



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



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ARTICLE INFO

Keywords:

Neuroscience
Cell biology
Biochemistry
Ophthalmology
Endocrinology
Proteins
Metabolite
Mouse
Retina
Hydroxyindole-O-methyltransferase
Melatonin

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 methyltransferases inhibited by NAS. Under present conditions, retinal HIOMT V_{max} accounted for by ≈ 40 fmol/h/mg protein, 2.6-hundreds-fold lower than the pineal counterpart, displaying equivalent K_{Ms} (≈ 10 μ M). 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 (Claustrat 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 methylation 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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– acetylserotonin-O-methyltransferase (ASMT) (Axelrod and Weissbach, 1960; Yu et al., 1993; Borjigin et al., 1999; Simonneaux and Ribelayga, 2003), according to a Bi–Bi kinetic scheme.

Once released from the pineal gland into the bloodstream at nighttime, MLT reaches all body districts and interacts with specific membrane-bound or intra-cellular receptors (Reiter et al., 2014). A variety of extra-pineal organs, tissues, and cells from vertebrates and mammals also generate appreciable amounts of MLT (Kvetnoy, 1999; Acuña-Castroviejo et al., 2014). Precisely, in mammals, extra-pineal MLT has been measured in: the retina (Quay, 1965; Cardinali and Rosner, 1971; Pévet et al., 1980; Heuther, 1993; Tosini and Menaker, 1996; Chanut et al., 1998; Fukuhara et al., 2004), lens (Abe et al., 1999), Harderian gland (Pévet et al., 1980; Djeridane et al., 1998), ovary (Itoh et al., 1997), cortical astrocytes (Liu et al., 2007), skin (Slominski et al., 2008), gut (Chen et al., 2011), immune cells (Carrillo-Vico et al., 2013) and many other tissues, organs and systems at the intra-cellular level (Kvetnoy, 1999). Such ubiquitous MLT distribution, its persistence after pinealectomy, and the presence of AANAT and HIOMT in some of these tissues/cell lines, prompted the notion that intracellular levels of MLT in extra-pineal tissues result do not result from its uptake from extracellular fluids, but rather reflect its *de-novo* biosynthesis (Reiter et al., 2010; Tan et al., 2010; Acuña-Castroviejo et al., 2014). The functional relevance of extra pineal MLT goes beyond its autocrine/paracrine activities to include receptor-independent mechanisms of action (Reiter et al., 2010). The defense against reactive oxygen and nitrogen species (ROS and RNS) or other endogenous free radicals (Reiter et al., 2014, 2017), may represent a primeval function of this molecule during life evolution (Hardeland et al., 2011; Acuña-Castroviejo et al., 2014; Reiter et al., 2017).

Enzymes of the MLT pathway, their expression, activity, and rhythmicity in several extra-pineal sites lack a full characterization yet; the retina has been the first non-pineal tissue investigated for the presence of HIOMT, due to the presumed common phylogenetic origin of photoreceptors and pinealocytes (Foster et al., 2003). These studies started more than 30–40 years ago, focusing on the expression of HIOMT in mammalian retinal slices/extracts by means of immunohistochemistry, Western-blot and enzyme kinetic analyses, but provided controversial results (Cardinali and Rosner, 1971; Cardinali and Wurtman, 1972; Bubenik, 1974; Wiechmann et al., 1985; Wiechmann, 1986; Bernard et al., 1995; Coon et al., 2002; Rath et al., 2016). On the other hand, both human Y-79 retinoblastoma (Wiechmann and Burden, 1999) and retinal ARPE-19 (Żmijewski et al., 2009) cell lines express the enzymes and produce MLT.

RT-PCR or Northern blot gene expression analyses detected small amounts of *Hiomt* mRNA transcripts in retinas from rodents, non-human primates and humans (Rodriguez et al., 1994; Bernard et al., 1995; Coon et al., 2002). Interestingly, despite the detection of retinal AANAT in the non-human primate *Macaca mulatta*, HIOMT activity has been found instead undetectable in this species, showing however low-level transcripts that moderately increased at night (Coon et al., 2002). Moreover, two independent circadian clocks modulate the rhythmic expression of *Aanat* and increase MLT similarly to the pineal gland in the retina of golden hamster (Tosini and Menaker, 1996), mouse (Tosini and Menaker, 1998) and guinea-pig (Macchia et al., 2003), supporting a role of HIOMT in the circadian MLT synthesis by the eye. These puzzling results make the production and function of MLT in the retina of mammals still misunderstood and underappreciated (Tosini et al., 2012).

