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Day and Night Dysfunction in Intraretinal Melatonin and Related Indoleamines Metabolism, Correlated with the Development of Glaucoma-Like Disorder in an Avian Model

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Abstract

As previous studies have suggested that melatonin and serotonin may be involved in the regulation of intraocular pressure, retinal concentrations of melatonin, 5-HT, and related indoleamines measured at day and at night were studied during the development of a glaucoma-like disorder with increased intraocular pressure in the *al* mutant quail. Indoleamine levels were determined by HPLC with electrochemical detection in 1-month-, 3-month-, and 7-month-old *al* mutant and control quails. Morphology and numbers of melatonin-synthesizing and 5-HT-containing cells, labelled immunohistochemically with an anti-hydroxyindol-0-methyltransferase (HIOMT) antibody and an anti-5-HT antibody, respectively, were studied. Major findings were that: (1) no significant changes in morphology of melatonin-synthesizing cells or in the morphology and density of 5-HT-containing amacrine cells were observed during the d velopment of glaucoma: (2) 5-HT metabolism was modified during the night at 1 month of age and during the day after 3 months; and (3) melatonin metabolism was modified during the night at 7 months and during the day after 3 months. These results demonstrate a relationship between the temporal evolution of this avian glaucoma and a dysfunction in indoleamine retinal metabolism.

Melatonin, once thought to be produced only by the pineal gland, appears now to be present in various nervous or non-nervous tissues such as the Harderian gland, gut, ciliary body and retina (review in 1–3). Melatonin is synthesized from serotonin (5-HT) via the consecutive actions of two enzymes: serotonin N-acetyl transferase (NAT) and hydroxyindole-0-methyl transferase (HIOMT). The synthesis is rhythmically regulated, and the highest levels are generally detected at night. These fluctuations are controlled by the rhythmic activation of serotonin N-acetyl transferase (4).

Melatonin is involved in a number of retinal events: it promotes the dark-adaptive electrophysiological responses in horizontal cells (5), elicits dark-adaptive retinomotor movements in cones and pigment epithelium (6), activates rod outer segment disk shedding (7) and inhibits evoked dopamine release (8, 9). In turn, dopamine modulates melatonin synthesis by serotonin N-acetyl transferase inactivation (10). Exogenous melatonin reportedly can modify intraocular pressure (11, 12), but this action of melatonin depends on experimental conditions. Intracameral melatonin increases intraocular pressure in cat (13) whereas topical applications, intravenous, intra-arterial and intravitreal injections are ineffective in rabbits (14). In humans, oral melatonin lowers intraocular pressure when administered in the evening (12). Because of the possible role of melatonin in the regulation of intraocular pressure, it is interesting to examine whether melatonin levels are modified in glaucoma: a widespread ocular disorder characterized, in most cases, by an elevation of intraocular pressure.

Our study was performed in an avian model, the hypopigmented *al* mutant of quail *Coturnix coturnix japonica* with a sex-linked recessive gene, which, 3 months after hatching, displays characteristics similar to a human glaucoma: altered cellular arrangement of the iridocomeal angle, elevated intra-

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ocular pressure, buphthalmy and ganglion cell degeneration (15–18). Recently, impaired retinal metabolism of dopamine correlated with tyrosine-hydroxylase immunoreactive cell loss was observed in this model (19). We have now assayed the retinal content of melatonin and related indoleamines: 5-HT (which may also be involved in the regulation of intraocular pressure (20)); 5-hydroxyindole acetic acid (5-HIAA); and N-acetyl serotonin (NAS) in this mutant quail. We also used an immunohistochemical approach to characterize 5HT-containing and melatonin-synthesizing cells to observe possible degeneration as previously observed for tyrosine hydroxylase-immunoreactive cells (19).

