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DISTRIBUTION OF 2-[^{125}I]IODOMELATONIN BINDING SITES IN THE BRAIN OF THE PIED FLYCATCHER (*FICEDULA HYPOLEUCA*) AND THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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Summary

Using *in vitro* autoradiography, the distribution of 2-[^{125}I]iodomelatonin (IMEL) binding sites in the brain of the pied flycatcher and the zebra finch was examined. The results show IMEL binding in retinofugal, tectofugal and thalamofugal brain areas of the visual system. Additionally, IMEL binding sites were detected in the ectostriatum, the thalamus, the mesencephalon and the limbic system. No IMEL binding could be demonstrated in the pineal gland, the hippocampus, the nucleus suprachiasmaticus, the visual wulst or the pituitary.

Key words: pineal gland, melatonin, melatonin binding sites, visual system, pied flycatcher, *Ficedula hypoleuca*, zebra finch, *Taeniopygia guttata.*

Introduction

The pineal gland is an endocrine organ, located in the epithalamus of vertebrates. It is the main source of the pinealindole melatonin (*N*-acetyl-5-methoxytryptamine), which is rhythmically secreted over 24 h, with highest pineal and peripheral blood melatonin levels occurring at night (Schneider *et al.* 1994*a*). This circadian rhythm is caused by circadian oscillators within the circadian system which are photoperiodically synchronized (Cassone, 1990). The circadian melatonin rhythm is abolished after pinealectomy (Underwood and Goldman, 1987; Schneider, 1993). Various effects, including arrhythmicity of the circadian pattern of activity (Gaston and Menaker, 1968), feeding (Gwinner, 1989) and body temperature (Binkley *et al.* 1971) have been reported after removal of the avian pineal gland. Transplantation of a pineal gland to the anterior chamber of the eye of an arrhythmic pinealectomized house sparrow *Passer domesticus* confers both the rhythmicity and the circadian phase of the donor bird (Zimmermann and Menaker, 1979). Daily melatonin injections in pinealectomized, arrhythmic European starlings *Sturnus vulgaris* and domestic pigeons *Columba livia* entrain the circadian activity pattern (Gwinner and Benzinger, 1978; Chabot and Menaker, 1988). These results indicate that, in starlings and pigeons, the daily and circadian secretion of melatonin imposes rhythmicity on brain centres involved in the generation of activity.

Other behavioural effects caused by the pineal gland have been demonstrated in the pied flycatcher *Ficedula hypoleuca* (Muscicapidae), a nocturnal migrant. Flycatchers migrate during the autumn from central Europe to their wintering areas in western Africa. Behavioural experiments with hand-raised pinealectomized pied flycatchers during their first autumn migration indicate that the pineal gland is important for species-specific direction finding using the magnetic field as an orientation cue (Semm *et al.* 1984*a*). Deficits in the magnetic orientation behaviour of pinealectomized pied flycatchers could be eliminated by daily melatonin injections (Schneider *et al.* 1994*b*), confirming that melatonin is the main output of the pineal gland responsible for the above result. Given the profound role that the pineal gland and melatonin play in the circadian and circannual organization of the pied flycatcher, the sites of melatonin action within the brain are of special interest. For the localization of 2-[^{125}I]iodomelatonin (IMEL) binding sites the *in vitro* binding of IMEL and autoradiography were used. This technique has been used successfully in chickens (Rivkees *et al.* 1989), house sparrows (Cassone and Brooks, 1991) and several species of mammal (Vanecek, 1988; Weaver *et al.* 1989; Laitinen *et al.* 1989). For comparison, another passerine bird, the zebra finch *Taeniopygia guttata*, was also investigated.

Materials and methods

Four pied flycatchers of both sexes were removed from their nest boxes at the age of 10 days and hand-raised. Four zebra finches of both sexes were obtained from a colony in the Zoologisches Institut, Universität Frankfurt, Germany. The

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animals were kept in individual cages with food and water available *ad libitum* under controlled photoperiodic conditions of 12 h:12 h L:D.

