

# Structural and Functional Evolution of the Pineal Melatonin System in Vertebrates

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In most species daily rhythms are synchronized by the photoperiodic cycle. They are generated by the circadian system, which is made of a pacemaker, an entrainment pathway to this clock, and one or more output signals. In vertebrates, melatonin produced by the pineal organ is one of these outputs. The production of this time-keeping hormone is high at night and low during the day. Despite the fact that this is a well-preserved pattern, the pathways through which the photoperiodic information controls the rhythm have been profoundly modified from early vertebrates to mammals. The photoperiodic control is direct in fish and frogs and indirect in mammals. In the former, full circadian systems are found in photoreceptor cells of the pineal organ, retina, and possibly brain, thus forming a network where melatonin could be a hormonal synchronizer. In the latter, the three elements of a circadian system are scattered: the photoreceptive units are in the eyes, the clocks are in the suprachiasmatic nuclei of the hypothalamus, and the melatonin-producing units are in the pineal cells. Intermediate situations are observed in sauropsids. Differences are also seen at the level of the arylalkylamine *N*-acetyltransferase (AANAT), the enzyme responsible for the daily variations in melatonin production. In contrast to tetrapods, teleost fish AANATs are duplicated and display tissue-specific expression; also, pineal AANAT is special—it responds to temperature in a species-specific manner, which reflects the fish ecophysiological preferences. This review summarizes anatomical, structural, and molecular aspects of the evolution of the melatonin-producing system in vertebrates.

**Key words:** melatonin; arylalkylamine *N*-acetyltransferase; pineal organ

## Introduction

Most of the biochemical, physiological, and behavioral events of living organisms are rhythmic; the daily and annual rhythms are among the most preeminent. This is the result of specific adaptations to the cyclic variations of the environment. The variations of photoperiod and temperature play a major role in the synchronization of daily and annual rhythms. In a

general manner, these rhythms are generated by internal clocks, which free run with a period of approximately 24 h (circadian rhythms) or 1 year (circannual rhythms). Organisms with an internal time-keeping system are able to predict and anticipate daily and annual changes so that the right event will occur at the right time. Whereas information accumulates concerning the function of the circadian clocks,<sup>1</sup> far less is known on the mechanisms of the circannual clocks. It has been suggested that circannual clocks are either based upon (i) a “counting” of circadian days, (ii) a sequence of interdependent physiological states, or (iii) one or more specific circannual oscillators.<sup>2</sup> A circadian system comprises all the different

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components by which light enters the organism and is transformed into a timed signal.<sup>3</sup> The core of the system is made of the clock machinery, which functions autonomously with a period close to 24 h. The endogenous activity of the clocks is synchronized to the prevailing 24 h light–dark (LD) cycle by light perceived through specific light sensors; in turn, the clock drives the rhythmic production of output signals. In vertebrates, the retina and the pineal organ play a crucial role in the transduction of the photoperiodic and thermoperiodic (ectotherms only) information. In addition, both organs produce melatonin, one major output of the circadian clocks.<sup>4,5</sup> Melatonin is produced in a rhythmic manner and is now well known as the daily and annual time-keeping hormone.<sup>1,3,4,6</sup> Generally speaking, retinal melatonin acts and is catabolized locally whereas pineal melatonin is released into the cerebrospinal fluid (CSF) and blood.<sup>3,7–10</sup> In other words, the hormonal time-keeping effects of melatonin in the organism result from the activity of the pineal gland. In all species investigated so far, plasma melatonin levels are high at night and low during the day, which provides the organism with a reliable indication of the respective durations of day and night. Seasonal changes in the LD cycle and temperature are reflected in the amplitude and duration of the nocturnal surge. It is an intriguing observation that, despite this constancy among vertebrates, the organization of the system that controls the nocturnal surge in pineal melatonin production has changed dramatically from fish to mammals. Here we review some structural, functional, and molecular aspects of the modifications undergone by the melatonin-producing system in vertebrates.