Results

Immunohistochemistry of 5-HT and HIOMT

In the retina, three cell types were observed which were immunoreactive to 5-HT. Cells with pyriform somata (7 μm mean diameter, measured on 25-μm thick vibratome sections) were located in the innermost part of the inner layer among amacrine cells (Fig. 1). A single primary dendrite or more rarely two, emerged from the vitreal pole of somata, and branched to form a plexus in stratum 1 of the inner plexiform layer. One or two vertical branches reached stratum 5 to form another plexus. Numerous small bipolar cells with a pear-shaped cell body (34 μm diameter) in the outermost part of the inner nuclear layer were also immunoreactive (Fig. 1). Thin processes emerged from both vitreal and scleral poles of their somata. The scleral processes arborized in the outer plexiform layer and the vitreal processes reached the outermost part of the inner plexiform layer. 5-HT immunoreactivity was also observed in photoreceptors: both in cones and in rods (Fig. 2).

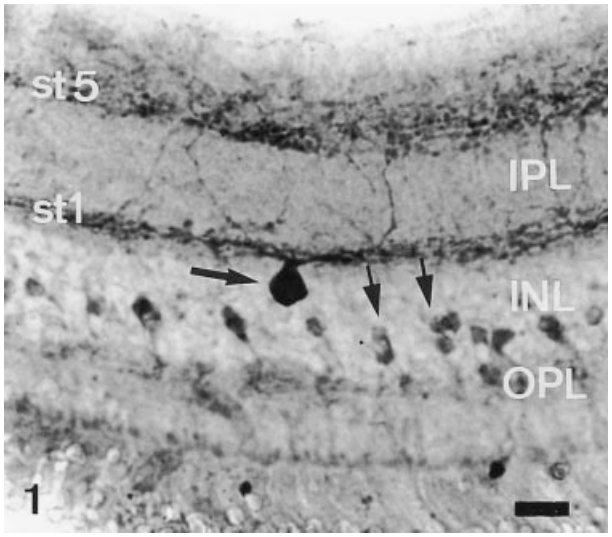

FIG. 1. 5-HT-containing amacrine and bipolar cells observed in a 25-μm thick vibratome section of 7-month-old mutant quail retina. A 5-HT amacrine cell (arrow) is located in the innermost part of the inner nuclear layer (INL) and its processes are distributed into two levels: stratum (st) 1 and 5 of the inner plexiform layer (IPL). 5-HT bipolar cells (small arrows) are located in the INL. Thin processes arborize in the outer plexiform layer (OPL) and in the IPL. The same pattern of 5-HT immunolabelling is observed in control quails. Scale bar: 10 μm.

HIOMT immunoreactivity was mainly located in photoreceptors (Figs 3, 4). Rare cell bodies were also labelled in the innermost part of the inner nuclear layer (Fig. 3). Their processes could not be distinguished. These cells were restricted to the peripheral retina.

The morphology and the localization of 5-HT and HIOMT immunoreactive cells remained unchanged in both strains at all stages. No degenerating cells were observed in mutant quails, and the densities of 5-HT-containing amacrine cells were similar to control quails (Table 1). However, the progressive increase in cell density observed from 1 to 7 months in control quails was not observed in mutant quail, in which the mean density of 5-HT immunoreactive cells appeared more heterogeneous as indicated by a higher standard deviation.

Biochemical assays

At 1-month of age, the retinal 5-HT content, when measured at night, was higher in mutant than in control quails. By contrast, in daytime, the 5-HT content was not significantly different between strains. At 7 months, whereas an increase in 5-HT contents was observed in control quails, no significant change was noted in mutant quail (Figs 5, 6).

The retinal 5-HIAA content always appeared higher during the day than at night in both strains and at all stages. However, no differences were observed between strains in both day and night levels except at 1 month when levels were higher in mutant than in control animals (Figs 5, 6). The 5-HIAA/5-HT ratio, an indicator of 5-HT metabolism, was higher by day (Table 2) than at night (Table 3) in both strains at 1 and 7 months. The ratio was also considerably increased in 7-month-old mutant quail during the day. NAS could not be detected during the day in any animal. At night, the NAS content was significantly higher at 7 months in control quail compared to younger stages, whereas no variation with age was observed in the mutant animal strain. The night NAS content was the same in both strains at 1 and 3 months whereas it was reduced in mutant quails only at 7 months (Fig. 5). At all stages and in both strains, melatonin content was higher at night than by the day (Figs 5, 6) except in 3-month-old mutant quails where no significant difference was noted between night and day. A

