A carnivore species (Canis familiaris) expresses circadian melatonin rhythm in the peripheral blood and melatonin receptors in the brain

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Dogs kept under controlled photoperiodic conditions of 12 h light and 12 h dark expressed a clear diurnal melatonin rhythm in the peripheral blood, with a swift peak restricted to the late part of the scotophase. The highest density of high-affinity, G-protein-linked 2-[¹²⁵I]iodomelatonin binding sites was found in the pars tuberalis of the pituitary gland. Binding sites were found also in the pars distalis, and light microscopy/high-resolution autoradiography showed that binding was located exclusively over the chromophobe and basophilic cells forming the adenohypophysis zona tuberalis, well developed in this species, and extending into the gland as a continuation of pars tuberalis. Cords of basophilic cells located in the pars distalis proper also expressed high receptor density. The cosinophil in the adenohypophysis and the neural lobe were devoid of binding. Heavily labeled were the external laminar and the mitral cell layers of the olfactory bulbs, but no binding was detected in the fiae nervi olfactorii or tractus olfactorii. The hypothalamic suprachiasmatic nuclei were discernible clearly. Quantitative autoradiography inhibition experiments revealed that the apparent melatonin inhibitory constant (IC₅₀) in all those areas was around 0.1 nmol/L, which is a physiologically appropriate value considering the peripheral blood melatonin levels. Co-incubation with guanosine 5’-O-(3-thiotriophosphate) (GTP·S) led to a consequential decrease in the binding density. The specific binding observed in other areas (hippocampus, frontal, parietal, occipital cortex and cerebellum) was rather weak, diffuse and could not be attributed to a particular layer; the apparent IC₅₀ for melatonin was about 1 μmol/L, and co-incubation with GTP·S did not modify the binding density. Collectively, these data show that the dog possess all the prerequisites for an efficient network adapted to photoperiodic time measurements. A circadian melatonin signal in the peripheral blood and an apparently functional readout receptor system located in key positions within the brain are both present in this species.

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Recognition of the oscillatory properties of the functions of the neural structures is among the most striking discoveries emerging in the modern era of brain research. The oscillatory function apparently is shared at all levels: gene expression; output patterns of neuronal networks and complex intercommunicating neuroendocrine systems; behavioral and adaptive responses of an individual and therefore of a species (1–3). The fluctuating nature of most of the biochemical, physiological and behavioral parameters is expressed most noticeably in oscillations occurring on a daily basis and persisting under constant conditions, with periods near 24 h in length, showing that they are driven by a “circadian clock”. The existence of mechanisms controlled on a circadian basis is fundamental for the adaptation of the species to the environment, and involve complex phenomena such as feeding, sleep-wake cycles and reproduction (4, 5).

Most carnivore species, including the dog, are not considered to be clearly circadian in terms of their physiology and behavior, and a number of parameters, such as the sleep-wake cycles, the core temperature or the urinary electrolyte and water excretion, apparently do not display conspicuous circadian rhythms. It has even been suggested that dogs possess a rhythmic organization completely different from that of most mammalian species (4), but there is a number of conflicting reports (6–9).

The dog is a common laboratory animal, employed in chronic experiments that often do not take into consideration the eventual impact of endogenous circadian oscillations on the systems-generated output. Therefore, knowledge concerning the ability of this species to measure time in the circadian and photoperiodic sense and to make the appropriate physiological adjustments is of crucial importance when performing long-term studies. This information concerning carnivores in general, and the dog in particular, is scarce and extremely controversial (4–9).

Because melatonin is a hormone expressing a
rhythmicity that is controlled directly by the circadian biological clock, it is now considered the most suitable marker for revealing the existence of oscillatory endogenous circadian rhythmicity (10). Moreover, the melatonin signal is decoded by high-affinity melatonin receptors in the brain that are located, in most of the species studied to date, in the hypothalamic suprachiasmatic nuclei, which is the site of the circadian pacemaker (11–14). No data concerning the possible existence of the melatonin signal in the peripheral blood or the presence of melatonin receptors in the brain of the dog have ever been reported.

