Nonpineal Melatonin in the Alligator (*Alligator mississippiensis*)

Abstract. *All living and most fossil representatives of the reptilian subclass Archosauria lack pineal bodies. Arrhythmic, low-level, nonpineal melatonin is present, however, in the blood of* Alligator mississippiensis. *Although pineal bodies have been implicated in circadian phenomena, these results suggest that arrhythmic melatonin in alligators may not be involved in circadian events and indicate that the pineal is not the only source of the hormone melatonin. The evolutionary loss of the pineal in Archosauria occurred during the Mesozoic, an era noted for its seasonal stability. Arrhythmic melatonin titers in alligators and pineal loss in alligators and other archosaurs may be related to Mesozoic seasonal stability.*

The presence of the indoleamide melatonin (*N*-acetyl-5-methoxytryptamine) was initially discovered by Lerner *et al.* (*1*) in the bovine pineal gland; subsequently, numerous studies have shown that melatonin is a characteristic hormone of pineal organs. The last enzyme involved in melatonin formation (hydroxyindole-*O*-methyltransferase) once was thought to occur only in the pineal gland, but since has been found in such extrapineal locations as the retina (*2, 3*), Harderian gland (*4*), and brain (*2*). Radioimmunoassay (*5, 6*) and immunocytochemical (*7*) procedures indicate that melatonin is present in these tissues and in the enterochromaffin cells of the intestinal tract (*8*), an additional site of serotonin synthesis. Melatonin also occurs in the blood of surgically pinealectomized animals. (*5, 6, 9*). These observations indicate that melatonin may not be a pineal-specific hormone. Since it is possible to detect melatonin after pinealectomy and since extrapineal sites for melatonin synthesis and secretion have been identified, it is appropriate to verify the presence or absence of nonpineal melatonin in a species that does not possess a pineal gland.

Our interest in alligators (*Alligator mississippiensis*) stems from the observations that (i) the presence or absence of melatonin has not been examined heretofore in any animal lacking a pineal organ; (ii) the presence of melatonin after loss of the hormone's major gland of secretion has not been questioned in an evolutionary sense; and (iii) among reptiles, circulating melatonin has been identified only in lizards (*10*) and turtles (*11*) which represent two of the four living orders in the class Reptilia.

Modern alligators have changed little from their Oligocene ancestors which shared a similar body form and aquatic ecology (*12*). The Crocodilia originated in the Middle Triassic. Embryological (*13, 14*) and paleontological (*15*) studies demonstrate that modern alligators and their Triassic ancestors lack pineal bodies. Also, we have not been able to demonstrate either pineal bodies or the presence of pineal cells in the alligator and the caiman. After validating a melatonin radioimmunoassay (*16*) for alligator plasma, we determined that the circulating plasma of alligators contains melatonin and that the melatonin titer shows no significant fluctuation over a 24-hour, light-dark (LD) period (one-way analysis of variance).

The alligators used for our study were raised in an outdoor enclosure at the St. Augustine Alligator Farm (St. Augustine, Florida) where they were entrained by natural photothermal periods. On day 1 at 1200 hours, five animals were moved, because of their size and temperaments, from the home enclosure to adjacent individual wooden chambers. The animals rested in the chambers until sampling began at 1425 hours. Every 3 hours thereafter, each animal was handled for approximately 1.5 minutes during which time samples (5 ml) of whole blood were removed percutaneously from a supravertebral vessel off the internal jugular vein at a location just posterior to the cranial suture of the supraoccipital and parietal bones, cephalad and medial to the first small dorsal scute (*17*). Eight samples were taken during one photoperiod (LD, 14:10) (samples 1, 2, 7 and 8 during the photophase and samples 3, 4, 5, and 6 during the scotophase) from each of the five animals. These samples were centrifuged at 2000 rev/min, immediately quick-frozen, and stored at −80°C until assay. Although body temperature was not recorded, maximum ambient temperature during the photophase was 35°C (at about 1400 hours) and minimum ambient temperature during the scotophase was 21°C (at about 0400 hours). Thus, animals were exposed to and entrained by a natural photoperiod (LD, 14:10) and thermoperiod (LD, 35°C : 21°C) with minimal disturbance due to handling. Periods of low-level lunar lighting and some peripheral lighting (60-W shielded bulb; nearest distance, 20 m) on buildings within the St. Augustine compound provided sufficient illumination while this sample was being withdrawn.