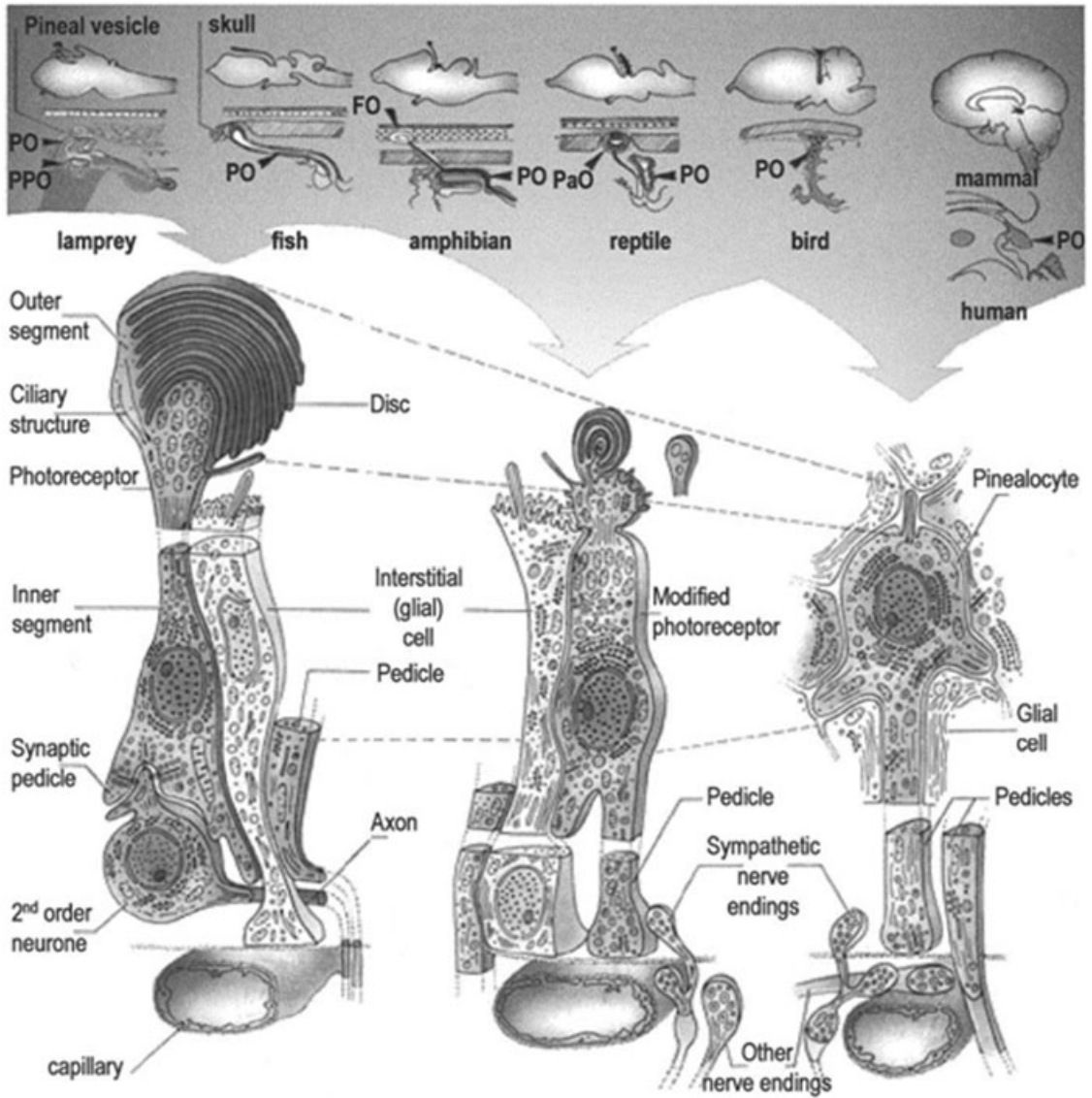
### Structural and Functional Evolution of the Melatonin-producing Units in Vertebrates

The structural and functional analogies that characterize the retina and the pineal organ of

vertebrates have been well emphasized.<sup>3,11–14</sup> However, while the retina displays more or less the same general organization in all vertebrates, the structure of the pineal gland has changed dramatically from ectotherms to mammals. This is the result of a unique evolutionary trend that led to the progressive replacement of the direct photosensitivity, as seen in non-mammalian vertebrates, by an indirect photosensitivity, as seen in sauropsids and mammals (Fig. 1).