TABLE 1. Densities of 5-HT Immunoreactive Cells. Comparison Between Mutant (MQ) and Control (CQ) Quails at All Ages.*

	5-HT-immunoreactive cell density				
	1 month	3 months	7 months	*p(1–3)*	*p(3–7)*
CQ	198 ± 11	260 ± 3	317 ± 10	0.02	0.01
MQ	252 ± 66	282 ± 20	256 ± 20	NS	NS
p(CQ-MQ)	NS	NS	NS		

*Comparison by Newman-Keuls, n = four retinas/ages (one retina = one quail) and 40 samples/retina. *p(CQ-MQ)*: statistical comparison between CQ and MQ at the same stage. *p(1/3)* and *p(3/7)*: statistical comparison between the age considered and the previous age. NS: not significant. No significant differences were observed between strains whatever the stages.

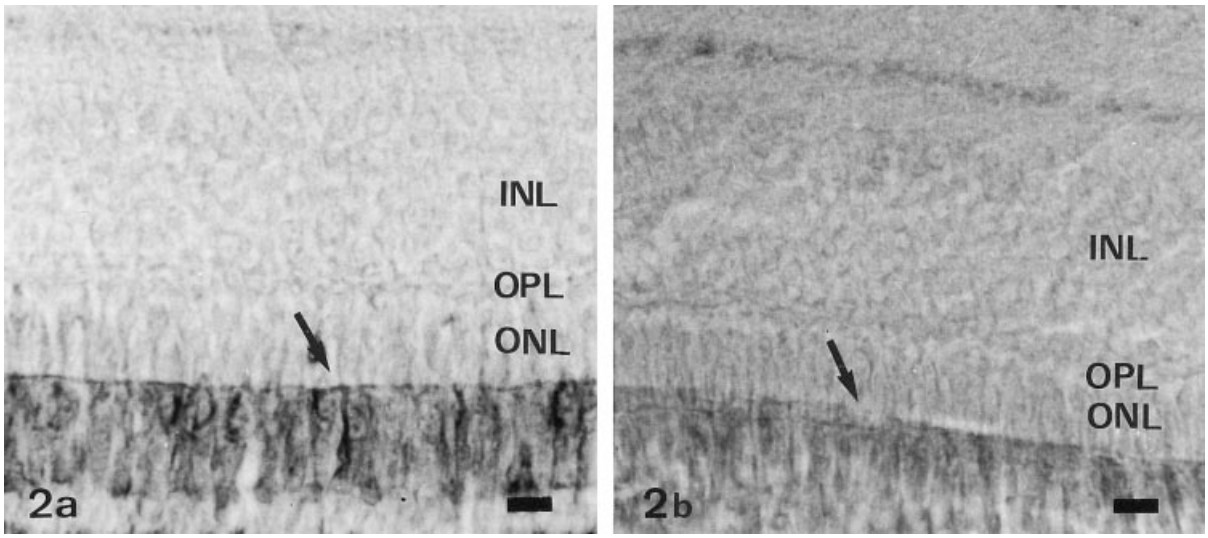

FIG. 2. (a,b) 5-HT containing photoreceptor cells observed in a 10 μm paraffin section of 7-month-old control (a) and mutant (b) quail retina fixed by Bouin fixative. Note that all photoreceptors are labelled. Scale bar: 10 μm.

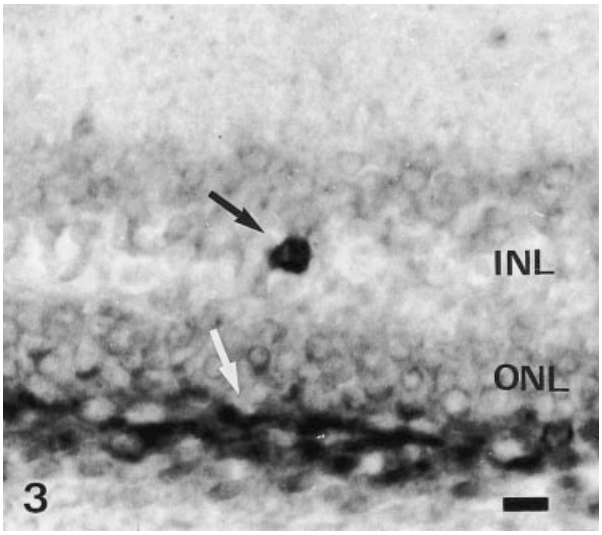

FIG. 3. HIOMT-immunoreactive cell (black arrow) in the inner nuclear layer (INL) observed in a semi-tangential 10 μm paraffin section of 7-month-old mutant quail retina. Processes are not visible. Photoreceptor cells (white arrow) are also labelled. Scale bar: 10 μm.