Instead, as a matter of fact, MLT may act in the retina as a multi-tasking effector, modulating the dark-adapted electroretinogram, photoreceptor renewing and disc shedding, pigment epithelium (RPE) turnover, ciliary movement, as well as neurotransmission, dopamine release and photoreceptor light sensitivity or viability (Dubocovich, 1983; Pang and Yew, 1979; Pierce and Besharse, 1987; White and Fisher, 1989; Baba et al., 2009, 2013). MLT has also been implicated in eye protection against diseases such as glaucoma and age-related macular degeneration (AMD) (Yi et al., 2005; Lundmark et al., 2007).

To our knowledge, no study has successfully determined HIOMT

activity in the retina of the mouse, a valuable animal model to investigate the physiopathology of MLT rhythms in the eye (Tosini et al., 2012; Hiragaki et al., 2014). This gap probably results from some laboratory mouse strains bearing genetic mutations affecting the enzymes of MLT biosynthesis, with significantly reduced or absent NAS and MLT production by the pineal gland in respect to other proficient strains (Kasahara et al., 2010). Intriguingly, *Hiomt* localization in the pseudo-autosomal region (PAR) of mouse sex chromosomes, may result in a high rate of genetic recombination (Kasahara et al., 2010). In humans, *HIOMT* also localizes within the PAR region (Yi et al., 1993), and display a high inter-individual enzyme variance (Bernard et al., 1995). Besides these genetic features, the possible expression of HIOMT isoforms, even truncated, has been proposed (Chen et al., 2018). It is thus possible that currently available analytical methods are not sensitive enough to detect HIOMT variants in the retinal counterpart, preventing the investigation of its function and regulation in the native tissue (Tosini and Menaker, 1998; Żmijewski et al., 2009; Tosini et al., 2012).

In the present work, we have addressed the question of the presence or absence of an active HIOMT enzyme in the mouse retina, as a preliminary investigation to understand the significance of its expression and role in the mammalian eye as well as to elucidate the regulatory pathways of MLT generation in this tissue. To this end, we used MLT-proficient mice were used (Kasahara et al., 2010) and measured HIOMT catalysis in retinal samples by the reference radiochemical assay (Sugden et al., 1986), taking the pineal activity as the control. We then tested different sample homogenization conditions and used HPLC to separate *de novo* formed MLT in the retina, as a measure of authentically NAS-deriving MLT (Abe et al., 1999). We also directly evaluated NAS and MLT levels in dark-adapted and light-exposed isolated retinas of A/J mice by HPLC coupled with electrochemical detection to support the local biosynthesis of this indoleamine, and also assessed the light sensitivity of the retinal MLT biosynthetic pathway.

2. Materials and Methods

2.1. Chemicals

All reagents used were of the highest analytical purity and HPLC-gradient grade. Standards of MLT, NAS, and SAM were 99% pure powders, purchased from Sigma-Aldrich, Milan, Italy. Water used for all experiments was of HPLC-gradient grade, treated by a purification Milli-Q equipment (Millipore, Milford, USA). We used the Bradford colorimetric assay for protein quantification (Bio-Rad). Perkin-Elmer, Milan, Italy, provided [³H]-SAM (specific activity: 80.7 Ci/mmol).

2.2. Animals

For all experiments, we used an MLT-proficient mouse strain, kindly provided by Dr. Wolfgang Berger (University of Zurich), which initially generated these mice by crossing MLT proficient AJ and melatonin-deficient C57BL/10 strains to identify spontaneous mutations responsible for cone-rod dystrophy in the latter strain (Wycisk et al., 2006). Mice used in this study were proficient for MLT and WT for the *Cacna2d4* gene, whose mutation leads to rod-cone dystrophy in both mice and humans; all mice had normal retinal morphology and function, as assessed by both ERG and immunostaining (Caputo et al., 2015).