The birds were anaesthetized using a 0.03 ml injection containing 0.01 ml of Ketamine (50 pg ml^{-1}), 0.01 ml of Rompun (50 pg ml^{-1}) and 0.01 ml of saline, injected into the pectoralis muscle, between 13:00 h and 15:00 h and transcardially perfused with 50 ml of cold saline (4 °C) and then 50 ml of 10 % sucrose in 0.1 mol l^{-1} phosphate buffer (pH 7.4). The brains were removed and quickly frozen in isopentane (−20 °C) before being stored at −80 °C. They were coronally sectioned at 20 μm intervals through their entire rostrocaudal extent on a cryostat at −20 °C and thaw-mounted onto gelatin-coated slides.

Before incubation with IMEL, slides with sections were preincubated in 0.02 mol l^{-1} phosphate-buffered 0.15 mol l^{-1} saline (pH 7.4) containing 0.1 % bovine serum albumin (PBS/BSA) for 1 h at 22 °C. Slides with adjacent sections were then incubated in either 100 pmol l^{-1} IMEL (specific activity 64.01–74 TBq mmol^{-1}) or 100 pmol l^{-1} IMEL in the presence of 1 mmol l^{-1} melatonin in PBS/BSA for an additional 1 h at 22 °C. Following incubation, sections were washed in PBS/BSA at 0 °C for 15 min and then for 15 min in PBS alone at the same temperature. Slides and sections were then dried and exposed to Amersham Hyperfilm b-max film for 7 days at −20 °C together with ^{125}I standards. Melatonin, IMEL, ^{125}I standards and Hyperfilm b-max autoradiographic film were purchased from Amersham. Following exposure, the films were developed using Kodak D-19, washed and fixed, while the sections were cleared and stained with Cresyl Violet. Anatomical localization of cerebral structures was accomplished using superimposition of a video-enhanced image of the Cresyl-Violet-stained section and the autoradiographical image using a computer-based image-analysis system (Viper). The autoradiographs were digitized and printed using a laser printer (Micro laser Pro 600, Texas Instruments). The anatomical nomenclature was derived from two stereotactic atlases for zebra finches (H.-J. Bischof, unpublished results) and pied flycatchers (Keller, 1991).

Table 1. *Abbreviations for IMEL binding sites in the avian brain*

Abbreviation	Structure
CO	Chiasma opticum
DLA	Nucleus dorsolateralis anterior thalami
DLL	Nucleus dorsolateralis anterior thalami, pars lateralis
E	Ectostriatum
EM	Nucleus ectomammilaris
GLV	Nucleus geniculatus lateralis pars ventralis
Hb	Nucleus habenularis
Imc	Nucleus isthmi, pars magnocellularis
IO	Nucleus isthmoopticus
Ipc	Nucleus isthmi, pars parvocellularis
LA	Nucleus lateralis anterior thalami
LM	Nucleus lentiformis mesencephali
LPO	Lobus parolfactorius
MLd	Nucleus mesencephalicus lateralis, pars dorsalis
NO	Nervus opticus
OMD	Nuclei nervi oculomotorii
RT	Nucleus rotundus
SGC	Stratum griseum centralis
SLu	Nucleus semilunaris
SP	Nucleus subpretectalis
TeO	Tectum opticum
TrO	Tractus opticus
VeM	Nucleus vestibularis medialis
VLT	Nucleus ventrolateralis thalami

The nomenclature is derived from H.-J. Bischof (unpublished results) and Keller (1991).

Results

The structure, nomenclature and abbreviations of the IMEL binding sites (sample sizes for all sites N=4) are given in Table 1. IMEL binding was present in retinorecipient structures of the retinofugal (nervus opticus NO, chiasma opticum CO, tractus opticus TrO), tectofugal (tectum opticum TeO, nucleus ectomammilaris EM) and thalamofugal (nucleus lateralis anterior thalami LA, nucleus geniculatus lateralis pars ventralis GLV, nucleus dorsolateralis anterior thalami DLA, nucleus dorsolateralis anterior thalami pars lateralis DLL) brain areas of the visual system. Additionally, IMEL binding sites were detected in thalamic (nucleus rotundus RT) and mesencephalic (nucleus isthmi pars magnocellularis Imc, nucleus isthmi pars parvocellularis Ipc, stratum griseum centralis SGC) relay nuclei, in the pretectal nuclei (nucleus subpretectalis SP, nucleus semilunaris SLu) and in the nucleus isthmo-opticus (IO) of the tectofugal visual systems. Finally, the ectostriatum (E), an integrative tectofugal centre in the telencephalon, nuclei of the limbic system (lobus parolfactorius LPO, nucleus habenularis Hb), and nuclei related to vision (nuclei nervi oculomotorii OMD) and audition (nucleus lentiformis mesencephali LM, nucleus mesencephalicus lateralis pars dorsalis MLd) bound IMEL. In the pied flycatcher alone, the nucleus ventrolateralis thalami (VLT) bound IMEL. The IMEL binding sites of the pied flycatcher are indicated in Fig. 1. In the zebra finch, additional IMEL binding sites were detected in the nucleus vestibularis medialis (VeM). The IMEL binding sites of the zebra finch are presented in Fig. 2.

