Differential expression of melatonin receptor subtypes MelIa, MelIb, and MelIc in relation to melatonin binding in the male songbird brain

Melatonin receptors in the songbird brain

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ABSTRACT

Previous autoradiography studies illustrated that several areas of the avian brain can bind the pineal hormone melatonin. In birds there are three melatonin receptor (MelR) subtypes: MelIa, MelIb, and MelIc. To date, their brain distribution has not been studied in any Passerine bird. Here we report the distribution of the mRNAs of melatonin receptor (MelR) subtypes in adjacent sections of the brain of two songbirds, the blackcap and the zebra finch, in parallel with that of 2-[^{125}I]-iodomelatonin (IMEL) binding sites in the same brains. The general pattern of receptor expression shown by *in situ* hybridizations of species-specific probes matched well that of IMEL binding. However, the three subtypes were area-specifically expressed with patterns similar in the two species. Some brain areas expressed only one receptor subtype, most brain regions co-expressed either MelIa with MelIb or MelIa with MelIc, whereas few areas expressed MelIb and MelIc or all three receptors subtypes. Since many sensory areas, most thalamic areas, and sub-areas of the cortex-analogue neopallium express melatonin receptors, it is likely that most sensory-motor integration functions are melatonin sensitive. Further, the area-specific patterns of expression suggests that the regulatory role of melatonin differs among different brain areas. Since sub-areas of well defined neural circuits such as the visual system or the song control system are equipped with different receptor types, we hypothesize a diversity of functions for melatonin in the control of sensory integration and behaviour.

INTRODUCTION

In birds melatonin regulates circadian and/or seasonal rhythms of locomotor activity, feeding, reproduction, singing, hatching, and immune function [reviewed in Bentley 2001; Cassone 1990; Gwinner and Brandstaetter 2001; Gwinner et al. 1997; Jansen et al. 2005]. Melatonin action at the central level is thought to be mediated mainly by specific membrane receptors that belongs to the G-protein coupled receptor superfamily. These receptors have been characterized pharmacologically and their brain distribution was studied in several avian species using the specific binding of 2-[^{125}I]-iodomelatonin (IMEL)[Aste et al. 2001; Bentley and Ball 2000; Cassone et al. 1995; Cozzi et al. 1993; Dubocovich et al. 1989; Gahr and Kosar 1996; Whitfield-Rucker and Cassone 1996]. IMEL binding sites were observed almost everywhere in the visual system (tectofugal, thalamofugal, and accessory optic pathways) of all birds, with some species-specific differences [for a detailed review see Aste et al. 2001]. In addition, dense IMEL binding was found in several nuclei of the neural song system that controls song learning and production in songbirds [Bentley and Ball 2000; Cassone et al. 1995; Gahr and Kosar 1996]. Thus, the melatonin binding sites are widespread in the avian brain, which is in line with the stronger influence of melatonin on rythms in birds compared with mammals [Reppert et al. 1995].

Studies based on IMEL binding can not tell apart melatonin receptor subtypes. Two subtypes of melatonin receptors, called MelIa and MelIc, were first identified in the domestic chicken [Reppert et al. 1995]. The chicken MelIa receptor subtype is homologous to the mammalian MelIa (i.e. human MT1), whereas MelIc has been described only in non-mammalian vertebrates [Barrett et al. 2003; Reppert et al. 1996], and recent phylogenetic studies have shown that MelIc is related to the melatonin-related receptor GPR50 of mammals [Dufourny et al. 2008] . The avian MelIb is homologous to the mammalian MelIb (i.e. human MT2) [Liu et al. 1995] [Jansen et al. 2005]. In the chicken, the expression of MelIa and/or MelIc was found to match the IMEL binding in most cases, however, some areas such as the entopallium showed IMEL binding but no or little receptor mRNA expression [Reppert et al. 1995]. The expression of MelIb has been reported only for specific regions of the zebra finch brain [Jansen et al. 2005].