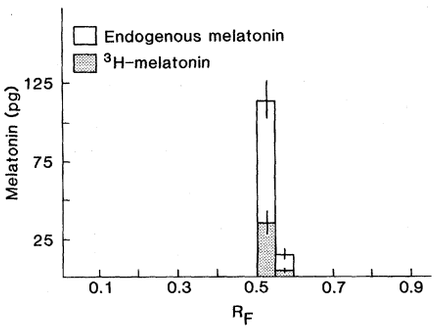

Fig. 1. Chromatographic identity was carried out on a pooled sample of alligator plasma (*19*). Radioimmunoassay and liquid scintillation spectrometry of the material eluted from the segmented chromatogram demonstrated that the [³H]melatonin comigrated with the immunoreactive material in the alligator plasma indicating that the extracted material was melatonin. Radioimmunoassay and subsequent analysis of triplicate, ten-point, two-fold sequential dilution series of either extracted alligator plasma or 1 ng of melatonin per milliliter in phosphate-buffered saline solution containing 0.1 percent gelatin demonstrated that the line derived by assay of extracted alligator plasma (slope, −1.75) was parallel to that derived from the buffer (1 ng/ml) (slope, −1.71).

The radioimmunoassay (RIA) was validated from pooled plasma samples by means of parallelism and chromatographic identity techniques (*18, 19*) modified for this study (Fig. 1). Alligator plasma extract and RIA control samples were assayed in triplicate by the non-equilibrium, double-antibody melatonin RIA (*16*). Data were analyzed by one-way analysis of variance.

The validation procedures indicate that melatonin is the molecule being measured by the RIA; nevertheless, there is still the possibility that a cross-reactant other than melatonin is responsible for the immunoreactivity in extracted alligator plasma.

We suggest that melatonin is present in blood of the alligator (Fig. 1), presumably produced by nonpineal sources. In addition, if mean values for photophase and scotophase are compared, rhythmic fluctuations in melatonin do not exist (Fig. 2). In all other species investigated, melatonin titers oscillate with peak titers occurring during the scotophase (*5, 6, 9–11, 18*). In this study, only the female alligator (animal B) exhibited melatonin titers that may have been rhythmic, indicating a potential sexual difference.

It is also possible that alligators may have rhythmic melatonin release during periods of the year with more than 10 hours of darkness; however, as is mentioned below, nocturnal melatonin synthesis seems to be controlled by the pineal body (*5, 6, 9*). Therefore, an increasing scotophase should have no effect on animals without pineal bodies.

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Hence, the lack of pineal bodies seems to be the most reasonable explanation for melatonin arrhythmia in alligators. The effect of increasing scotophase length on melatonin levels in alligators remains to be examined.

In animals that have pineal bodies, persistent, baseline levels of melatonin usually can be measured after surgical pinealectomy—a procedure that tends to reduce the amount of melatonin during scotophase to those more characteristic during photophase which are little affected by the procedure (*5, 6, 9*). Apparently, high scotophase titers of melatonin result from a surge of pineal melatonin added to a basal level of melatonin secreted by nonpineal sources. This possibility appears to be indicated by the nonrhythmic, low levels of melatonin in alligators. Potential sites of melatonin synthesis in alligators likely include the Harderian glands, which are especially large in members of the Crocodilia (*20*), and other extrapineal locations (*5, 6, 8*).