Mice were born and raised in the local animal house under 12:12 dark:light (D:L) schedule, with light onset at 7:00 am corresponding to circadian time 0 (Zeitgeber ZT0), and free access to food and drinking water, accordingly to institutional guidelines of the Association for Research in Vision and Ophthalmology (ARVO) statement for treatment and care of experimental animals. Diet composition was maintained constant before and during the study. As concerns HIOMT enzyme assay, we used, as a control, C57BL/6 mice, a strain lacking MLT production due to mutations lowering both AANAT (Ebihara et al., 1986) and HIOMT (Kasahara et al., 2010) activity.

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 (100 µl/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 14,300xg 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, accordingly to the reference method (Axelrod and Weissbach, 1960; Sugden et al., 1986). Unless specified, each tube contained 100 µl of the sample (0.6–0.9 mg protein), either 50 or 0 µM NAS, 1–25 µM cold SAM, and 0.1 µM [³H]-SAM (corresponding to 379,922 dpm) in a final volume of 200 µl 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 200 µl 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

Table 1

The 3 different experimental conditions used for tissue homogenization procedures.

Experimental condition	PBS	pH	DTT	Triton-X100
Buffer <i>a</i>	50 mM	7.9	1 mM	0.00 %
Buffer <i>b</i>	50 mM	7.9	1 mM	0.02 %
Buffer <i>c</i>	50 mM	7.9	0 mM	0.02 %

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 0.5 µM (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 (2.5–4–7.5–10–18.5–25 µM). 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 (RPLC), 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 Chromeleon HPLC software (Dionex, Thermofisher Scientific). The injection valve (Reodyne) connected to a 100 µl 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, 1mM Na-EDTA, pH 5. The analytical column was a C18 Atlantis T3, 150 × 4.6 mm, packed with 5 µm particles (Waters SpA, Milford, MA, USA) assembled with a Nova-pack C18 20 × 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 femtomoles 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 Bio-Rad assay kit).

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 $\times g$ for 30' 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 \times 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 \pm SEM. HIOMT Michaelis-Menten kinetic parameters (K_M , μM , and V_{max} , 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 $P = 0.05$.

Table 2

HIOMT assay radioactive counts in pineal glands and retinas from A/J/C57BL/10 and C57BL/6 mice – influence of homogenization procedure and sub-cellular compartments.

Buffer	a		b		c	
	50	0	50	0	50	0
NAS (μM)	50	0	50	0	50	0
Pineal A/J/C57BL/10	9,775–10,405 ^S	1,673–1,815 ^S	10,000–10,336 ^S	4,014–4,014 ^S	11879–11087 ^H	4930–5010 ^H
Pineal C57BL/6	NA	NA	NA	NA	7804–7708 ^H	5543–5739 ^H
Retina A/J/C57BL/10	691–923 ^S	1,036–1,408 ^S	7,983–7,179 ^S	8,436–8,480 ^S	3,415–2,763 ^S	1,855–2,016 ^S
Retina C57BL/6	NA	NA	5,713–5,327 ^P	4,457–4,457 ^P	2,175–2,090 ^P	1,751–1,816 ^P
			NA	NA	4085–4105 ^H	2705–1600 ^H

Values are [^3H]-*dpm* counts of 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: ^S Supernatant; ^P Pellet, ^H Homogenate.

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 $30\text{--}32^{\circ}\text{C}$), 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-proficient MLT C57BL/6 mouse strain (Kasahara 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