A detailed study of the expression pattern of melatonin receptor subtypes and of IMEL binding in the same brains is of primary importance for a number of reasons. First, because subtypes differ in their affinity for IMEL [Reppert et al. 1996], differences in IMEL binding between brain regions could represent differences in receptor concentration or differential expression of receptor subtypes. Second, knowledge about differential expression of receptor subtypes is necessary to study regional action of melatonin and melatonin receptor regulation. A number of papers have shown that MelIa and MelIb exert different functions in vivo in mammals. For example, in the SCN of rats MelIa mediates the inhibition of neuronal activity whereas MelIb mediates the phase shifts in the circadian rhythms of activity [Liu et al. 1997]. Third, there is evidence from mammalian melatonin receptors that receptor subtypes interact to form heterodimers [Ayoub et al. 2004] [Jockers et al. 2008] and the co-expression of different subtypes changes substantially the modalities of melatonin action. Because of the close homology between avian and mammalian melatonin receptors it is likely that similar processes mediate melatonin signalling in birds.

Equally compelling are the grounds for studying the distribution of melatonin receptor subtypes in bird species other than the domestic fowl. The domestic fowl is only remotely related to the Passeriformes, which represent almost the half of all bird species [Cracraft 2001]. Further, there are melatonin sensitive phenotypes that are missing in Galliformes such as the capacity of learning complex vocalizations - i.e. song [Bentley et al. 1999] [Jansen et al. 2005] and nocturnal migration [Fusani et al. 2011; Fusani and Gwinner 2004, 2005]. These have been intensely investigated in Passerine birds. Melatonin might be implicated in thermoregulatory adaptation during migration [Carere et al. 2010; Fusani et al. 2011]. Recent studies have suggested a direct role of melatonin in controlling the pattern of song [Deregnaucourt et al. 2012], and it has been suggested that melatonin modulates sub-oscillators that are located within the song nuclei and control circadian rhythms of singing and calling [Wang et al. 2012].

In this work, we report the distribution of MelIa, MelIb, and MelIc in the brain of two representative songbird species, the zebra finch (*Taeniopygia guttata castanotis*) and the blackcap (*Sylvia atricapilla*), for which partial sequences of the three melatonin receptor subtypes were cloned. The zebra finch is one of the most studied songbirds and a model species for studies from neurobiology to behavioural ecology, and is a

98 non-photoperiodic opportunistic breeder originating from Australia. The blackcap is a seasonal breeder with
99 long-distance migratory population. In addition, the blackcap is a nocturnal migrant, i.e. during the migratory
100 periods birds of this species becomes night active. The expression of MelIa, MelIb, and MelIc was compared
101 with the IMEL binding on parallel sections of the same individual birds. Our study shows that melatonin
102 receptor subtypes are differentially expressed in the songbird brain, providing basic knowledge to understand
103 melatonin function in the avian brain.
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105 METHODS

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107 **Animals and tissue collection**

108 We selected a northern population of blackcaps that are long-distance migrants [Berthold et al. 1990]. The
109 birds were trapped at Tovetorp Zoological Research Station, Sweden, at the beginning of September. Birds
110 were captured with permission of the Swedish Environmental Protection Agency. The birds were transported
111 to the Max-Planck-Institute for Ornithology, Andechs, Germany, housed in individual cages, and exposed to
112 a decreasing day length simulating southward migration until 18 October, when the day length reached 12 h.
113 From this day onwards, the birds were kept in a 12-h photoperiod (light on: 06:00) until the end of the
114 experiments. All birds were housed in the same room at 20 ± 1 °C. Food and water were given ad libitum
115 and were renewed every day right after lights on. In November, while they were showing migratory
116 restlessness [see Fusani and Gwinner 2004], six male blackcaps were killed by decapitation at 24:00 hr (± 30
117 min), the brain was rapidly dissected, frozen on liquid nitrogen, and stored at –80 °C. Zebra finches were
118 from the colony kept at the Free University of Amsterdam, the Netherland. The birds were kept in indoor
119 aviaries under a 14L:10D schedule, with lights on at 06:00 hr, and a temperature of 22 ± 1 ºC. Food and
120 water were given ad libitum and were renewed every day right after lights on. Six adult males were sampled
121 between 09:00 and 11:00 hr. All brains were cut with a cryostat in parasagittal sections of 20 µm that were
122 thaw-mounted on Superfrost slides in 5 parallel series, air-dried and stored at –80 °C. One series was used
123 for Nissl staining, three series for the *in situ* hybridization of MelIa, MelIb, and MelIc, and one series for
124 IMEL binding (see below). Nissl-stained sections were used for identifying brain regions. All the
125 experimental procedures were done in accordance to the guidelines of the relevant Dutch and German
126 agencies.