Alligators appear to have a relatively precise activity rhythm entrained by light (*21*) and perhaps temperature. Although the pineal body and melatonin have been implicated in circadian (*22*) and diurnal activity rhythms (*23*), noncyclic, nonpineal melatonin secretion in alligators suggests that a circadian role for this indoleamide in alligator biology may be questionable, although a circannual role cannot be excluded.

The skulls of fossil vertebrates clearly indicate that epiphyseal structures such as the parietal eye and pineal body were common features in evolution (*15, 24*). Fossil and modern vertebrate pineal organs present three basic conditions: E-2 (parietal eye and pineal present), E-1 (pineal present), and E-0 (parietal eye and pineal absent). Epiphyseal systems are absent (E-0) in only a few modern vertebrates other than crocodilians, for example, edentates and sirenians (dugongs) (*25*). The E-2 condition, although common in evolutionary history, is primarily restricted to lizards, the Tuatara (*Sphenodon punctatus*), some amphibians, and lampreys. The E-1 condition is nearly ubiquitous among modern vertebrates (*25*). Although the evolutionary prevalence of the epiphyseal system may underscore its importance, the E-0 condition in some vertebrates suggests that (i) other undefined systems have evolved to replace the recognized functions of the pineal body, (ii) enough overlap in brain function exists (*26*) that other systems have assumed or resumed the role of the pineal, or (iii) some combination of anatomy, physiology, and ecology renders retention of the pineal nonessential in certain environmental conditions. The third possibility is most reasonable.

Alligators are the surviving representatives of the archosaurs, the subclass of reptiles to which dinosaurs belong. The ancestors of alligators and most dinosaurs are E-0 vertebrates (*14*) which evolved in the latter half of the Triassic at a time when climates were less seasonal than during the Permian, a trend which would continue through the Middle Cretaceous (*27*). We suggest that, in response to these conditions, the circadian, reproductive, and thermoregulatory aspects of pineal regulation were de-emphasized to the point at which the extrapineal synthesis of melatonin reported herein for E-0 crocodilians was adequate for daily, circannual, reproductive, or thermoregulatory processes. The present-day persistence of crocodilians in tropical, subtropical, and some warm temperate regions may be indicative of an ancestral adaptation to the particular Mesozoic climates wherein their E-0 condition originated. If the crocodilian E-0 condition did originate in response to selection in a warm, less seasonal Mesozoic world, further insight into the evolution of vertebrates without pineal bodies might be gained by examining the history of certain endothermic E-0 vertebrates. For example, edentates and dugongs, whose ancestors can be traced to the Eocene, are E-0 endotherms. Vertebrates with small E-1 pineal morphology, such as the opossum, whose ancestors appear in the late Cretaceous fossil record, may also provide interesting physiological evidence which may be linked to specific environmental conditions. However, it should be noted that small organ size may not reflect less involvement of an organ in physiology, or that large size reflects selection for a relatively greater contribution from large organs.

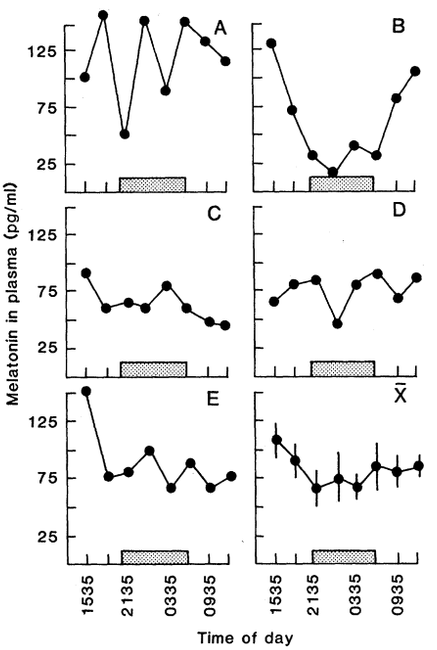

Fig. 2. Radioimmunoassay of eight plasma samples taken over a 24-hour period from each of five alligators revealed that these animals did have circulating melatonin even though they lacked a pineal organ. Interestingly, animal B (the only female) did show what appeared to be a daily rhythm in plasma melatonin (scotophase, stippled block). The mean ($\bar{X}$) plasma melatonin titer calculated from all animals for each time point over the 24-hour sampling interval did not show a significant variation from day to night.