In ectotherms, the pineal gland displays adaptation to light perception. It is a vesicle located just below the skull in an area where the bone is thinner and the surrounding tissues are less pigmented thereby facilitating light entry (Fig. 1).<sup>2,3</sup> In big fish, such as bluefin tuna *Thunnus thynnus*, where the brain is located deep in the head, the pineal vesicle enters a translucent cartilaginous tube that drives it toward the skull. The vesicle is connected to the roof of the diencephalon by a stalk. In most species, the lumen is opened to the third ventricle and is thus filled with CSF. In lizards and birds the pineal gland is folliculated whereas in ophidians and mammals it appears as a compact gland.<sup>11</sup> This progressive change in structure is paralleled by a progressive internalization of the gland; in humans, the gland occupies one of the most central parts in the brain (Fig. 1). A similar anatomical variety has been observed in some teleosts,<sup>15</sup> although it is not yet known whether this reflects a convergent evolutionary trend or species-specific adaptations. The anatomical modifications are paralleled by a unique and dramatic evolution of its cell types.

In ectotherms, photoreceptor cells, neurons, and interstitial (glial) cells make up the pineal parenchyma. The photoreceptor cells resemble the retinal cones of the retina. Their apical part protrudes into the pineal lumen; the basal part contacts the second-order neurons (Fig. 1),<sup>3,5</sup> which send their axons to the brain through a pinealofugal nerve. Neither the photoreceptors nor the neurons can contact the basal lamina and surrounding vesicles from which they are isolated by the glial



**Figure 1.** Evolution of the pineal organ in vertebrates. The upper panel shows the anatomical location of the pineal gland in the vertebrate brain (in black). The lower panel shows the cell types of the epithelium in different representatives from fish to mammals. Note the disappearance of the photoreceptive pole and of the second-order neurons as well as the appearance of the sympathetic innervations. FO, frontal organ; PaO, parietal organ; PO, pineal organ; PPO, parapineal organ. Adapted from Ref. 11.

cells, which occupy the whole length of the pineal epithelium. In brief, the pineal epithelium of fish and frogs resembles a retina, which would possess very few or no interneurons (such as bipolar, amacrine, or horizontal cells). Photoreceptor cells *per se* are no longer observed in mammals and ophidians; the mammalian and ophidian pinealocyte exhibits no

outer and inner segments, which typically make up part of the photoreceptor structure (Fig. 1). In addition, neurons are no more detected and glial cells are only a small proportion of the pineal cell population. Intermediate situations are seen in lizards and birds, where cone-like photoreceptors are seen together with photoreceptor cells showing

disorganized outer segments and no synaptic contacts with neurons (Fig. 1); the proportion of each varies from one species to another. It is noteworthy that photoreceptors that do not contact neurons also exist in some teleost fish.

The anatomical and structural data together with functional histological and electrophysiological studies<sup>8,12,16</sup> clearly indicate that the pineal organ of ectotherms is a direct light sensor, which functions as a luminance detector. This is not the case anymore in mammals. However, the mammalian pineal remains, albeit indirectly, sensitive to light.<sup>17</sup> Light reaches the pineal organ through a complex nervous pathway that involves a specific subset of retinal ganglion cells, the axons of which constitute the retinohypothalamic tract (RHT). The RHT connects with the central circadian clocks of the suprachiasmatic nuclei of the hypothalamus (SCN), and from there the information is conveyed, successively, to the paraventricular nuclei, the intermediolateral column in the upper thoracic spinal cord, and the superior cervical ganglia, which finally send their sympathetic axons to the pineal organ. In other words, the direct photosensitivity of the pineal organ of ectotherms has been progressively replaced by an indirect photosensitivity in more distant vertebrates.

How can this evolutionary trend be explained? What mechanisms underlie the progressive and concomitant loss of photoreception by the pinealocyte and of the pinealofugal neurons? Although particularly pronounced in mammals, a similar trend is observed in the different vertebrate lineages, although at different degrees of complexity. This suggests that the potential to give either a direct or an indirect light-sensitive organ exists early in the vertebrate lineage. In this respect it is interesting that (i) an inverse (causal?) relationship has been observed in birds between the development of the sympathetic innervation and the disappearance of the pinealofugal neurons<sup>18</sup> and (ii) norepinephrine prevents expression of the visual pigment rhodopsin in cultured neonate rat pinealocytes and quail photoreceptors.<sup>19–22</sup>