In the present study we investigated whether the domestic dog (Canis familiaris) possesses the essentials of a network suited to biological timing, i.e. a circadian melatonin signal in the peripheral blood and high-affinity melatonin receptors in the brain.

Materials and methods

Animals and tissues

All procedures used in this study were in accordance with the pertinent laws of the country and were approved by the institutional committee for laboratory investigations. Six young adult Beagle dogs (four females, two males; 15 months old) were kept in individual rooms with water available ad libitum and food supplied twice a day under controlled photoperiodic conditions of 12 h of light and 12 h of darkness (LD 12 : 12), with lights off at 18:00 h. The females were anestrous at the time of the experiment and all dogs were kept on the LD 12 : 12 photoperiod for at least 1 month before the beginning of the experiment. Blood (about 3 ml) was drawn from the jugular vein at 3-h intervals in heparinized (75 IU), evacuated blood-harvesting tubes (Venoject, Terumo Europe, Leuven, Belgium). During the scotophase, the blood was collected with the aid of dim red light. The samples were chilled immediately on ice, centrifuged and the plasma stored frozen at −20°C until assayed for melatonin (see below).

The brains of three male dogs of mixed breed (18–32 months old) were acquired courtesy of the Department of Clinical Physiology and Hypertension, University of Milan. All were healthy normotensive animals that had served as controls in previous studies and had not been subjects of experimentation for the last 2 months. The anesthesia, induced around the middle of the light phase under LD 12 : 12 consisted of intramuscular ketamine (10 mg/kg body weight) followed by intravenous ketamine (2 mg/kg) and then euthanasia by exsanguination. The brains were perfused with ice-cold saline, rapidly removed with the pituitary glands attached and the dissected areas frozen in cold isopentane (−30°C); the samples were stored at −70°C.

Materials

2-[^125]I]Iodomelatonin (SA, 1600–2000 Ci/mmol) was purchased from Amersham (Aylesbury, Buckinghamshire, UK). Drugs and chemicals were obtained from Sigma Chemical Co. (St Louis, MO), unless stated otherwise. 2-[^127]I]Iodomelatonin was obtained from RBI (Natick, MA). Anesthetics and the solutions used during the blood and brain tissue collection were from Farmitalia-Carlo Erba, Milano, Italy. The autoradiographic film X-OMAT S was from Eastman Kodak (Rochester, NY). Hyperfilm MP was from Amersham.

Determination of melatonin in the plasma

The plasma samples were thawed and analyzed by radioimmunoassay (15), validated for dogs plasma using rabbit anti-melatonin antibody R/R/19540-16876 kindly provided by Dr J-P Ravault (Nouzilly, France) and 2-[^125]I]Iodomelatonin as a labeled ligand. Dextran (0.05%)-coated charcoal (0.5%) was used to separate the unbound hormone, and aliquots of the supernatants were counted on a Packard Multy Prias-I gamma counter set up to a counting error of 2%. The results were calculated by using a computerized approach (logit/log data transformation). The sensitivity of the assay was 2.5 pg/tube, with a linearity range of 10–80 pg/tube, and the incubation volume was 500 µl. The slope of the standard curve was −1.32; the slope of a serial twofold dilution of plasma containing a high melatonin level was −1.27 and the recovery was 92%. The mean interassay and intra-assay (N = 6) coefficients of variation were 5.6% and 12.8%, respectively.