TABLE 2. 5-HIAA/5-HT Ratios in Control (CQ) and Mutant (MQ) Quails in Daytime (d) Conditions at Each Age.*

	5-HIAA/5-HT				
	1 month	3 months	7 months	*p(1–3)*	*p(3–7)*
CQd	0.44 ± 0.05	0.58 ± 0.11	0.38 ± 0.03	NS	0.02
MQd	0.48 ± 0.03	0.46 ± 0.08	0.73 ± 0.08	NS	0.02
p(CQ-MQ)	NS	NS	0.001		

*Comparison by Newman-Keuls test, n = 10 pairs of retinas for each condition at each age. *p(CQ-MQ)*: statistical comparison between CQ and MQ at the same age and light conditions. *p(1/3)* and *p(3/7)*: statistical comparison between the age considered and the previous age. NS: not significant. 5-HIAA/5-HT ratio does not change in both strains at all ages except in 7-month-old MQ where an increased ratio.

TABLE 3. 5-HIAA/5-HT Ratios in Control (CQ) and Mutant (MQ) Quails in Night Time (n) Conditions at Each Age.*

	5-HIAA/5-HT				
	1 month	3 months	7 months	*p(1–3)*	*p(3–7)*
CQn	0.22 ± 0.02	0.54 ± 0.06	0.22 ± 0.02	0.001	0.001
MQn	0.23 ± 0.02	0.42 ± 0.04	0.24 ± 0.01	0.001	0.001
p(CQ-MQ)	NS	NS	NS		

*Comparison by Newman-Keuls test, n = 10 pairs of retinas for each condition at each age. *p(CQ-MQ)*: statistical comparison between CQ and MQ at the same age and light conditions. *p(1/3)* and *p(3/7)*: statistical comparison between the age considered and the previous age. NS: not significant. 5-HIAA/5-HT ratio does not change in both strains.

progressive increase in melatonin content was observed from 3 to 7 months in control quails in both day and night samples. Compared to control quails, the night melatonin content was reduced in mutant quails at 7 months, while the day content remained higher in mutant quails after 3 months (Figs 5, 6).

Discussion

5-HT and HIOMT-immunoreactive cells

The function of 5-HT in the retina remains poorly understood. Endogenous retinal 5-HT contents are high in non-mammals but low or null in mammals (21–23) except in Prototherians (24). In non-mammals, 5-HT is thought to be contained mainly in amacrine and bipolar cells (23). The 5-HT immunoreactive cells that we observed in quail retina were subpopulations of amacrine and bipolar cells similar to those previously observed in chicken retina (25, 26), but the mean diameter of their somata appeared smaller (3 μm