Norepinephrine treatment was not efficient if applied after expression had started. These interesting observations suggest that the establishment of the sympathetic innervation could be one triggering process that inhibits photoreceptor development. In keeping with this, it is interesting that, in the chicken pineal gland, mammalian-like pinealocytes are found in the perifollicular areas of the gland where the sympathetic terminals end whereas modified photoreceptors are located at the central part of the follicle, not reached by the nerve endings. This and early ontogeny structural studies bring support to the idea that pinealocytes first develop as photoreceptor cells and then follow, depending on the linkage, an ontogenetic regression process.<sup>3,11,12</sup> An alternative hypothesis suggests that evolutionary changes in the regulatory linkage of developmental processes, associated to changes in cellular functions in the embryonic pineal field, have shaped the different types of photoreceptors seen today in the vertebrate pineal gland.<sup>12</sup> The observation that the pineal organ of an ancestor vertebrate, the lamprey, possesses all three types of cells (i.e., photoreceptors, “modified” photoreceptors, and pinealocytes) displaying regional distribution does not help in solving the enigma but indicates that the potential to give rise to the different types of photoreceptors exists in early vertebrates.

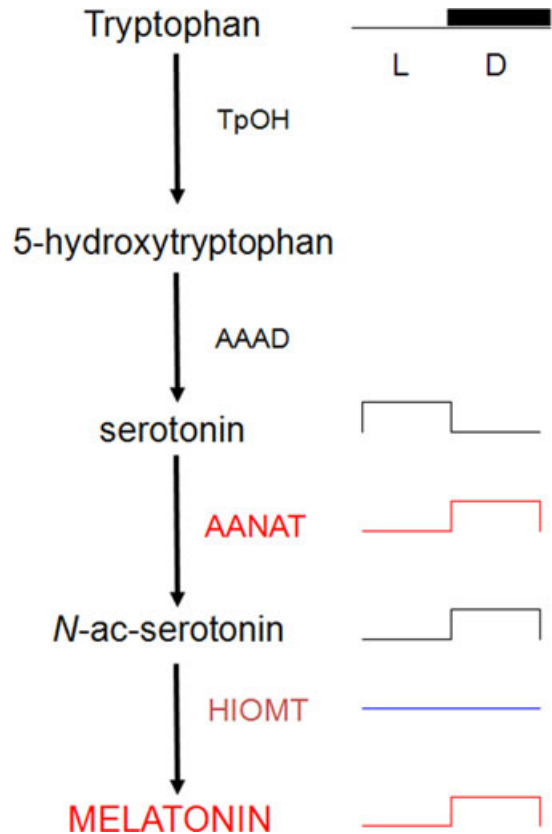
The profound modifications that have affected the pineal organ of vertebrates have led to the loss of a direct light sensitivity and of the neuronal message sent to the brain. In other words, the pineal no longer functions as a detector of rapid light changes in distant vertebrates. However, it still detects changes related to day length and night length durations through the nocturnal secretion of melatonin. It is interesting that despite the profound structural and functional changes that the chief vertebrate cells have undergone, they all have kept the ability to produce melatonin at night. This was achieved because the mechanisms that control this production have also changed dramatically.

## Molecular Evolution of the Melatonin-producing System in Vertebrates

### Pathway and Sites of Melatonin Biosynthesis

Basically, the melatonin biosynthesis pathway is the same in the retina and pineal organ of vertebrates. Melatonin is synthesized from tryptophan in four enzymatic steps (Fig. 2).<sup>23–25</sup> Tryptophan hydroxylase catalyzes the conversion of tryptophan into 5-hydroxytryptophan, which is then decarboxylated by the aromatic amino acid decarboxylase to produce serotonin. Arylalkylamine *N*-acetyltransferase (AANAT) converts serotonin to *N*-acetylserotonin, which is then *O*-methylated by the action of the hydroxyindole-*O*-methyltransferase (HIOMT) to produce melatonin. The oxidative deamination of serotonin, catalyzed by monoamine oxidase, leads to the formation of other indole compounds, including 5-hydroxyindole acetic acid and 5-hydroxytryptophol, which are also good substrates for HIOMT. Melatonin may also be deacetylated *in situ* to produce 5-methoxytryptamine and 5-methoxytryptophol in the fish pineal organ and vertebrate retina.<sup>26,27</sup>