No IMEL binding was found in the pineal organ, nucleus suprachiasmaticus, eminentia mediana, adeno- and neurohypophysis, nucleus angularis, nucleus triangularis, hyperstriatum accessorium and hyperstriatum ventrale, visual wulst, hippocampus, nucleus mesencephalicus lateralis, neostriatum, nucleus Edinger-Westphal, nucleus ovoidalis, archistriatum or nucleus tuberis, structures which bound IMEL in other bird species (chicken *Gallus domesticus*, Dubocovich and Takahashi, 1987; Rivkees *et al.* 1989; Cassone, 1990;

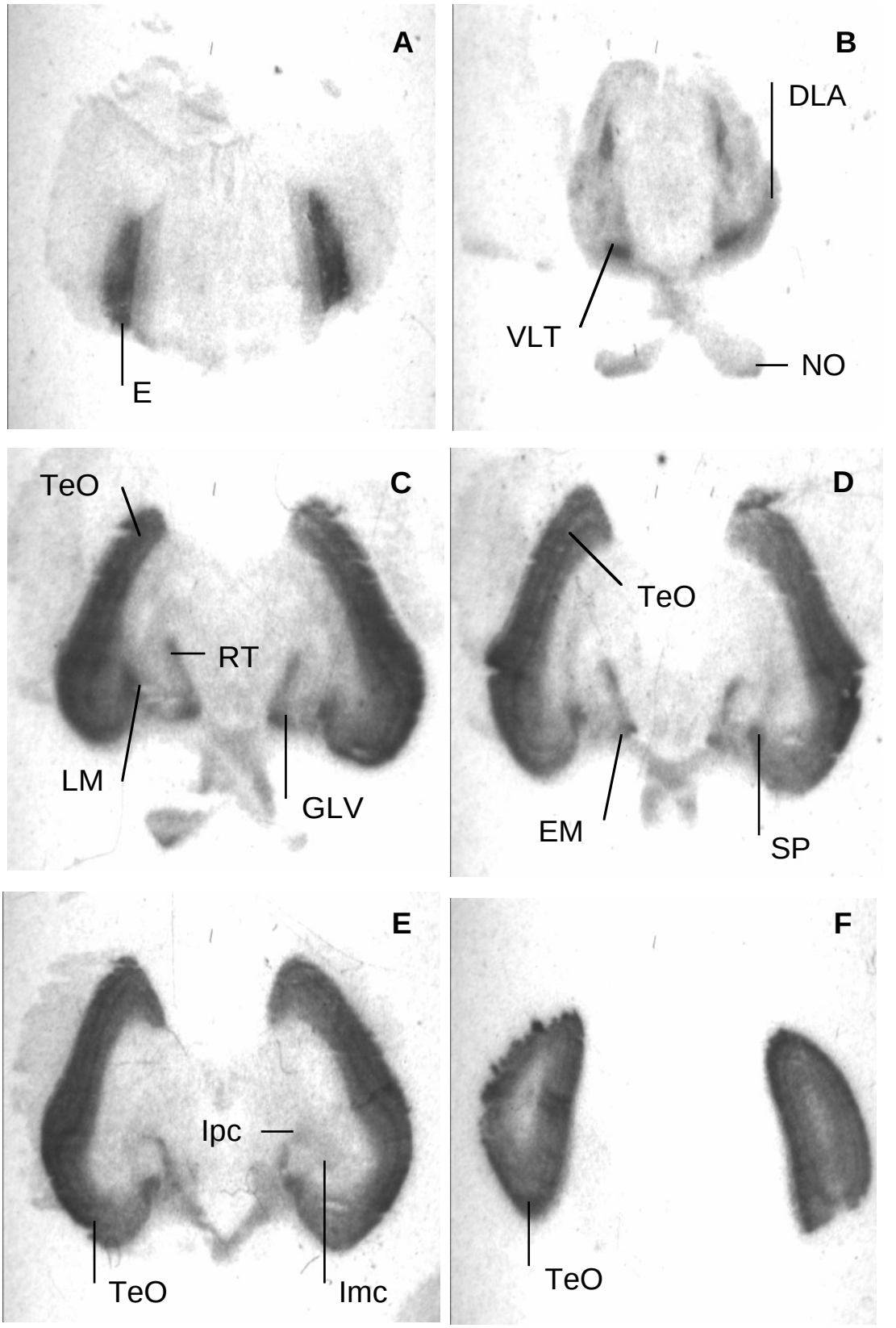

Fig. 1. (A–F) Video-digitized images of autoradiographs from pied flycatcher brain sections incubated in 100 pmol l^{-1} 2-[^{125}I]iodomelatonin. Images are arranged rostrocaudally in sequential order from A to F. Magnification: A, ×12; B–D, ×16; E,F, ×15. Note that, in the pied flycatcher, the nucleus ventrolateralis thalami bound IMEL. SeeTable 1 for the structure nomenclature and abbreviations.

budgerigar *Melopsittacus undulatus*, Java sparrow *Padda orizivora*, northern cardinal *Richmondena cardinalis*, Cassone, 1990; house sparrow *Passer domesticus*, Cassone, 1990; Cassone and Brooks, 1991).

The strongest IMEL binding was observed in visual areas such as the optic tectum (N=4). No non-specific IMEL binding was measured in sections incubated with 100 pmol l^{-1} IMEL in the presence of 1 mmol l^{-1} melatonin (N=4).