127

128 **IMEL binding**

129 The binding sites of melatonin in the bird brain were localised by in vitro autoradiography for 2-[^{125}I]-
130 melatonin (IMEL) as previously described [Gahr and Kosar 1996]. Briefly, 20 µm mounted sections were
131 incubated for 1 hr at room temperature with 20 pM IMEL (NEX 236, PerkinElmer Life and Analytical
132 Sciences, Inc., Boston, MA, specific activity 2,200 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) with 4mM

CaCl2 in either the presence (non-specific binding) of absence (total binding) of 1 μM melatonin (Sigma). No IMEL binding was found in any brain area in the presence of 1 μM melatonin. After incubation, the slides were rinsed once with ice-cold Tris-HCl buffer for 2 min, then twice rapidly, and were dried on a warm plate. Autoradiograms were produced by exposing Kodak Biomax MR films to the slides for 7 days at – 18 °C. The films were then developed with Kodak D19 for 5 min at 18 °C, rinsed with water and fixed with Kodak Fixer.

Cloning of MelIa, MelIb, and MelIc

We isolated mRNA from brain tissue with the Dynabeads mRNA DIRECT kit (Deutsche Dynal GmbH; Hamburg) and used the reverse transcription SUPERSCRIPT II Reverse Transcriptase kit (Life Technologies). For the PCR, the forward primer (5'-GSMATHGCYATCAACMGSTA-3') for MelIa and MelIc was from the end of trans-membrane region 3 and that for MelIb (5'-GCYGAYCTGGTGGTGGCCTT-3') (according to[Reppert et al. 1995]). The reverse primer of Mel-1A, Mel-1B and Mel-1C (5'-CARCTGTTRAAATABGCCAT-3') was from the middle of trans-membrane region 7 (according to [Reppert et al. 1995]). Fragments were sub-cloned into PGEM-7. Linearization for the sense probes was with HindIII for all three receptors (T7 polymerase) and with EcoRI (MelIb, MelIc) and XhoI (MelIa) for the anti-sense probes (SP6 polymerase) from the middle of trans-membrane region 2.

***In situ* hybridisation**

Probes were prepared using the sequences for zebra finch and blackcaps MelIa, MelIb, and MelIc described in the previous paragraph. The synthesis and labeling of the probes with ^{35}S-CTP (1250 Ci / mmol; NEN) was performed using the Riboprobe System (Promega, Madison, WI) according to the manufacturer's instructions. The *in situ* hybridization procedure has been described in detail previously [Metzdorf et al. 1999]. After hybridization, the slides were dipped into Kodak NTB-2 nuclear track emulsion. The duration of the exposure (15 days) had been optimized to give clear, non-saturated specific labeling. After development, sections were counterstained with the Nissl-stain thionin and observed under light- and dark-field illumination. The areas to be analyzed were digitized with an image analysis system (Imatec, Munich,

Germany). The level of expression was scored on a 4-level arbitrary scale: "-" = no labeling; "+" = scattered labeled cells; "++" = many labeled cells; "+++" = many intensely labeled cells.

RESULTS

Because the general pattern of 2-iodomelatonin binding in the avian brain has been reported in detail in previous studies including various songbird species [Aste et al. 2001; Bentley and Ball 2000; Cassone et al. 1995; Cozzi et al. 1993; Dubocovich et al. 1989; Gahr and Kosar 1996; Whitfield-Rucker and Cassone 1996], we focus here on the differential expression of the three melatonin receptor subtypes in the same brain regions, and on the match between 2-iodomelatonin binding and melatonin receptor expression. In particular, we focus on areas which are either known to be melatonin-sensitive, or are being intensively investigated as part of a model system, or have a strong 2-iodomelatonin binding and/or melatonin receptor expression. Thus, there are brain areas expressing melatonin receptors which are not explicitly mentioned in the present work, in particular when expression is scattered. Anatomical descriptions were based on recent reviews of the nomenclature [Reiner et al. 2004; Chen et al. 2013; Jarvis et al. 2013].

