised as indicated in the Material and Method section. (Ns): HIOMT Km means were not significantly different between pineal glands and retinas, Student t-test, p > 0.05 (**); HIOMT Vmax means were significantly lower in retinas vs. pineal glands, Student t-test, p < 0.0001.

Table 3
Comparison between HIOMT enzyme kinetic parameters, measured in A/J/ C57BL/10 mouse tissues:

| A/J mouse tissues | HIOMT, Km (μM) | HIOMT, Vmax, fmoles/h/mg protein |
|-------------------|----------------|---------------------------------|
| Pineal gland      | 9.3 ± 0.52     | 9.550 ± 912                     |
| Retina            | 9.8 ± 0.26     | 36.5 ± 4.3**                   |

Data are presented as the mean ± SEM of 3 separate experiments. Pineal gland was homogenized in buffer a, whereas retinas in buffer c; pineal and retinal HIOMT were appraised as indicated in the Material and Method section. (Ns): HIOMT Km means were not significantly different between pineal glands and retinas, Student t-test, p > 0.05 (**); HIOMT Vmax means were significantly lower in retinas vs. pineal glands, Student t-test, p < 0.0001.

4. Discussion

The development of reproducible methods to measure both HIOMT activity and endogenous MLT levels in the mammalian retina is a key step to address the controversies on their local expression and functions. We succeeded herein, for the first time, to measure HIOMT kinetic rate in mouse retinal crude homogenates, using HPLC to separate NAS-derived MLT in the MLT-proficient A/J/C57BL/10 mouse strain (Kasahara et al., 2010). Coupling HPLC resolution with high-sensitive electrochemical detection for the determination of MLT, an approach already validated in the rodent retina (Harumi et al., 1996; do Carmo Buonfiglio et al., 2011), increased the sensitivity of the assay, by lowering the detection threshold from 150 fmol MLT (radioactive or fluorimetric immunoassays, Itoh et al., 1997), down to about 5 femtomoles. By this technique, HIOMT kinetic analysis in the retina returned comparable SAM Km for both retina and pineal tissues of A/J mice, close to those previously measured in the rat pineal gland (Sugden and Klein, 1983).

HPLC coupled to a double detector system, employing in-line fluorometric and electrochemical detectors (data not shown), considered one of the most valid detection devices applied to accurately measure indoles in body fluids (de Almeida et al., 2011), additionally confirmed the identity of MLT and NAS peaks. It seems therefore that mouse HIOMT has similar activity in pineal gland and retina, at variance with the occurrence of distinct isoforms reported in the rat Harderian gland (Cardinali and Wurtman, 1972). However, the issue remains to be clarified: the effects of subcellular fractioning and buffer composition observed in retinas of crossbreed A/J and C57BL/10 mice when using the reference radiochemical assay could reflect distinct regulatory or expression patterns in the eye, or result from distinct subcellular localizations of enzyme isoforms. Considering that immune-detection of retinal HIOMT in other species have met with conflicting results (Bernard et al., 1995; Coon et al., 2002; Rath et al., 2016) and that, to our knowledge, mouse anti-HIOMT antibodies are not available yet, it is challenging to address the subcellular localization of HIOMT in the mouse. In addition to the high HIOMT genetic variability (Kasahara et al., 2010), the possible expression of tissue-specific HIOMT isoforms, even truncated (Chen et al., 2018) may also contribute to the interindividual and between tissue variability. Our results stimulate the development of more specific molecular approaches and isolation strategies of the protein responsible of [3H]-MLT formation, such as more robust RT-PCR and Western blot methods, able to confirm the identity of the HIOMT protein in the...
Our results also promote the study of HIOMT activity in human retina. Although the human retinoblastoma cell line Y79 and human ARPE-19 cells express AANAT, HIOMT and produce MLT (Bernard et al., 1995; Zmijewski et al., 2009), the elevated genetic variance resulting from similar chromosomal organization in humans and mice, together with low-abundance expression may adversely impact HIOMT determination in the human retina. Indeed, similar to the mouse, human HIOMT is localized in the pseudoautosomal region PAR of the X chromosome (Yi et al., 1993) used for meiotic crossing over. PAR localization associated with a high frequency of allelic variants, some of which show a considerably reduced enzymatic activity (Pagan et al., 2011).

Intriguingly, HIOMT allelic variants in humans have been linked to psychiatric disorders (Etain et al., 2012; Talarowska et al., 2014), autism spectrum disorders (Jonsson et al., 2010) or sleep and neurodevelopmental disturbances (Botros et al., 2013), but limited data are available for eye diseases. To identify a link between MLT and eye pathology in humans, high-sensitive HIOMT assays, as the one applied in this study, together more selective molecular biology and immune-detection techniques, should be employed in human models too, including the use of multilayered retinal organoids (Nakano et al., 2012; Zhong et al., 2014). Improved knowledge about HIOMT properties will shed light on eye pathophysiology related to MLT proficiency in the retina of the various mammalian species, possibly defining new therapeutic approaches.