Melatonin biosynthesis takes place in the chief cells of the pineal gland.<sup>3,5</sup> Indeed, compounds as well as enzymes of the pathway all co-localize in the photoreceptors (cone-like in ectotherms and modified in sauropsids) or pinealocytes (mammals). Similarly, biosynthesis occurs in the photoreceptor cell layer of the retina in all vertebrates. In addition, expression of *Aanat* has also been reported to occur in a subset of cells in the inner nuclear and ganglion cell layers of trout, chicken, and monkey, although apparently at much lower levels than in the photoreceptor cells.<sup>7,28,29</sup> In trout, the same cells also express *Hiomt*, consistent with the idea that melatonin biosynthesis is not an exclusive property of the photoreceptor cell line. AANAT expression has also been found in extraretinal



**Figure 2.** Melatonin biosynthesis pathway. The right panel shows (top) a 24-h light (L)/dark (D, black bar) cycle and the (bottom) corresponding variations of the indole compounds or enzymes. AAAD, aromatic amino acid decarboxylase; AANAT, arylalkylamine *N*-acetyltransferase; HIOMT, hydroxyindole-*O*-methyltransferase; TpOH, tryptophan hydroxylase. (In color in *Annals* online.)

and extrapineal areas of fish and frog brains (authors' unpublished data).<sup>30,31</sup> The functional significance of this expression remains to be elucidated (see below).

### Photoperiodic and Circadian Control of Melatonin Biosynthesis

The melatonin rhythm relies mainly on the rhythmic activity of AANAT, both in the retina and pineal gland (Fig. 2).<sup>32,33</sup> This is why the enzyme deserves the nickname “timezyme.”<sup>33</sup> Indeed, in all species so far investigated, the alternation of light and darkness synchronizes the rhythmic activity of AANAT activity and

melatonin production, and there is an inverse relationship between the levels of serotonin and those of *N*-acetylserotonin. AANAT is responsive to light; light exposure of dark-adapted animals results in a rapid decrease of enzyme activity and consequently of melatonin secretion. In teleost fish this is achieved directly by virtue of the light sensitivity of its photoreceptor cells. Photoreceptor depolarization at night leads to a concomitant increase in intracellular calcium ( $[Ca^{2+}]_i$ ) and cyclic AMP (cAMP); both contribute to increase AANAT activity. Illumination results in photoreceptor hyperpolarization, decrease in  $[Ca^{2+}]_i$  and cAMP, and subsequent enzyme degradation through proteasomal proteolysis.<sup>34</sup> A similar intracellular pathway operates in the mammalian pinealocyte; however, the nocturnal increase is triggered by norepinephrine released from the sympathetic terminals at night.<sup>13,25</sup> Light caught through the eyes turns off the system and AANAT follows proteasomal degradation. Variations on the theme have been described in the chicken.<sup>35</sup>

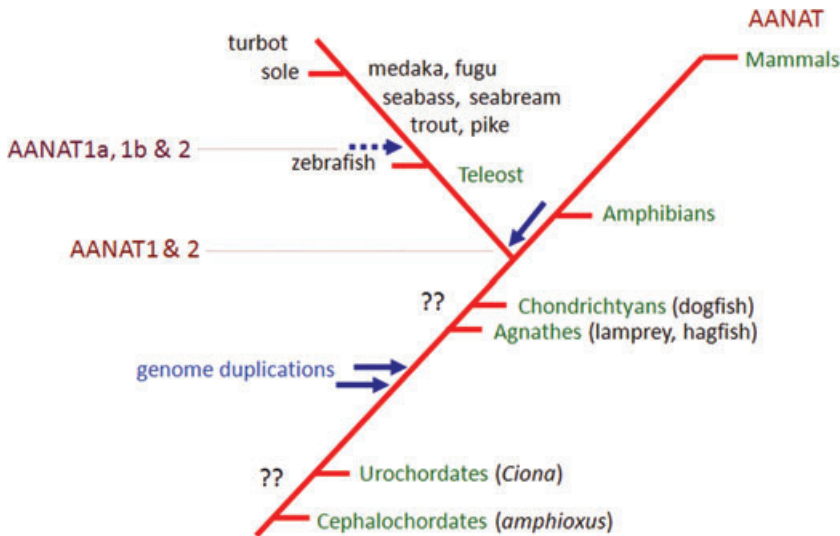
In addition to this on/off type of effect, the LD cycle also acts on the melatonin rhythm through synchronizing the phase of the circadian clocks that drive *Aanat* expression. In fish, each photoreceptor cell possesses a clock machinery so that under constant darkness AANAT gene expression as well as AANAT activity continue to oscillate with a period close to 24 h *in vivo* and *in vitro*.<sup>36</sup> This is because the AANAT gene is a direct output target of circadian clock genes.<sup>37</sup> This occurs only *in vivo* in mammals; in addition to losing the photoreceptive machinery, the pinealocyte has lost the circadian machinery. Rather, it is the clock machinery of the SCN (which activity is synchronized by light caught through the eyes) that controls pineal *Aanat* expression.<sup>13,25</sup> The melatonin rhythm of vertebrates thus reflects both the phase of the circadian clock and the environmental lighting. The difference between fish and mammals is that the fish photoreceptor contains the three elements that make a full circadian system: the photoreceptive unit,