General aspects of melatonin receptor expression in the songbird brain

All major brain subdivisions (forebrain, midbrain, hindbrain) express all three receptor types with the exception of the cerebellum, which contains strongly labelled MelIc mRNA neurons in the Purkinje cell layer and some weakly labelled MelIa in the vermis (Table 1). The labelled cells in most cases have a neuronal appearance, and the staining was consistent between animals. Some brain areas expressed only MelIa, MelIb, or MelIc, many brain regions co-expressed either MelIa with MelIb or MelIa with MelIc, and few areas expressed MelIb and MelIc or all three receptors subtypes (Fig. 1 - 3 2; Table 1). There were no major differences in the overall pattern of receptor expression between zebra finches and blackcaps (Table 1), with the exception of the song system (see below). Thus, the pattern of melatonin expression does not appear to differ between the migratory and the non-migratory species. Melatonin binding sites and melatonin receptor expression were found in brain areas that are part of various sensory systems including the visual, the olfactory, vestibular and (beak) somatosensory system, and motor systems such as song control circuit

(Table 1). Since basically all parts of the forebrain express at least one receptor type (Table 1), the entire bird cortex analogue appears to be sensitive to melatonin.

Co-expression categories of MelIa mRNA, MelIb mRNA and MelIc mRNA.

We find brain areas expressing none of the receptor types (e.g. Area L pallii; N. mesencephalicus lateralis, pars dorsalis; the Septum), brain areas expressing only one type, either MelIa (e.g. N. isthmo-opticus, Fig. 1D-1F) or MelIb (e.g. N. suprachiasmaticus [Fig. 1A-1C], nucleus N. robustus arcopallialis [Fig. 1G-1I]) or MelIc (e.g. Purkinje cell layer, N. nervi hypoglossi, Table 1). More typical is the co-expression of MelIa with MelIb or MelIa with MelIc; less common was the co-expression of MelIb and MelIc or of all three receptor types (Table 1; Fig. 1J-1O).

The extent of co-expression that we report depends of course on the level of spatial resolution of our analysis: E.g., the tectum opticum expresses all three receptor types but each of its layers appears to express either MelIa or a combination of MelIa and MelIb or MelIa and MelIc (Fig. 1M-1O & Fig. 4B). The methodology employed does not allow ascertaining whether a single cell expresses more than one receptor subtype.

Specific expression pattern in various neural systems and brain regions

All sensory systems express at some level at least one receptor type (Table 1). In the visual system, MelIa is the dominant receptor type throughout all components of all ascending sub-systems (tectofugal, thalamofugal, and accessory optic pathway; Fig 1D-1F), although some areas express also MelIc RNA or MelIb RNA (Table 1; 1M - 1O). In the olfactory system, MelIb and MelIc are found in the lamina granularis (LGr) whereas MelIa in present in the lamina mitralis layer (LMi) of the bulbus olfactorius (Table 1; Fig. 1J - 1L, Fig. 4). In the vestibular system, the N. vestibularis expresses MelIa and MelIc. In the somatosensory system, we find all three subtypes but in different regions of the N. basorostralis pallii (Table 1). In the auditory system, the main areas of the ascending auditory system, such as the N. mesencephalicus lateralis, pars dorsalis, the thalamic N. ovoidalis, and the forebrain Area L pallii 2, equivalent to layer IV of the primary auditory cortex of mammals, do not express melatonin receptors. All receptor subtypes are, however, found in regions of the association- cortex-like pallial regions that are involved in higher order

auditory processing such as the Nidopallium caudomediale (NCM) and the Mesopallium caudomediale (MCM) (Table 1).