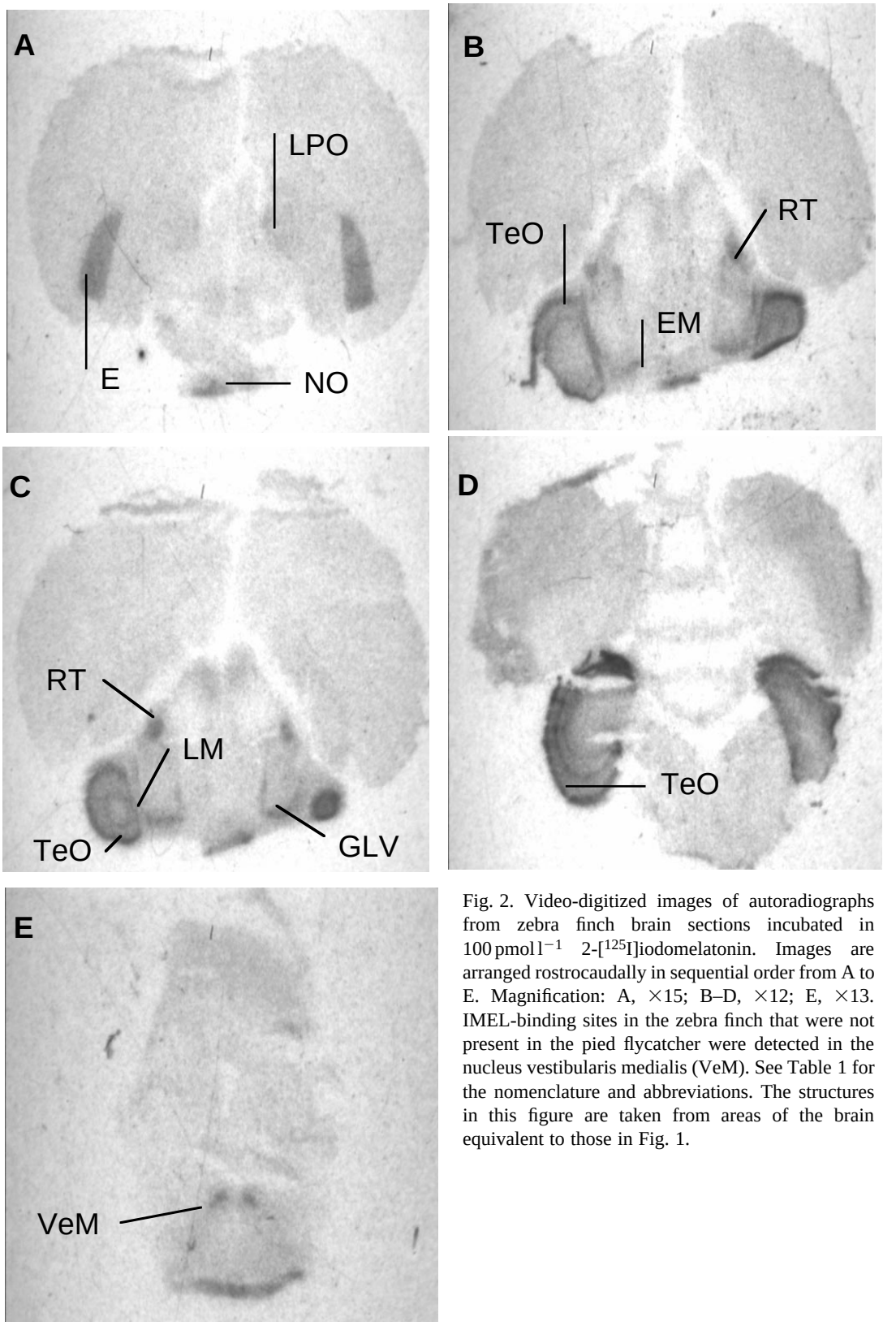

Fig. 2. Video-digitized images of autoradiographs from zebra finch brain sections incubated in 100 pmol l^{-1} 2-[^{125}I]iodomelatonin. Images are arranged rostrocaudally in sequential order from A to E. Magnification: A, ×15; B–D, ×12; E, ×13. IMEL-binding sites in the zebra finch that were not present in the pied flycatcher were detected in the nucleus vestibularis medialis (VeM). See Table 1 for the nomenclature and abbreviations. The structures in this figure are taken from areas of the brain equivalent to those in Fig. 1.

Discussion

The results of the present study confirm earlier reports that IMEL binding sites are widely distributed in the avian brain (Cassone, 1990). The data reveal intensive IMEL binding in many brain structures related to vision at several levels of neuronal organization. Brain areas which were labelled with IMEL are structures involved in receiving and mediating visual information, including tectofugal and thalamofugal structures and nuclei, associated with visual–motor reflexes. Ascending auditory nuclei as well as limbic structures and those associated with arousal behaviour bound IMEL. It is not known whether these binding patterns reflect binding on perikarya or fibres or what cell types bind the radioligand (Cassone and Brooks, 1991).