the clock, and the melatonin-producing unit (the clock output). In mammals, these three elements are located in distinct and specialized areas: the eye, the SCN, and the pineal gland, respectively. There are exceptions to this schematic presentation. For example, no functional circadian machinery could be evidenced in the pineal organ of salmonid fish *in vitro*.<sup>38–40</sup> However, pineal AANAT activity, as well as serotonin and melatonin content, do oscillate on a circadian basis in trout maintained under constant darkness *in vivo*.<sup>41</sup> This would suggest that the circadian organization that controls pineal melatonin secretion is likely to depend on a retinal/brain pathway in trout, as is the case in mammals. Further studies are needed in trout and other fish in order to determine whether this reflects a convergent evolutionary proper to salmonid fish species.

### AANAT along Vertebrate Evolution

As a crucial enzyme in the regulation of melatonin secretion, AANAT has deserved special attention. First cloned in mammals,<sup>42,43</sup> AANAT nucleotide and amino acid sequences are now available from a number of vertebrate representatives, including birds,<sup>44</sup> frogs,<sup>30</sup> and teleost fish (Fig. 3).<sup>3,5</sup> These AANATs form a family within the superfamily of acetyltransferases, which share in common a binding site for acetyl coenzyme A.<sup>45</sup> Ancestor forms of these AANATs are found in bacteria, fungi, unicellular green algae, as well as in the amphioxus (prechordate) genome but not in other Eukaryotes. These AANATs lack specific regions involved in regulation, binding, and catalysis<sup>45</sup> and might have different functions.<sup>13</sup> How and when did vertebrates acquire AANAT? Was this concomitant with the appearance of melatonin synthesis or was the function of the primitive AANAT different? These are among the questions that remain unresolved.<sup>45</sup>

It is interesting that teleost fish, unlike all other vertebrates, possess two AANAT subfamilies, namely AANAT1 and AANAT2 (Fig. 3). More distant teleost fish may even express two



**Figure 3.** Evolution of AANAT subtypes in vertebrates. The blue arrows point to (possible) whole genome duplications. (In color in *Annals* online.)

subtypes (a and b) of the AANAT1 subfamily. The AANAT1 subfamily is homologous to the AANAT found in tetrapods. They are expressed preferentially in the retina and discrete brain areas. AANAT2 is more specifically expressed in the pineal organ and has no equivalent in other vertebrates.<sup>5,46</sup> It is believed that a whole genome duplication that occurred close to the origin of teleost fish<sup>47–49</sup> is responsible for the appearance of the AANAT1 and AANAT2 subfamilies and another round of duplications might have been responsible for the occurrence of two AANAT1 isoforms in the more distant teleost fish.<sup>45</sup> It has been suggested that *rapid evolution*, a common feature of teleost fish that results from large progenitor size and short generation time, might also have facilitated rapid genetic changes in response to environmental pressures. A more complete picture should arise after a large-scale search for AANAT genes has been performed in teleost and other fish. This interesting hypothesis is supported by the specific temperature responses displayed by the AANAT enzymes.