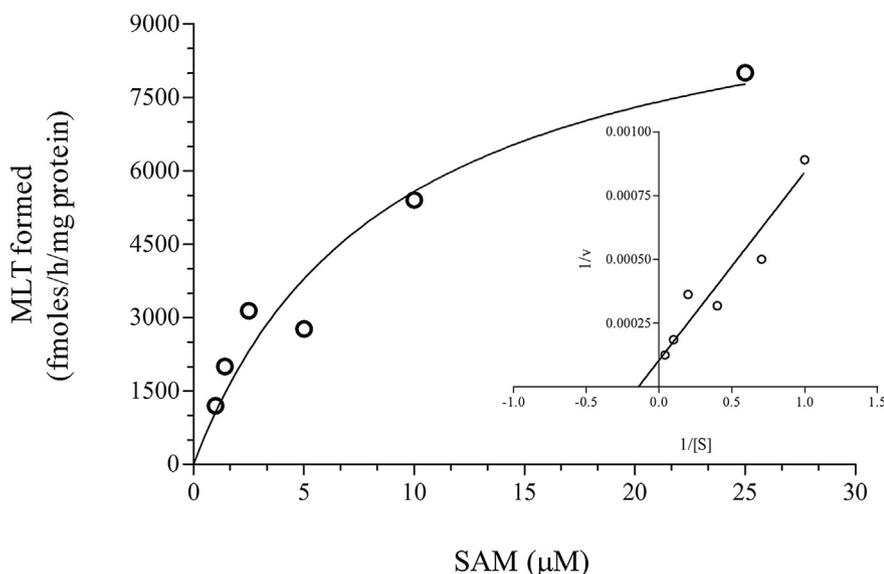


Fig. 1. HIOMT saturation and Lineweaver-Burk plot analyses in mouse pineal gland homogenates. Representative Michaelis-Menten curve of mouse pineal gland HIOMT, homogenized in Buffer *a* and assayed under radiochemical standard assay conditions (Sugden et al., 1986) using SAM concentrations ranging from 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.

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 unlabeled 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 V_{max} and apparent K_M 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 V_{max} values more than 2-hundreds-folds higher than the retinal extracts, without apparent differences of the Michaelis-Menten constants, K_M s.

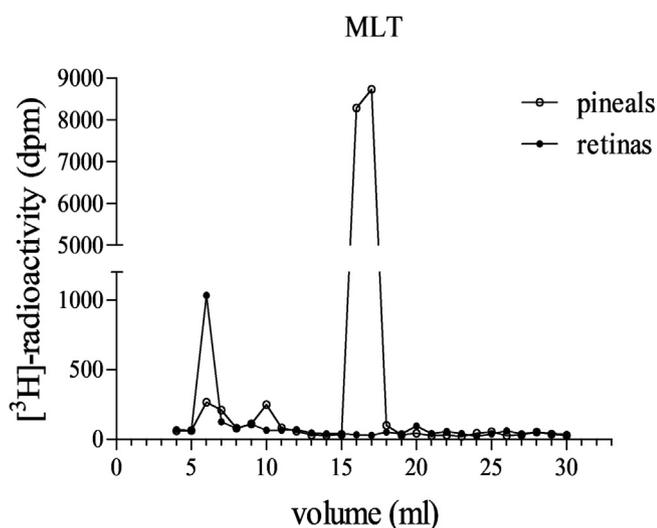


Fig. 2. Chromatograms of the extracted pineal and retinal homogenates. Data-points plot radioactive counts of HPLC-eluted fractions, following injections of samples derived from the supernatants of either retina (filled circles; 5,933 dpm injected) or pineal glands (open circles; 23,000 dpm injected) 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).

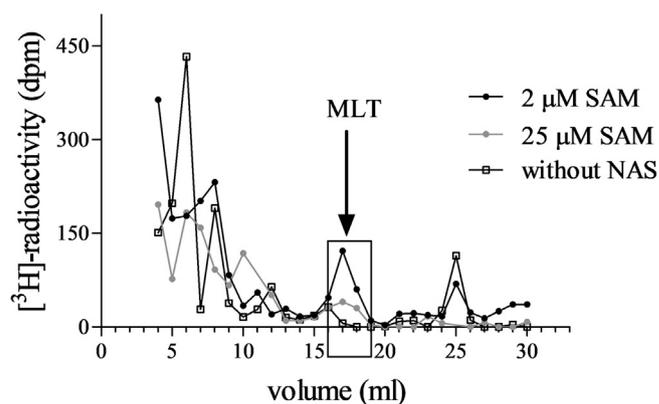


Fig. 3. Chromatograms of extracted retinal homogenates. Data points plot counts of HPLC-eluted fractions following injections of HIOMT assay products carried out in permeabilized membranes of retinas homogenized in Buffer *c*. The HIOMT assay was carried out at 30–32 °C for 75 min. Symbols plot assays 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).