Autoradiographic studies

Sections (20 µm) were cut in a cryostat at −15°C and thaw-mounted on chrome-alum gelatin-coated slides. The sections were left overnight at 4°C in a desiccator and kept thereafter at −70°C for no longer than 3 days. The whole hypothalamus and the overlaying thalamus, up to the ventral border of the corpus callosum, was examined at intervals of 100–600 µm. Additionally, a wide range of coronal and parasagittal sections from the olfactory bulbs, prefrontal, frontal, parietal, temporal and occipital cortices, hippocampus, medulla oblongata and the cerebellum were analyzed. The basic autoradiographic method employed has been described in detail elsewhere (14). Additionally, following the last wash, the sections were fixed in 4% paraformaldehyde for 5 min. The quantity of the labeled ligand in the mapping experiments was about 60 pmol/l. The non-specific binding was determined in adjacent sections in the presence of 1 µmol/l unlabeled 2-iodomelatonin. In the inhibition experiments, melatonin was used as a competitor in the range 1 × 10⁻¹¹–1 × 10⁻⁶ mol/l. When the effects of
200 µmol/l guanosine 5’-O-(3-thiotriphosphate) (GTPγS) were evaluated the quantity of the labeled ligand was 30 pmol/l. The dose of GTPγS employed was calculated on the basis of preceding in vitro ligand binding experiments with melatonin receptors (16), to obtain 50% inhibition of the specifically bound 2-[^125]Iodomelatonin with the same batch of GTPγS. Equimolar doses of ATP or ATPγS were without affect. Following exposure, the autoradiograms were inspected visually, and, subsequently, semi-quantitative analysis was performed on a computerized image-analysis system. The background-subtracted raw optical densities of the film images generated from sets of slides processed under identical experimental conditions were compared in order to express the results. The relative inhibition values for melatonin were calculated by using non-linear fitting strategies. All data were gathered from two or three independent determinations per area per animal in duplicate.

Adjacent brain sections were stained with Cresyl Violet and examined by light microscopy to verify the location of the structures according to an atlas of the dog’s brain (17). A number of sections containing the pituitary gland were stained with hematoxylin–acid fuchsin–orange G–light green, allowing for a differentiation between the pituitary cell types. In certain cases, micromanipulating apsition of the autoradiographic film on the same section slide following staining was employed to pinpoint the exact location of the binding. In addition, a high-resolution autoradiographic method employing emulsion-coated coverslips (18) was used to detect binding at the cellular level.

Results
Melatonin peripheral blood levels
The peripheral blood melatonin concentrations during the photophase were extremely low, oscillating around 5 pg/ml. These values did not change significantly in the early part of the scotophase (up to 7 h in the dark). An apparently swift peak in the melatonin content was registered in the later part of the dark period, the values exceeding daytime levels by five or six times (Fig. 1). Thus, a clear circadian rhythm of the hormone was registered, the melatonin signal apparently being restricted to the later part of the night.

Autoradiographic studies
The specific binding on the autoradiograms was detected in discrete brain regions as dark patches and areas; this is in contrast to the non-specific binding, which was extremely low, homogeneous and equal to the background of the sections, and often indistinguishable from the background of the autoradiographic film.

2-[^125]Iodomelatonin labeled a number of brain areas, but a few sites expressed a neat, well-delineated competitive binding the later experiments (see below) verified as being suggestive of a functional receptor: the olfactory bulbs, the hypothalamic supraoptic nuclei and the pars tuberalis of the pituitary gland; labeling was found also in certain areas of the pars distalis of the adenohypophysis. These findings were consistent in all the animal brains processed.

In the olfactory bulbs a clear stratification of the specific binding could be observed in the series of parasagittal sections. While the filae nervi olfactorii, the tractus olfactorius and the substantia grisea periventricularis were devoid of binding, the external plexiform lamina and mitral cells lamina were labeled heavily. The lamina granularis was marked diffusely (Fig. 2).

In the anterior hypothalamus, the only structures that bound iodinated radioactive melatonin were the supraoptic nuclei (Fig. 3). The rest of the anterior hypothalamus and the area preoptica were devoid of binding.