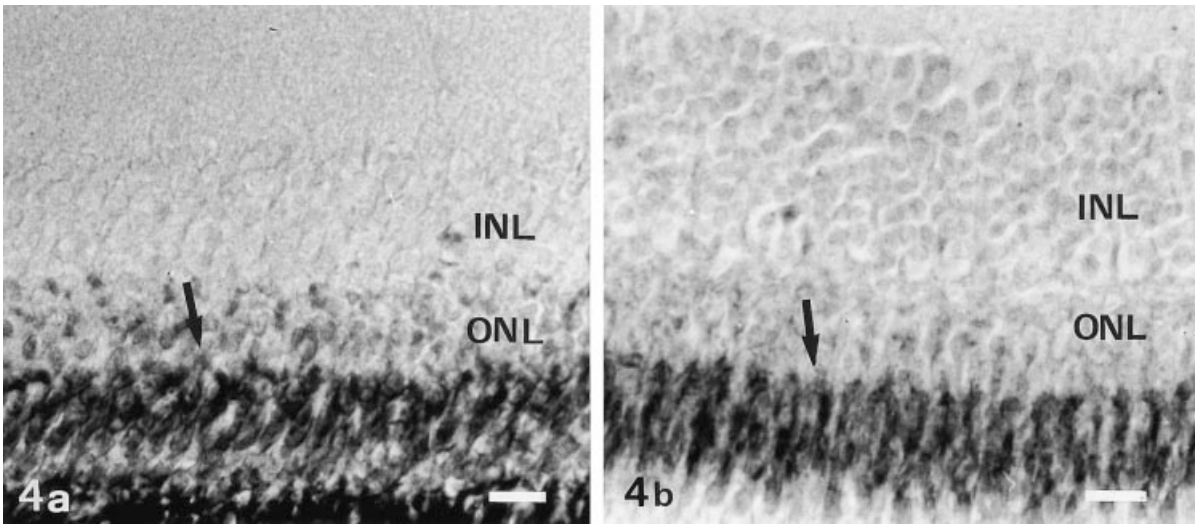

FIG. 4. (a,b) HIOMT-immunoreactive photoreceptor cells (rods and cones) in 10-μm paraffin section of 7-month-old mutant (a) and control (b) quail retina. Note that all photoreceptor are labelled. Scale bar: 10 μm.

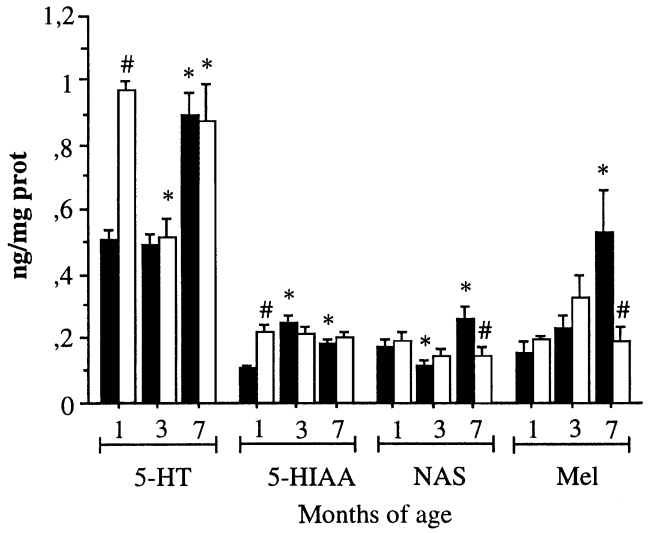

FIG. 5. 5-HT, 5-HIAA, NAS and melatonin concentrations in control (CQ, □) and mutant (MQ, ■) quail in nighttime conditions at different ages. Comparison by Newman-Keuls test ($n=10$ pairs of retinas for 5-HT, 5-HIAA and NAS; $n=$ five pairs of retina for melatonin) for each condition at each age. #, significant differences between CQ and MQ; *, significant differences between the age considered and the previous age ($P<0.05$). 5-HT: note the higher concentration in 1-month-old MQ compared to 1-month-old CQ. 5–HIAA: apart from a higher content of 5-HT in 1-month-old MQ compared to CQ, no significant difference is noted between MQ and CQ at later age. NAS: the increased concentration observed in 7-month-old CQ is not detected in MQ. Melatonin: the same pattern as for NAS is observed.