IMEL binding studies in chickens *Gallus domesticus*, pigeons *Columba livia*, ringed turtle doves *Streptopelia risoria*, budgerigars *Melopsittacus undulatus*, Java sparrows *Padda orizivora*, northern cardinals *Richmondena cardinalis* and house sparrows *Passer domesticus* indicate a rather similar binding pattern (Cassone, 1990).

A comparison between the IMEL binding areas in the pied flycatcher and the zebra finch with those reported for the house sparrow, which are all passerines, and for the chicken (Galliformes), shows a higher degree of similarity between the flycatcher, the finch and the sparrow than the chicken (Rivkees *et al.* 1989; Cassone and Brooks, 1991). The most important difference is that IMEL binding sites were detected in auditory regions only in the chicken but not in the house sparrow. In contrast to these findings, the present results show IMEL binding in the nucleus mesencephalicus lateralis, pars dorsalis, which is believed to be associated with the auditory system (Cassone, 1990). Moreover, no IMEL binding was observed in the nucleus suprachiasmaticus, which bound IMEL in sparrows (Cassone and Brooks, 1991). As in the house sparrow, no IMEL binding sites could be detected in the pineal gland, the hypophysis, the hippocampus, the hyperstriatum and the visual wulst of pied flycatchers and zebra finches (Cassone and Brooks, 1991).