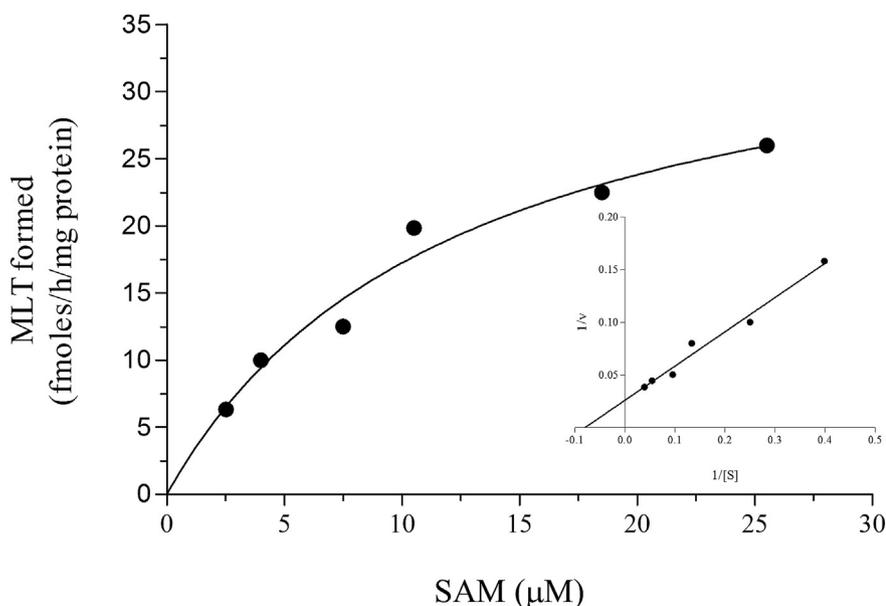


Fig. 4. HIOMT kinetics in mouse retinal homogenates. Representative Michaelis-Menten curve and Lineweaver-Burk plot (insert) of retinal HIOMT as a function of SAM, using Buffer c and HPLC separation. HIOMT activity (75 min incubation at 32 °C) was assayed with 0.9 mg of mouse retinal proteins, increasing concentrations of SAM (corresponding to specific activities within the range of 20.6–1.65 Ci/mmol) and 50 μM NAS. Homogenates were assayed for protein concentration by the Bradford method (Bio-Rad protein assay kit).

Table 3

Comparison between HIOMT enzyme kinetic parameters, measured in A/J/C57BL/10 mouse pineal gland and retina.

A/J mouse tissues:	HIOMT, K_M , μM	HIOMT, V_{max} , fmol/h/mg protein
Pineal gland	9.3 ± 0.52	9,550 ± 912
Retina	9.8 ± 0.20 ^(Ns)	36.5 ± 4 ^(****)

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 K_M means were not significantly different between pineal glands and retinas, Student *t*-test, $p > 0.05$ (***) HIOMT V_{max} 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, due to the interference of an unrelated peak eluting at about 17 min, preceding MLT peak at 17.5 min (Fig. 5A, B). In dark-adapted retinas of animals sacrificed at ZT23, we consistently found oxidation peaks at about 4.5 and 17.5 min after injection (Fig. 5A), corresponding to elution times of NAS and MLT standards, respectively (Fig. 5C): retinal NAS levels ($n = 3$ replicates, 1.48 ± 0.35 pMoles retina⁻¹) were nearly twice those of MLT ($n = 3$ replicates, 0.75 ± 0.2 pMoles retina⁻¹). On the other hand, we found reduced MLT and NAS levels in retinas exposed to room light after isolation from dark-adapted mice (Fig. 5B), implying that light rapidly suppressed the retinal synthesis of these compounds.

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 K_M s 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