Posteriorly, strong well-delineated binding was detected in the pars tuberalis of the pituitary gland and some areas of the pars distalis. No binding was observed in the dense vascular network supplying this area. The median eminence and the neural lobe of the distal pituitary were devoid of binding. The rest of the hypothalamus and the overlying thalamus also did not express levels of binding above the background (Fig. 4).

The labeling of the pars distalis represented considerable interest because of the peculiarities in the morphology of the pituitary in this species (19). Following staining with hematoxylin–acid fuchsin–orange G–light green, we were able to distinguish very well the pars tuberalis, as well as the so-called zona tuberalis that consists of strands of pars tuberalis cells that intrude the pars distalis of the adenohypophysis. Micromanipulation apsition of the autoradiographic film over the same stained section demonstrated clearly
that the greatest part of the diffuse binding was located over the chromophobe and basophilic cells of zona tuberalis, and that the clusters of acidophils representing a prominent part of the pars distalis proper apparently were not labeled (Fig. 5). The high-resolution, emulsion-coverslip technique revealed that the diffuse binding in the pars distalis proper was associated with the cords of basophilic cells, and the acidophils essentially were devoid of binding (Fig. 6). No specific binding was detected in the medulla oblongata. However, weak specific binding was observed in a number of extrahypothalamic areas, including the hippocampus and the frontal, parietal, temporal, occipital and cerebellar cortices. The label, however, was not displaced completely by the unlabeled 2-iodomelatonin, and the binding appeared to be non-competitive.

In view of the evident dissimilarity in the competitive binding distribution in the different brain areas, the additional experimental series included determination of the inhibitory constant (IC50) of melatonin, the endogenous ligand for the putative receptor site. Indeed, quantitative autoradiography demonstrated clear-cut differences: the IC50 for melatonin in the laminar layers of the olfactory bulbs, the suprachiasmatic nuclei and the adenohypophyseal pars tuberalis and pars distalis were below 0.1 nmol/l while in the remaining tested areas the apparent IC50 was around 1 μmol/l.

Moreover, incubation of the tissue sections with the labeled ligand in the presence of 200 μmol/l non-hydrolyzable guanosine triphosphate analog GTPγS resulted in significant reduction or virtual elimination of the binding in the areas expressing picomolar affinity for melatonin. The diffuse, apparently non-competitive binding observed in other brain locations (see above) was not affected by GTPγS. The data are summarized in Table 1.

Statistical analysis was performed by Fisher's test.

Table 1. Distribution of 2[125I]iodomelatonin binding in the brain of the dog, the calculated IC50 for melatonin in the competition studies and the effects of guanosine 5’-O-(3-thiotriphosphate) (GTPγS) on the apparent binding density.*

| Area  | Control | +GTPγS | IC50 |  
|-------|---------|--------|------|------|
| OB    | 0.86    | ND     | 0.093 x 10^-9 |  
| SCN   | 0.28    | ND     | 0.082 x 10^-9 |  
| PT    | 0.93    | 0.041*** | 1.015 x 10^-9 |  
| PD    | 0.24    | ND     | 0.1 x 10^-3   |  
| POA   | ND      | Not tested | Not tested |  
| Th    | ND      | Not tested | Not tested |  
| Hipp  | 0.04    | Not tested | Not tested |  
| FC    | 0.27    | 0.19   | 1.1 x 10^-6   |  
| PC    | 0.18    | 0.20   | 1.2 x 10^-6   |  
| TC    | 0.16    | 0.14   | Not tested   |  
| OC    | 0.11    | 0.14   | Not tested   |  

*Data are given as mean raw optical densities of the background-subtracted autoradiographic film. The standard deviation (SD) values were distributed homogeneously and the coefficient of variation (CV) amounted to less than 15%; ***p < 0.001 (see text for details).

†OB: olfactory bulb; SCN: suprachiasmatic nuclei; PT: pars tuberalis; PD: pars distalis; POA: preoptic area; Th: thalamus; Hipp: hippocampus; PC: parietal cortex; TC: temporal cortex; OC: occipital cortex.