Although the role of HIOMT-generated MLT in the retina remain to be defined, the much lower HIOMT’s $V_{max}$ values in the retina than in the pineal gland of mice would seem to limit the physiological relevance of the retinal enzyme. However, several reflections arise from present results. Indeed, we clearly show here that a functional and MLT-producing HIOMT is detectable in the mouse retina, a result of relevance in light of previous conflicting reports on the expression and function of this enzyme in the retina (Bernard et al., 1995; Coon et al., 2002; Rath et al., 2016). Furthermore, the reported patterns of HIOMT activity (Rodriguez et al., 1994; Zhang et al., 2017; Chen et al., 2018), reveal the presence of HIOMT-like (HIOMTL) enzymes (Ried et al., 1998; Zhang et al., 2017) or hydroxyindole-MTs with distinct substrate-specificity (Tan et al., 2016), as well as different feature and expression between MLT-containing tissues, leading to the proposal of alternative MLT synthetic pathways in several extra-pineal tissues (Tan et al., 2016). Furthermore, HIOMT activity may catalyze the formation of other methoxyindoles from 5-HT, in particular, 5-methoxytryptophol, as observed in the retina and pineal gland of golden hamsters (Pevet et al., 1980). Some authors proposed that retinal MLT formation by HIOMT supports the powerful autocrine/paracrine and protective functions of this indoleamine, such as the protection of photoreceptors from oxidative stress induced by light exposition (Marchiafava and Longoni, 1999) during the dark phase (Zmijewski et al., 2009; Blasiak et al., 2016).

We report herein tissue-specific features of retinal HIOMT. Indeed, as previously observed by other authors in rodents and primates (Bernard et al., 1995; Coon et al., 2002), we measured HIOMT activity in the pineal gland of the MLT-proficient AJ/C57BL/10 strain, while failing to detect it in the supernatant fraction of the retina of these same animals, by means of the reference radiochemical assay described by Sugden et al. (1986). Besides, when we analyzed either different sub-cellular compartments or crude homogenates in PBS buffers containing 0.02% Triton...
X-100 to permeabilize cell membranes, and/or the redox-reducing agent DTT (Sudgen and Klein, 1987), we managed to detect HIOMT activity in the retina: in the pellet component or in both pellet and soluble fractions when using buffers b and c, respectively. Intriguingly, using soluble fractions of the retina generated using the reference buffer a or the Triton X-100- and DTT-containing buffer b we found higher radioactive counts in the absence than in the presence of NAS, suggestive of indoleamine-unrelated methylation. At the same time, buffer conditions permissive for HIOMT measurement in the mouse retina reduced the operation of the pineal enzyme, by increasing non-speciﬁc components. The entrapment of HIOMT in lipid micelles during retinal homogenization, modifying its accessibility to substrates, could contribute to these results, but does not fully explain the NAS effects observed when using buffers a or b. We thus hypothesized that spurious SAM-dependent methyltransferases (MTs) could be present in both the pineal gland and retina. These interfering MTs, activated by DTT and inhibited by NAS, would prevail over HIOMT in the soluble fraction of retina but have a marginal expression in pinealocytes. Distinct subcellular localizations may enhance the opposite activity pattern of HIOMT vs. competing MTs in the retinal and pineal tissues. Consistent with this hypothesis, compared to MLT-proliﬁcent mice, the MLT-defective C57BL/6 mouse strain had sharply reduced HIOMT activity in crude homogenates of the pineal gland, but not of the retina, when prepared in buffer c. These ﬁndings may indicate the expression of distinct MTs in these two areas and, as a consequence, the inability of the radiochemical reference method to accurately measure NAS-deriving MLT formation in retinal samples.

Some authors have questioned the relevance of retinal MLT production, since NAS displays protective actions on its own, by activating the neurotrophic receptor Trkb (Jang et al., 2010). Beyond NAS synthesis, AANAT has been proposed as the primary physiological player in the retina (Klein, 2007; Coon et al., 2002), because the enzyme also acts as a detoxifying enzyme that prevents the accumulation of toxic all-trans-retinal derivative (2E) (Klein, 2007). Present results highlight that AANAT activity may play an important additional role by its ability to enhance MLT formation in the retina.

5. Conclusions

This study demonstrates, for the ﬁrst time, the presence of NAS-dependent and MLT-producing HIOMT in the mouse retina. To determine HIOMT saturation kinetic parameters in this tissue, different homogenization conditions and HPLC resolution were evaluated, to offset the interfering contribution of enzyme activities inhibited by NAS. Under our experimental conditions, retinal and pineal SAM K_M values were similar, showing however much lower V_max values in the retina than in the pineal gland. Despite the low basal HIOMT activity found in the retina, this result represents a valuable starting point to characterize this enzyme in the eye as well as to investigate the tissue-speciﬁc remodeling of the MLT-synthesizing pathway in different mammalian species, including humans. Insights into retinal HIOMT would provide a more detailed comprehension of the respective role of the pineal- or retinal-derived MLT in maintaining ocular chemical homeostasis.

Declarations

Author contribution statement

Laura Betti, Lionella Palego: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Gian Carlo Demontis: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Fabiana Miraglia: Analyzed and interpreted the data.

Gino Giannaccini: Conceived and designed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conﬂict of interest.

Additional information

No additional information is available for this paper.

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