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19. High pressure liquid chromatography (HPLC) was used as a second identity validation for melatonin. Ten 1-ml samples of alligator plasma, one containing [^{3}H]melatonin (5000 count/min; New England Nuclear), were extracted with chloroform, and washed in sodium bicarbonate buffer (*p*H 10.25) and distilled water. The samples were concentrated and dried with N_2 gas. The dried extract was resuspended in 250 μl of chloroform and filtered through a 0.45-μm Teflon filter. The filtrate was injected onto a Waters μBondapak NH_2 HPLC column (30 cm) with a 10-minute linear solvent gradient (50 percent tetrahydrofuran and 50 percent isooctane to 100 percent acetonitrile containing 1 percent acetic acid). Every 15 seconds, 0.5-ml fractions were collected, dried with N_2 gas, and resuspended in 350 μl of phosphate-buffered saline gel buffer; the ^{3}H was determined by liquid scintillation in each resuspended 100-μl portion. Two 100-μl portions were analyzed by RIA for melatonin (Fig. 1). [^{3}H]Melatonin is present in larger amounts in fraction 31 (7:30 to 7:45 minutes) or fraction 32 (7:45 to 8:00 minutes).
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28. We thank the St. Augustine Alligator Farm, St. Augustine, Fla., for providing the facilities and alligators for this study. We particularly express our appreciation to David Drysdale. The RIA's of this study were funded by NIH grant NS 12257. The melatonin antibody and radioiodinated analog were supplied by Drs. Gordon Niswender and Mark Rollag.

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Neuronal Control of Acetylcholine Receptor Turnover Rate at a Vertebrate Neuromuscular Junction

Abstract. *The turnover rate of acetylcholine receptors at neuromuscular junctions in mice increases progressively after denervation and, after 15 days, reaches a half-time of 30 ± 5 hours. Denervation thus causes the clustered junctional acetylcholine receptors to assume the rapid turnover characteristic of extrajunctional receptors before innervation.*

It is well documented that nerves influence the metabolism and physiology of skeletal muscle (*1–3*), although the mechanisms behind this are unknown. One expression of neuronal control is the progressive decrease in the turnover of acetylcholine (ACh) receptors which occurs at the neuromuscular junction (NMJ) after innervation (*4–6*). Two distinct turnover rates for ACh receptors in muscle have been described: for extrajunctional receptors (before innervation and after denervation), a half-time of about 1 day (*6–11*), and for adult innervated junctional receptors, a half-time of about 10 days (*7–16*). Recently, however, a half-time of 2 to 3 days for denervated adult junctional ACh receptors was reported (*12–13*). In the present study we sought to determine whether the denervated junctional ACh receptors demonstrate a third stable turnover rate or whether the reported half-time of 2 to 3 days represents a point in a time-dependent decay of neuronal influence on the turnover rate of junctional ACh receptors after denervation.