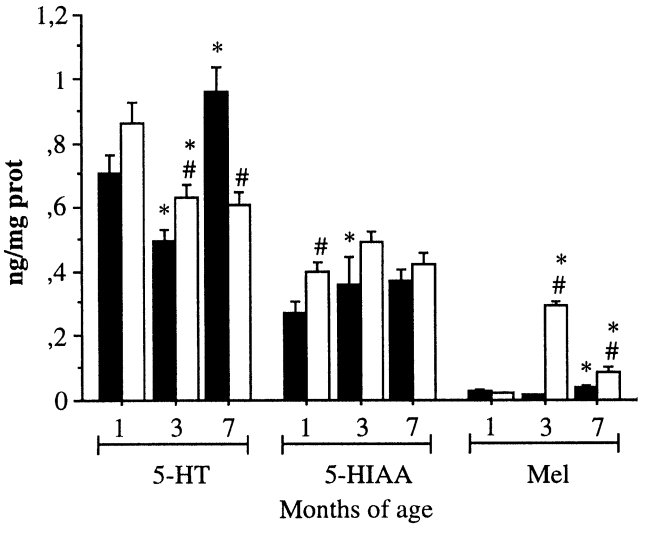

FIG. 6. 5-HT, 5-HIAA, and melatonin contents of control (CQ, ■) and mutant (MQ, □) quail in daytime conditions at each age (NAS was not detected during the day). Comparison by Newman-Keuls test ($n=10$ pairs of retinas for 5-HT, 5-HIAA and NAS; $n=$ five pairs of retinas for melatonin) for each condition at each age. #, significant differences between CQ and MQ; *, significant differences between the stage considered and the previous stage ($P<0.05$). 5-HT: note the lower 5-HT content in 7-month-old MQ compared to 7-month-old CQ. 5-HIAA: similar to nocturnal conditions, note the higher content of 5-HIAA in 1-month-old MQ compared to 1-month-old CQ. Melatonin: note the low levels of melatonin in CQ at all ages whereas in 3-month-old MQ, the concentration of melatonin is considerably increased. Melatonin concentration is decreased in 7-month-old MQ but remains twofold higher than in 7-month-old CQ.

instead of 6 μm for bipolar cells, and 7 μm instead 12 μm for amacrine cells). In chicken, a population of amacrine cells has been reported to be immunoreactive to an anti-phenylalanine hydroxylase antibody which also recognizes the 5-HT synthetic enzyme tryptophan-hydroxylase (27, 28). It has thus been proposed that 5-HT may be a neuro-transmitter in amacrine cells (26). In contrast, as no biosynthetic enzyme in bipolar cells was detected, it has been proposed that 5-HT is only accumulated by these cells (26) and possibly used as a melatonin precursor (29). This seems unlikely in the quail since HIOMT immunoreactivity was not observed in bipolar cells. Alternatively, the 5-HT in bipolar cells might serve as a 'borrowed transmitter' or a co-transmitter (26, 30, 31).

Numerous photoreceptors are labelled by the anti-5-HT antibody. 5-HT synthesis in such cells has been suggested in chicken due to the presence of tryptophan-hydroxylase activity in the outer retina (32), and in rats because photoreceptors contain aromatic L-aminoacid decarboxylase, an enzyme involved together with tryptophan-hydroxylase in 5-HT synthesis (33). Tryptophan-hydroxylase activity and mRNA have also been reported in isolated photoreceptors of *Xenopus leavis* (34). Because high levels of tryptophan-hydroxylase mRNA are generally found in regions of the central nervous system where melatonin is synthesized (35), we propose that 5-HT in quail photoreceptors may serve as a melatonin precursor. This suggestion is strengthened by HIOMT immunoreactivity in photoreceptors.

The quail retina, like that of the chicken, exhibits a high concentration of melatonin (36). Although HIOMT can also methylate 5-HT and 5–HIAA, its affinity is 10–20-fold higher for NAS, suggesting that HIOMT immunoreactivity is a good marker for melatonin-synthesizing cells. HIOMT immunoreactivity is mainly observed in quail photoreceptors. Since no unlabelled photoreceptors were observed, both cones and rods appear to be able to synthesize melatonin. The rare HIOMT immunoreactive cells in the amacrine cell layer could not be identified because their processes were not observed, but they resembled those described by Guerlotté *et al.* (37) in the chicken retina.

The development of glaucoma does not significantly affect the morphology or the density of the 5-HT-and HIOMT-immunoreactive cells. No cellular degeneration was observed contrary to the previously reported disappearance of tyrosine-hydroxylase immunoreactive amacrine cells (19).