Among behavioural control systems, we focused on the song control system of songbirds. This system does not express MelIa (Table 1; Fig. 1G). The other receptor types, MelIb and MelIc, are expressed within some nuclei but with an area-specific pattern. In particular, MelIb is strongly expressed in the pallial nuclei HVC (proper name) and N. robustus arcopallialis (RA) (Fig. 1H), whereas the brainstem song control nuclei nXIIts (N. nervi hypoglossi, pars tracheosynringealis) and the N. retroambigualis express MelIc (Table 1), as reported before for the zebra finch [Jansen et al. 2005]. The N. lateralis magnocellularis nidopallii anterioris (LMAN) and N. medialis magnocellularis nidopallii anterioris (MMAN) do not express melatonin receptors. The shell region of MMAN expresses MelIc and binds melatonin. There were subtle differences in the pattern of expression between zebra finches and blackcaps in that some MelIc expressing cells were found in HVC of blackcaps but not in zebra finches (Table 1; Fig. 5). However, these small differences could depend on endogenous and/or environmental factors as the blackcaps and the zebra finches came from two different laboratories and were not kept in identical conditions.

Some limbic regions involved in the control of reproductive behaviour such as the Area praeoptica medialis and the N. ventromedialis hypothalami show strong co-expression of MelIa and MelIb but little or no expression of MelIc. Interestingly, the N. suprachiasmaticus, the site of the main avian clock, expresses only MelIb mRNA.

Relationship between IMEL binding and melatonin receptors expression.

Most investigated regions that show IMEL binding express at least one type of melatonin receptor, with the exception of two visual regions, the nucleus isthmus parvocellularis and nucleus isthmus magnocellularis (Table 1). Vice versa, most regions that express melatonin receptor show some level of IMEL binding. In relation, in contrast to earlier reports [reviewed in Aste et al. 2001], we find strong IMEL binding and receptor expression in the entopallium, the avian equivalent of layer IV of the primary visual cortex, and cerebellar Purkinje cells. Thus, we conclude that most mRNA expression that we found in these areas relate to the local expression of melatonin receptor proteins.

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244

245 DISCUSSION

246 The mapping of the melatonin receptor subtypes MelIa, MelIb, and MelIc in two species of songbirds, the
247 zebra finch and the blackcap, provides new insights in the modalities of action of melatonin in the avian
248 brain. Since most regions that express melatonin receptor show some level of IMEL binding, we suggest that
249 the mRNA mapping of the three melatonin receptors reflects the distribution of the proteins of MelIa, MelIb
250 and MelIc. We found that the three receptor subtypes are differentially expressed in the brain in various
251 combinations, indicating that melatonin action is finely tuned in an area-specific fashion. Furthermore, at the
252 neural systems level, the different pattern of co-expression of the receptor types in different systems, such as
253 the visual and vocal ones, indicates a complex sensitivity of the entire sensory and vocal control system to
254 melatonin. We would like to stress that this work is based on the differential staining of parallel sections of
255 the same animals, which allowed us to describe patterns of co-distribution with a precision of at least 100
256 μm. Double and triple labelling of the same neurons might show even more complicated situation with
257 neurons expression either one or two or three receptors within some but not other brain regions.
258

259 **Functional role of melatonin receptor subtypes**

260 The observation that a two- or three-subtype pattern of expression is common to most regions suggests that
261 different receptor subtypes have different functions, as demonstrated for the homologous mammalian
262 melatonin receptors [Liu et al. 1997]. In addition, different receptor subtypes could form heterodimers to
263 mediate melatonin action [Ayoub et al. 2004]. The mammalian homologous of MelIc, GPR50, does not bind
264 melatonin but is able to form heterodimers with both MT1 and MT2 [Levoye et al. 2006]. In particular,
265 GPR50 inhibits the high-affinity agonist binding of MT1 but not of MT2. Because of the strong IMEL
266 binding found in several brain areas expressing only MelIc such as the vocal nucleus nXIIts and the Area
267 ventralis tegmenti, and in brain areas co-expressing MelIc and MelIa such as the N.oculomotorius (nIII) and
268 the Bulbus olfactorius, it is likely that the avian MelIc receptor does not have such an inhibitory function and
269 is a true melatonin receptor.
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272 **Melatonin receptors in songbirds versus other avian taxa**