Concentration of 2[125I]iodomelatonin was 30 pmol/l.

Concentration of 2-[125I]iodomelatonin was 30 pmol/l with addition of 200 μmol/l GTPγS.

†Melatonin was employed in the dose range 1 x 10^-13 - 1 x 10^-6 mol/l, incubated with 60 pmol/l 2-[125I]iodomelatonin.

ND = not detectable above the background.

Discussion

The results of the in vivo experiments performed in this study clearly demonstrated that the domestic dog expresses diurnal cyclicity in melatonin synthesis and release, depicted by a well-pronounced circadian rhythm of the indole in the peripheral blood. The feature of the rhythm, however, had apparent characteristics suggestive of species specificity. The pattern
of pineal melatonin production and peripheral blood concentrations vary significantly among species, even within the same order. In that sense, the melatonin pattern in the dogs studied under the conditions of the present experiments (LD 12:12) resembled those observed in the Syrian hamster, Mongolian gerbil and the house mouse, i.e. the pattern consisted of an apparently sharp, brief rise late in the dark period, a type A pattern melatonin production (for a review, see Ref. 20). Considering that a detailed description of the melatonin pattern in the dog was not the aim of the current study, we avoided subjecting the animals to a more frequent sampling protocol and the results were evaluated as adequate evidence of the presence of the melatonin circadian signal in this species.

We employed 2-[125I]iodomelatonin to study the distribution and the properties of the putative melatonin receptor in the brain of this species. The distribution pattern of the high-affinity 2-[125I]iodomelatonin binding, established in the mapping experiments, precluded an extensive pharmacological and biochemical characterization of the binding site using in vitro ligand–receptor binding and signal-transduction experiments with isolated membrane preparations or tissue explants. Therefore, the trials concentrated on quantitative autoradiography as the only feasible, presently available means of answering a number of questions related to the properties of the binding site. Although the samples were exposed together with radioactive standards, we refrained from converting the optical densities to mol/mg protein because of the morphological diversity in the structures examined (e.g. pars tuberalis and pars distalis vs olfactory bulbs and the suprachiasmatic nuclei). It is well known that quantitative comparative determinations between neural and non-neural tissues result in highly incorrect estimates (21).

As demonstrated by the results, a limited number of areas expressed picomolar binding affinity for melatonin, namely, the laminar layers of the olfactory bulbs, the hypothalamic suprachiasmatic nuclei and the pars tuberalis and pars distalis of the adenohypophysis. The calculated IC50 appeared physiologically appropriate, taking the peak peripheral blood melatonin levels (see Fig. 1 and Table 1). Notably, only in these loci was the binding of 2-[125I]iodomelatonin down-regulated by co-incubation with GTPγS, implying that the putative receptor site is similar to that described in other species (22–25) and is linked to a regulatory G-protein as a first step in its signal-transduction pathway (26). The flimsy binding observed in a number of neocortical areas expressed micromolar affinity for melatonin, and GTPγS had no effect on the apparent binding density. It is known now that 2-[125I]iodomelatonin can be taken up in a non-competitive manner by certain cell types (27, 28). Another possibility is that, in addition to the melatonin receptor, 2-[125I]iodomelatonin labels with lower

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*Fig. 3.* Autoradiographs generated from two consecutive coronal sections through the anterior hypothalamus. The specific binding (b) is undoubtedly confined to the suprachiasmatic nuclei, in contrast to the extremely low non-specific binding (a). The drawing (c), prepared from a slide of an adjacent cresyl violet-stained, paraformaldehyde-fixed section, indicates the morphology. B: pial blood vessels; LMET: lamina medullaris externa thalami; OC: chiasma opticum; V: ventriculus tertius. The arrowheads indicate the positions of the suprachiasmatic nuclei, their apparent borders being depicted with a dashed line. Scale bar = 1.5 mm.
affinity other signalling proteins that are not linked to a G-protein in their transduction pathway and their physiological response to the endogenous melatonin signal is uncertain. Moreover, there are inherent problems with quantitative autoradiography, especially related to exposure times to the autoradiographic film. Sites with low receptor density (e.g. the molecular layer of the cerebellum in some species) may result in underexposure under conditions of standard autoradiography, thus compromising the quantification (Stankov, unpub. data).