In this regard, teleost fish AANAT2 is of special interest. Initial studies have shown that temperature is likely to play an important role in the control of AANAT activity and melatonin

production<sup>3,5</sup>; temperature cycles are able to synchronize a rhythm in melatonin release by cultured fish pineal glands through controlling AANAT2 activity. However, unlike other external (LD cycle) or internal (hormones, neurotransmitters) factors that act through specific membrane-bound or nuclear receptors,<sup>3,5</sup> temperature targets the AANAT2 enzyme itself. Indeed, when studied in parallel, the response curve to temperature of cultured pineal organs and corresponding recombinant enzymes display the same profile.<sup>46</sup> Maximal AANAT2 activity depends not only on the species but also (usually) on the correspondence to the fish-preferred temperature (trout, 12°C; pike, 20°C; seabream, 27°C; zebrafish, 30°C).<sup>46,50–54</sup> In contrast, the activity of the corresponding retinal AANAT1 enzyme increases linearly (up to 25°C in trout and 37°C in pike and seabream). The results would indicate that AANAT2 optimal activity is very close to the thermal preferences/geographic distribution of the species. As a thermosensor, AANAT2 allows melatonin levels to reflect ambient temperature, thus providing further indication of the time of day, or year, or position in the water column. This is a remarkable adaptation to the environment. It was made possible by gene

duplication followed by discrete mutations in the AANAT2 peptide sequences favored by the environmental pressure specific to each species. Such mutations are likely to affect enzyme flexibility and mobility, thus the response to temperature.<sup>55-58</sup>

The differences in peptide sequence affect the enzyme kinetics as well. In a general manner, recombinant retinal AANAT1 has a relatively high substrate affinity and a low activity rate and is inhibited by high substrate and product concentrations.<sup>50,51,53,54</sup> In contrast, recombinant pineal AANAT2 exhibits a low substrate affinity and a high activity rate and is not inhibited by substrates or products. The two recombinant enzymes also exhibit differential substrate preference. Retinal AANAT1 acetylates a range of arylalkylamines, including indole- and phenylethylamines, while pineal AANAT2 preferentially acetylates indole-ethylamines. This indicates the two AANATs may have different tissue-specific functions. Pineal AANAT2 specializes in the production of large amounts of melatonin and perhaps other methoxyindoles (e.g., 5-methoxytryptamine) for endocrine purposes. The function of retinal and brain AANAT1 might be more complex in fish. In addition to contributing to the local production of melatonin, AANAT1 might also be involved in the production of acetyl-dopamine and acetyl-serotonin. This is relevant in view of the now well-established relationships between dopamine and melatonin in the retina and brain.<sup>59,60</sup> Acetyl-dopamine might be a catabolic product or an active compound with still unknown functions.

It is interesting that the kinetics of the mammalian AANAT appear closer in terms of substrate selectivity to teleost fish AANAT2 whereas phylogenetic analysis indicates a closer relationship with AANAT1. This has allowed the preservation of the primary function (melatonin production) in addition to allowing species-specific requisites (response to temperature).

## Conclusions

The organization of the circadian system of vertebrates has changed dramatically over evolution. Nonmammalian vertebrates are characterized by a network of more or less powerful and interconnected circadian systems, including the eye, pineal gland, and probably brain.<sup>4</sup> Mammals display a more "linear" organization where the different components of the circadian system are scattered in specialized areas, i.e., photoreception in the eyes, clock machinery in the brain, melatonin output in the pineal gland.<sup>4</sup> Despite the fact the pineal gland has undergone dramatic changes from early vertebrates to mammals, the nocturnal pattern of melatonin production has been maintained in all vertebrates so far investigated, emphasizing the importance of the delivered information. Changes in the pineal gland are seen at the anatomical, cytological, and molecular levels. They resulted in a complete loss of the direct photoreception and circadian functions; instead, a complex neural pathway, involving the eyes, the clocks of the SCN, and sympathetic innervations to the gland, brings the photoperiodic information. Within the gland, the melatonin regulatory pathways target AANAT, the key enzyme responsible for the melatonin rhythm. In all vertebrates, AANAT responds to the photoperiodic information; in ectotherms it also responds to temperature changes. In this regard, teleost fish are special because they possess several AANAT genes, which display tissue-specific expression. Also, the pineal AANAT2 responds to temperature in a species-specific manner, which reflects the fish ecophysiological preferences. The basis of this temperature dependence is not known. What evolutionary mechanisms have led to these modifications of the circadian organization in general and of the pineal gland in particular? When did AANAT appear in vertebrates and was its primitive function already related to melatonin production? These are among the questions that need to be addressed in the future.



### Conflicts of Interest

The authors declare no conflicts of interest.

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