Indoleamine content

In control quails, 5-HT, NAS and melatonin contents evolved concurrently during the 7 months following hatching: little or no change was observed between 1 and 3 months, followed by a marked increase at 7 months. A similar evolution in control animals has been reported for dopamine and was interpreted as a late maturation of aminergic pathways (19). Since no change in 5-HIAA content was observed with increasing age, the late maturation may concern the indoleamine system involved in melatonin synthesis. However, indole levels also appear to be sensitive to seasonal fluctuations (38) and the increase observed may be due, at least partially, to this variation. For this reason, results reported by different authors cannot always be compared. In this study, retinas of both strains were rigorously collected at the same period of the year for each age to obtain comparative data on indoleamine levels for a given age.

5-HT content differed between strains initially in nighttime at 1 month whereas changes in daytime occurred only at 3 and 7 months. Thus, despite the lack of apparent morphological change in indoleamine-containing cells, a dysfunction in the indoleamine system is present in mutant quails before the appearance of clinical signs of glaucoma.

5-HT may be involved in two distinct functions in the avian retina: neurotransmission and melatonin synthesis. 5–HT is considered to be a neurotransmitter at least in a subpopulation of amacrine cells. These cells release 5-HT mostly during light adaptation (21, 39). The released 5HT is then probably catabolized into 5-HIAA as attested by the higher 5-HLAA : 5-HT ratio observed during daytime. However, at night, 5-HT may be mainly involved in melatonin synthesis. Accordingly, in control quails, 5-HIAA, content is lower at night while NAS and melatonin contents are higher. The same pattern is observed in mutant quails suggesting that diurnal serotoninergic neurotransmission is not affected.

The nocturnal level of melatonin did not increase with age in mutant quail, unlike control quail. A parallel profile was demonstrated for its direct precursor, NAS. Moreover, although melatonin contents were higher at night than by day at 1 month in mutant quails, this difference became attenuated later and in spite of elevated diurnal contents of melatonin at 3 and 7 months, NAS content remained undetectable. The increase in melatonin content observed after 3 months in mutant quail may indicate a breakdown in its rhythmic metabolism (either in its synthesis as noted above, and/or its degradative mechanisms).

Intraocular pressure, eye growth and melatonin synthesis are rhythmic events (40–42) and all three are impaired during the development of glaucoma in mutant quail. The correlation between imbalance of retinal melatonin synthesis, elevated intraocular pressure and buphthalmy fits well with the involvement of retinal melatonin in the control of intraocular pressure and eye size. Indeed, subcutaneous administration of melatonin has been shown to increase eye weight and intraocular fluid content in the hamster (43), and a significantly higher melatonin content is observed in the retina of another avian model of glaucoma induced by light (44). However, melatonin is also rhythmically synthesized by the ciliary body (2) and further experiments are needed to document the respective role of retina and ciliary body in providing the eye with melatonin.

Material and methods

One hundred and two mutant quails, gene symbol *al*, and 102 normal control quails *Coturnix coturnix japonica* were provided by the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). The birds were kept under a 14 light : 10 dark photoperiod (light on at 6.00 a.m.; light off at 8.00 p.m.) from hatching until they were killed by decapitation. Mutant quails were killed at 1 month, in May (before the appearance of clinical signs of glaucoma), at 3 months, in August (when the first pathological signs were apparent, especially increased intraocular pressure), and at 7 months, in December (when glaucoma was well established). Control quails were killed according to the same schedule. The experiments were performed in accordance to the legal requirement in the UK.

Immunohistochemistry

Twenty-four retinas (four from each strain obtained from four quails, at the three stages) were prepared for immunohistochemistry; for each animal, one retina was prepared for whole mount and the other for sections. All birds were decapitated at 2.00 p.m. and enucleated. Retinas were dissected free from pigment epithelium. Those prepared for paraffin sections were fixed in Bouin fixative for 48 h at 4 °C, then cut in 10-μm sections. Those prepared for whole mounts or vibratome sections (25 μm) were fixed in 4% buffered paraformaldehyde for 4 h. Sections and whole mounts, were incubated, respectively, for 24 h or 72 h in rabbit antiserum against 5-HT (Immunotech, France); diluted 1 : 500 or against HIOMT (generously provided by Dr P. Voisin, Poitiers, France); and diluted 1 : 1,000 in phosphate buffer saline (PBS). Immunoreactivities were demonstrated by the avidin-biotin peroxydase technique (ABC kit, Vectastain, USA). Controls were made by omitting the primary antiserum or by replacing it by non-immune rabbit serum at the same concentration as the antibody. No labelling was observed in control sections.