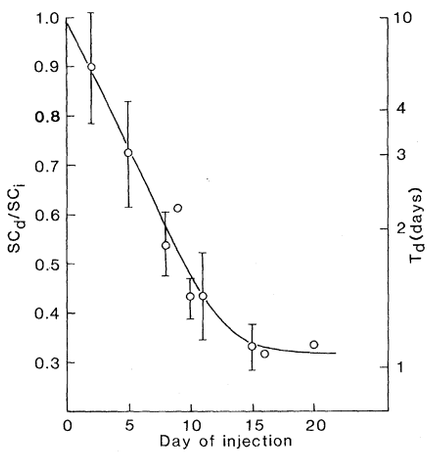

Fig. 1. Ratio of residual radioactivity at denervated (SC_d) and innervated (SC_i) NMJ's of mice injected with ^{125}I-labeled α-BGT at various times after denervation and killed 2 days (t) later. The average half-time for turnover of denervated junctional receptors (T_d) was approximated from the ratio of the two exponential decays, $SC_d/SC_i = (2 \exp - t/T_d)\, SC_{d_0}/(2 \exp - t/T_i)SC_{i_0}$, with an average half-time for turnover at innervated junctions of 10 days (*11*) and given that the radioactivity in the two junctions at time zero is the same $[(SC_{i_0}) = (SC_{d_0})]$. Errors bars represent standard errors of the means for groups of three to seven animals. Data points without error bars represent values for single animals.

We assessed the turnover of ACh receptors by measuring the loss of radioactivity bound to the NMJ's after an injection of ^{125}I-labeled α-bungarotoxin (α-BGT), the inhibitor of the nicotinic ACh receptor of vertebrate muscle (*17*). This assay gives a reliable measure of the degradation of both extrajunctional and junctional ACh receptors (*1, 9, 14–16, 18–20*) rather than of mere dissociation of α-BGT from the receptors. For the innervated NMJ's the validity of this assay obtains in part from the demonstration that various metabolic inhibitors cause a decrease in the rate of both the loss of radioactivity after labeling (*9, 14–15*) and the recovery of neuromuscular response (*21*) after inactivation with α-BGT. In preliminary studies we found that actinomycin D similarly decreases the rate of loss of radioactivity from denervated NMJ's. Furthermore, up to 16 days after denervation, structural localization and absolute density of radioactive sites after inactivation at the NMJ's by ^{125}I-labeled α-BGT is the same as that at innervated junctions, and at both kinds of junctions the loss of radioactivity mirrors the appearance of new labeled binding sites (*13*). Because of this steady state at the NMJ, degradation reflects metabolic turnover of receptors.

We used mouse sternomastoid muscle since it has an easily accessible nerve and a well-defined end-plate band and was previously used in our laboratory for electron microscope (EM) autoradiography studies of receptor localization (*20*) and turnover after denervation (*13*). We anesthetized the mice (27 to 37 g) with Nembutal and denervated one of the two sternomastoid muscles by removing 1 to 2 mm of nerve as close to the muscle as possible.

At different times after denervation, groups of these animals were injected with ^{125}I-labeled α-BGT (4.1 μg per 100 g of body weight, intraperitoneally) (*22*). Two days later, the animals were killed under anesthesia by intracardial perfusion with 4 percent paraformaldehyde and the two sternomastoid muscles were removed and stained for acetylcholinesterase in order to identify the end-plate

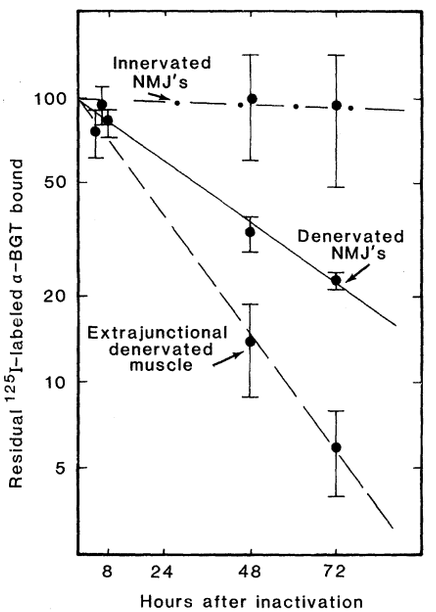

Fig. 2. Decay of radioactivity bound to innervated and denervated sternomastoid muscles after injection of labeled α-BGT. All curves are normalized to 100 percent at the time of injection.

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