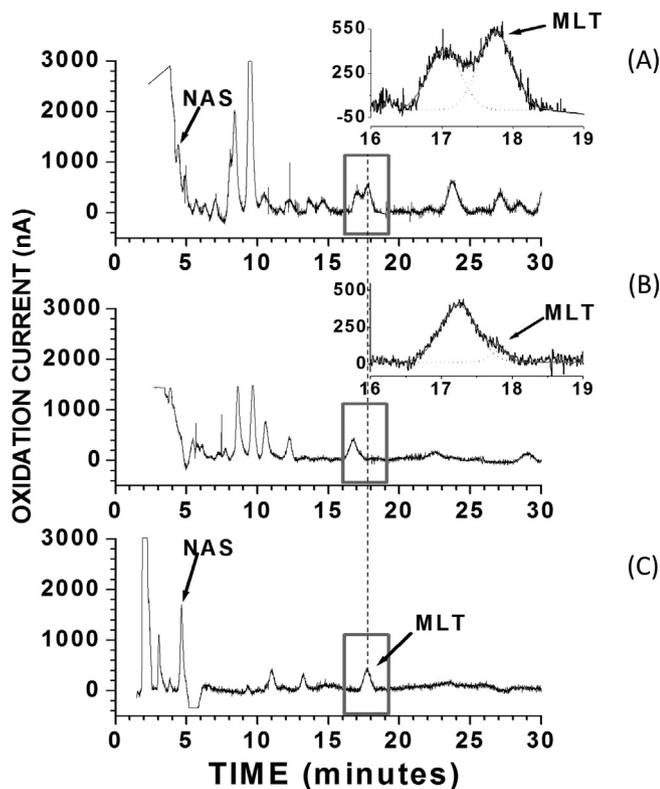


Fig. 5. Chromatograms of endogenous NAS and MLT in dark and light adapted retinal homogenates. A/J/C57BL/10 mice (N = 9) were dark-adapted from ZT12, and retinas isolated at ZT23 under dim red light cultured for 1.5 h in saline (see Material and Methods), either in darkness or in room light. A) HPLC chromatogram plotting oxidation currents in samples obtained from darkness maintained mouse retinas, after TFA and solid-phase Sep-pack extraction steps, as indicated in the Material and Methods section. The gray box identifies the MLT peak, plotted at an expanded scale in the inset on the right, with the gray line representing the resolving MLT fit from the interfering peak, by the sum of two Gaussian functions. Arrows point to oxidation peaks of retinal MLT, its precursor NAS and respective retention times. B) Similar to A), but for retinas exposed to room light before the experimental procedure. C) Elution profile of NAS and MLT standards treated as retinal samples with TFA and solid-phase Sep-pack extraction (2.5 pmol injected). The vertical dotted line shows MLT peaks' correspondence among the three chromatograms A, B, and C.

mammalian retina.

Our results from dark- and light-adapted isolated retinas provide additional support for the presence of a photosensitive two-step (AANAT/HIOMT) biosynthesis of MLT in the mouse retina: 90 min light exposure leads to a rapid decrease in both NAS and MLT retinal levels. This finding represents a starting point to define the modulation of retinal HIOMT function by light, circadian clock or catecholamine release vs. the pineal ones (Ribelayga et al., 1999; Baba et al., 2009, 2013; Giancesini et al., 2015). One open issue waiting for clarification is the homeostasis of the retinal milieu, and its alteration in eye pathologies (Samples et al., 1988; Alcantara-Contreras et al., 2011; Blasiak et al., 2016). An additional open issue is the degree of synchronization between retinal and pineal MLT biosynthesis. Present enzyme kinetic experiments, conducted in dark-adapted animals sacrificed at a fixed time, ZT2, should be extended to other circadian times, under different conditions and in different mouse strains, as shown in the work of Tosini and Menaker (1998). Likewise, the light-sensitivity of HIOMT activity should change at different circadian times in the mammalian retina. It is conceivable that finely tuned regulatory mechanisms control HIOMT expression and functionality in the retina, involving translational and post-translational modifications of the protein. These regulatory mechanisms would rapidly suppress or enhance retinal HIOMT activity, so that

the enzyme would be fully active in a limited time window in this tissue, preventing the formation of too low or too high MLT levels in the presence of light (Tosini and Menaker, 1998).

Advancing our current understanding of HIOMT regulation via AANAT, second messengers and protein-kinase phosphorylation could help define the role of MLT in photoreceptors and other subtypes of retinal cells and, perhaps, the significance of HIOMT in subcellular fractions. Furthermore, the study of both pineal and retinal HIOMT activities in MLT-proficient and -deficient mouse strains and their crossing (as A/J/C57BL/10 mice), could reveal the contribution of MLT-related phenotypes to retinal pathophysiology.

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; Żmijewski 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 (Pévet et al., 1980). Some authors proposed that retinal MLT formation by HIOMT supports the powerful auto-oxid/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 (Żmijewski 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 A/J/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-proficient 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 neurotrophin 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 (A2E) (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-deriving 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.

Funding statement

This work was supported by the university funds of the University of Pisa; Gian Carlo Demontis was supported by a grant from the Italian Ministry of University and Research (MIUR) Prin 20094CZ3M2_003.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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