5-HT immunoreactive amacrine cells were counted on whole mounted retinas using an X–Y plotter coupled to a light microscope. The population of 5-HT immunoreactive amacrine cells was so dense that a complete mapping of each retina was not practical. Cells were counted in 40 samples of 1 mm^2 per retina, distributed 10 per quadrant, and 50 : 50 in the central *versus* peripheral retina. The mean cell densities of the four retinas of each strain were averaged in each of the three stages examined. The mean cell densities in mutant and control quails were compared using the Newman-Keuls test.

Biochemical assays

Retinas of both strains were collected at 2.00 p.m. and at midnight in identical conditions. At midnight, mutant and control quails were killed in darkness and quickly dissected in light (the rapid dissection in light did not modify the nocturnal elevation in melatonin content observed in avian retina). Retinas were collected at each age, from each strain, under each nocturnal and diurnal condition. Concentrations of 5-HT, melatonin and related indoles as NAS and 5-HIAA were measured using high performance liquid chromatography (HPLC) with electrochemical detection. For quantification of 5-HT, 5-HIAA and NAS, our validated method was used (45). For this, 10 pairs of retinas (one quail = one pair of retina) were sonicated in 0.2 M perchloric acid containing 0. 1% $Na_2S_2O_5$ and 0.1% EDTA. Homogenates were then centrifuged (15,000 ***g***, 5 min, 4 °C) and the clear supernatants were stored at −80 °C before injection (20 µl) into the HPLC system. Separation of 5-HT, 5-HIAA and NAS was achieved using reverse phase chromatography (Beckman Ultrasphere C18.5 µm column, 150 × 4.6). The mobile phase (pH 3.75) consisted of water/methanol (90/10 v/v) containing 0.1 M KH_2PO_4, 0.01 mM EDTA and 5 mM heptane sulphonic acid (45). For melatonin quantification, our recently published method was used (46). For this, pools of two retinas from the same quail were sonicated in 1 ml ice-cold 0.2 M PBS buffer (pH 6.5). Homogenates were then extracted with 5 ml of CH_2Cl_2 after alkalinization (100 µl 2N KOH). 6-fluoro-tryptamine was used as internal standard. After centrifugation (1,000 ***g***, 10 min, 4 °C), the organic layer was evapored under nitrogen. The residue was dissolved in 100 µl phase mobile and 60 µl were injected into the HPLC system. For melatonin measurement, the chromatographic conditions were identical to that given for 5-HT excepted the mobile phase, which contained 0.5 mM octane sulphonic acid instead of heptane sulphonic acid, and 20% acetronitrile instead of methanol (46). Electrochemical detection was performed with an EG & G model 400 amperometric detector, at a working potential of +0.80 V (glassy carbon electrode), relative to an Ag/AgCl electrode. 5-HT, 5-HIAA and NAS were quantified by peak height measurement relative to a single point standard as there is a linear response over the range of observed peak heights. Melatonin content was calculated using the peak height ratio (melatonin/internal standard) in each sample, compared to a standard curve (linear response between melatonin and peak height ratio in the range 25–1,000 pg melatonin). The amount of protein in retina was determined in the pellet (for 5-HT, 5-HIAA and NAS quantification) or in the pellet and the aqueous phase (for melatonin), using the Bradford method (47). Results were expressed in ng/mg protein. For each method, detailed validation parameter about limit of sensitivity, intra-and inter-assay variations have been published (45, 46). For quantification of melatonin in particular–concentration of which is lower than that of other indole compounds–it can be noted that the recovery was 94–97%. The quantification limit, using a 5 : 1 signal to noise ratio, was 10 pg/tube for standards and 15 pg/tube for retina. In terms of precision, for evaluation of the within- and between-assay coefficients of variations (CV), samples of pooled retina at low and high concentrations were prepared and analysed three times the same day (within CV) and on four different days (between CV). The CV were never greater than 15% (data not shown). Individual data were analysed using a global anova, followed, when significant ($P < 0.05$), by individual inter-age comparisons with the parametric Newman-Keuls test.

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