The distribution of IMEL binding in the avian brain is much more widespread than the relatively restricted IMEL binding areas of mammals. In rats *Rattus norvegicus*, Djungarian hamsters *Phodopus sungorus* and sheep *Ovis aries*, IMEL binding sites predominate in three cerebral structures, the nucleus suprachiasmaticus, the pars tuberalis of the adenohypophysis and the area postrema (Cassone, 1990). Secondary binding sites include the pineal gland, the thalamic nucleus paraventricularis, the subiculum and, in sheep, the hippocampus (Vanecek *et al.* 1987; Weaver *et al.* 1988, 1989; Morgan and Williams, 1989; Vanecek, 1988; Reppert *et al.* 1988). This suggests that in birds, but not in mammals, a wide array of sensory and integrative functions in the brain are under circadian influence *via* the circadian secretion of melatonin (Cassone, 1990).

Because the duration of melatonin secretion is roughly proportional to the daily dark period, the melatonin cycle also provides time-of-year information in species exposed to naturally changing day lengths (Goldman, 1983). Pinealectomy abolishes the circadian and circannual melatonin rhythm (Underwood and Goldman, 1987; Schneider, 1993). Different effects, such as arrhythmicity of the circadian pattern of activity, feeding and body temperature, have been reported after pinealectomy (Gaston and Menaker, 1968; Gwinner, 1989; Binkley *et al.* 1971) and could be explained by the loss of the circadian melatonin rhythm (Vakkuri *et al.* 1985). Transplantation of a pineal gland or daily melatonin injections into pinealectomized birds can counteract the effects of pinealectomy (Zimmermann and Menaker, 1979; Gwinner and Benzinger, 1978; Chabot and Menaker, 1988).

Other behavioural effects caused by the pineal gland have been found in the pied flycatcher. These birds migrate during the autumn from central Europe to their wintering areas in western Africa. Laboratory experiments with hand-raised flycatchers indicate that the magnetic field of the earth is used as a navigational 'inclination' compass if no visual orientation cues are available (Beck and Wiltschko, 1981, 1982). Later experiments with pinealectomized flycatchers during their first autumn migration indicate that the pineal gland is important for species-specific orientation behaviour using the magnetic field as an orientation cue (Semm *et al.* 1984*a*). It has been hypothetized that the pineal gland is not directly involved with orientation, because other pinealectomized birds, such as the homing pigeon, can orient using their magnetic compass system (Maffei *et al.* 1983; Semm *et al.* 1987). Instead, the pineal gland and its hormone, melatonin, are involved in the ontogenetic process for expressing genetically encoded directional information on the migratory direction using the magnetic field as an external cue (Schneider *et al.* 1994*b*).

Magnetically sensitive brain areas in the pigeon, such as the habenular nuclei, parts of the optic tectum and the optic tract, the pineal gland and the nucleus ectomammilaris, have been identified using the [^{14}C]deoxyglucose technique or electrophysiological methods (Mai and Semm, 1990; Semm and Demaine, 1986; Semm *et al.* 1984*b*). Most of these magnetically sensitive areas in the brain of the pigeon overlap with IMEL binding sites (Cassone, 1990; Semm *et al.* 1984*b*; Semm and Demaine, 1986; Mai and Semm, 1990). However, the functional significance of this relationship between melatonin binding and magnetic sensitivity is unknown. Several studies indicate that melatonin is involved in the modulation of neuronal excitability (Mason and Rusak, 1990; Meissl *et al.* 1990). Different mechanisms coupled with dopaminergic, noradrenergic or GABAergic neuronal transmission have been suggested (Zisapel and Laudon, 1982; Rosenstein and Cardinali, 1986; Stankov and Reiter, 1990; Stankov *et al.* 1992). The information regarding the molecular mechanisms by which the melatonin receptors act on cell physiology is rather sparse. Several lines of evidence indicate that the effects of melatonin are mediated by pertussis-toxin-sensitive inhibitory GTP-binding proteins (G_is) associated with cyclic AMP synthesis (Carlson and Reppert, 1989). In rats, it has been suggested that an *N*-methyl-D-aspartate (NMDA) receptor is involved in the regulation of melatonin receptor

density in the nucleus suprachiasmaticus (Gauer *et al.* 1994). However, future research on melatonin receptors in the avian brain is required to improve our understanding of the differences and similarities in the mechanisms of the circadian system of birds and mammals.

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