The high-affinity, GTP-regulated binding observed in the hypothalamic suprachiasmatic nuclei of the dog strongly suggest that in this species the endogenous pacemaker makes use of the melatonin signal as a zeitgeber. This discovery is significant, given the recently reported (29, 30) apparent lack of binding in the same nuclei of two mustelid species; findings that had led to speculations that in carnivores there is no need for central processing of the melatonin signal. Our present results clearly suggest that this is not the case, at least with the dog. In the current study, we employed an improved autoradiographic approach (final paraformaldehyde fixation) that resulted in much better morphology upon staining and a crisper autoradiographic image. It remains to be determined if the negative findings in mustelids are the result of receptor degradation and/or ligand diffusion or, more likely, they represent species differences.

The dog has been considered generally as not clearly circadian, on the basis of conspicuous lack of diurnal rhythmicity in a number of physiological variables (for a review, see Ref. 4), but there are a number of contradictory reports (6–9). Our present data depict the first concise evidence that the dog possesses all the prerequisites for an elaborate system adapted to efficient circadian photoperiodic time measurements. These findings can help to clarify the conflicting data regarding

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Fig. 2. 251I]Iodomelatonin binding in a series of anteroposterior sequential coronal sections through the base of a dog's brain at the level of the pituitary gland. Section (b) is 500 µm and (c) is 980 µm distant from (a). The level of the non-specific binding is shown in (d), adjacent to (a). Drawing (e) is prepared from a slide of a hematoxylin–acid fuchsin–orange G–light green-stained section used to generate the autoradiographic image in (c), and schematically indicates the gross morphology. CF: columna fornicis; ME: eminentia mediana; NL: processus infundibuli (neurolobe); OT: tractus opticus; PD: pars distalis; PT: pars tuberalis; ST: stria terminalis; v: ventriculus tertius. Note that, apart from PT and PD, the rest of the hypothalamus and the overlaying thalamus are devoid of binding. Scale bar = 3 mm.
circadian studies in the dog (4, 6–9): they suggest also that fundamental experimental approaches should be re-examined when using canids in long-term studies.

The dog seemingly utilizes the melatonin signal and the readout receptor system also for other purposes, indirectly associated with circadian time measurements and related to the photoperiod, e.g. reproductive competence. The dog and other canids are overtly seasonal (31, 32) and apparently photoperiodic in their reproduction (31, 33, 34). In this context, the presence of melatonin receptors in the pars tuberalis and the zona tuberalis of the adenohypophysis might be of critical importance. These areas of the proximal adenohypophyseal tract are remarkably vascularized and receive blood from the portal circulation, which then enters the pars distalis proper from the zona tuberalis (19, 35), and both areas are rich in gonadotrophs having the same origin, i.e. the pars tuberalis.

Micromanipulation apposition of a high-sensitivity film over the same section, differentially stained to distinguish between the adenohypophyseal cell types, as well as the emulsion-coated coverslip technique allowed for a confident detection of the binding distribution in the pituitary (see Figs. 5 and 6). Both approaches were considered better alternatives to coating the sections with autoradiographic emulsion, a method having a number of inherent problems (21). To our knowledge the present data portray the first experimental evidence that the specific, high-affinity, G-protein-regulated binding site in the pars distalis of the adenohypophyseal tract is associated exclusively with the clusters of basophils and neutrophils in the zona tuberalis and that the eosinophil populations are essentially free of receptors. Thus, melatonin should be expected to participate directly in the regulation of gonadotropin synthesis and release.

Binding of 2-[\(^{125}\)I]iodomelatonin has been reported in the olfactory epithelium of fetal and adult Siberian hamsters (36) and in isolated membranes from the olfactory bulbs of other species (37, 38), but no evidence for the presence of a functional signal-transduction pathway in the olfactory bulb has been presented before, and no data concerning the discrete distribution

Fig. 5. Higher magnification (19×) of an autoradiograph generated from a section through the median eminence and pituitary (a). The autoradiographic film (a) was apposed by micromanipulation over the microscopic slide (b), following staining with hematoxylin–acid fuchsin–orange G–light green, to produce a composite image (c). Schematic drawing (d) is prepared from a slide of the stained section (b) to indicate the morphological features. ME: eminentia mediana; PT: pars tuberalis; PD: pars distalis; ZT: zona tuberalis. Clearly, the ME and the dense vascular network of the hypophyseal portal system are devoid of binding. Note the dense binding over the pars tuberalis and the diffuse binding over the same cell type (predominantly chromophobes, forming the so-called zona tuberalis), invading the clusters of acidophil cells of the pars distalis.
Fig. 6. (a) Frontal section of the basal part of a dog's brain, showing the median eminence (me), the pituitary neural lobe (nl), the pars tuberalis (pt) and distalis (pd) of the anterior pituitary and the third ventricle (tv). The section was incubated with 60 pmol/2-[125I]iodomelatonin and used for emulsion-coated coverslip quantitative autoradiography. The rectangle depicts the location of the area shown in (c) and (d). Bar = 1 mm. (b) Bar graph showing the grain density (mean±SEM) in the dog's pituitary and median eminence. A high concentration of grains is found in the pars tuberalis of the anterior pituitary (p < 0.001 vs lobeus posterior). In addition, the grain density is high above the basophilic cells, located as cords in the pars distalis proper (p < 0.01 vs eosinophilic cells). (c) High magnification of the area indicated by the rectangle in (a). Cords of basophilic cells (ba) are seen in close proximity to an area with larger eosinophilic cells (ec). Bar = 50 µm. (d). The same area as shown in (c). The focus is on the coverslip, allowing us to see the grains in the emulsion. Note that the binding is located over the small basophilic cells (ba) and that the large eosinophils (ec) are essentially free of binding. Bar = 50 µm.
of the putative receptor in the complex morphological organization of the olfactory bulb have ever been published.

The afferent nerve fibers originating in the olfactory epithelium, interact with dendrites coming from second-order neurones, i.e. the mitral cells and the glomerular cells, to shape the granular lamina. Dendrites from both cell types also form an extensive dendro-dendritic synaptic network with dendrites from glomerular cells in the large external plexiform lamina. Notably, the strongest 2-[125I]-iodomelatonin binding was found in this latter bulbar layer and the mitral cell lamina. The glomerular lamina expressed weaker, though well discernible specific binding (see Fig. 2). Therefore, melatonin should be expected to participate in the processing of the primary olfactory information.

The hypothalamus is among the areas of major efferent output from the olfactory bulbs, through the olfactohypothalamic tract (39), and although there are no functional data concerning the dog, olfactory bulbectomy influences the gonadal responses to photoperiod in other species, such as the Syrian hamster and the rodent (40–42). The Syrian hamster becomes insensitive to melatonin or short photoperiods in terms of gonadal status (40, 41), with concomitant changes in the prolactin, testosterone and gonadotropin levels (43).

In conclusion, the present data are the first demonstration that the dog possesses all the prerequisite sites for an elaborate system adapted to efficient photoperiodic time measurements. The melatonin signal is present on a circadian basis in the peripheral blood; an apparently functional receptor complex is situated in several brain locations, providing a framework for temporal programming of biological activity and involved in the control of accurately timed neuroendocrine events that are necessary for adaptation of the body physiology to the changing environment.

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