Since melatonin receptor expression was similar between a migratory, seasonal breeder (the blackcap) and an non-migratory, opportunistic breeder (the zebra finch), the melatonin sensitivity of avian brain functions does not appear to be associated to the life history of the species. We are aware that this study was limited to two species, however, the lack of substantial differences between the two species examined for most brain regions is suggestive. Given that blackcaps and zebra finches are not closely related within the order Passeriformes [Barker et al. 2004] it is likely that the distributions reported here reflect songbird-typical pattern of melatonin expression at the gross morphological level. Whether this pattern is similar to other avian taxa remains to be ascertained. The only other study of melatonin receptor distribution in birds, analysing the chicken brain, included only MelIa and MelIc [Reppert et al. 1995]. The distribution of MelIa and MelIc between homologous brain areas of the chicken and blackcaps and zebra finch are rather similar, with a few exceptions (cfr. Table 1 with Table 1 of [Reppert et al. 1995]. The main difference concerns the expression of melatonin receptors in the entopallium of the two songbird species that was not reported in the chicken. Thus, in the chicken entopallium, IMEL binding might be related to MelIb. Some differences of melatonin binding sites between passerine birds and Galliformes species reported previously, for example the lack of IMEL binding in the nucleus of Edinger-Westphal, in the cerebellum, and in the hyperstriatum (hyperpallium) of Passeriformes [Aste et al. 2001; Cassone et al. 1995; Schneider 1995], were not confirmed by the present study in the zebra finch and blackcap. Nevertheless, since brain structures of songbirds that evolved recently, in particular the song control system, express melatonin receptors, we can expect taxon-specific patterns of melatonin sensitivity among birds.

Melatonin receptor and song

Seasonal changes in the photoperiod affect the duration of night melatonin action and the level of circulating melatonin [Fusani and Gwinner 2004; Gwinner 1996], which in turn could be important for differential cellular responses. To date, the role of melatonin receptors in most brain areas of birds is unknown. Seasonal changes of the song control system are mainly due to seasonal variations on the circulating levels of testosterone, yet there are phenomena that appear to be directly dependent on the photoperiod, such as the rate of neuronal death [Kirn and Schwabl 1997]. In addition, simulating of long or short nights through exogenous administration of melatonin in house sparrows kept in constant light influences the size of the

song nuclei independently of the reproductive state [Cassone et al. 2008]. In particular, HVC and RA are smaller in birds with a long melatonin-night, equivalent to short photoperiods, than in birds with a short melatonin-night, equivalent to long photoperiods [Cassone et al. 2008]. Recent studies have indicated that melatonin may have direct effects on the song and call patterning in zebra finches and Japanese quails [Deregnaucourt et al. 2012; Jansen et al. 2005], possibly mediated by melatonin receptors in vocal neurons [Jansen et al. 2005]. It is known that there are plastic changes within the song nuclei that depend on the sleep pattern, i.e. song deteriorates after sleep and is recovered during the day, allowing modifications of learned motor patterns [Deregnaucourt et al. 2012; Shank and Margoliash 2008]. However, recent work in zebra finches has shown that melatonin is capable of influencing circadian rhythms of vocal activity independently of locomotor activity, suggesting that there are clocks within the song system that are modulated by melatonin [Wang et al. 2012]. In agreement with the latter hypothesis, the differential expression of melatonin subtypes in separate brain regions suggests that melatonin does not simply switch the brain to a 'night' status but act on target areas to regulate behavioural patterns according to area-specific receptivity.

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413 FIGURE LEGENDS

414

415 Figure 1. Expression of melatonin receptor subtypes in the brain of blackcaps. Darkfield images of in situ
416 hybridization for MelIa (left: A,D,G,J,M), MelIb (center: B,E,H,K,N) and MelIc (right: C,F,I,L,O) in parallel
417 parasagittal sections at the level of the (top to bottom) suprachiasmatic nucleus (SCN: A,B,C), N. isthmo-
418 opticus (Nio: D,E,F), N. robustus archopallialis (RA: G,H,I), bulbus olfactorius (OB: J,K,L), and tectum
419 opticum (TO: M,N,O). Right is frontal, top is dorsal. Scale bar is 100 µm for all images except for the N.
420 isthmo-opticus (D,E,F) for which the scale bar is 50 µm.

421

422 Figure 2. Autoradiography pictures of 2-[^{125}I]-melatonin (IMEL) binding in the brain of a blackcap from the
423 lateral (A) to the medial (I) brain. Almost all brain areas show some IMEL binding. Main areas showing
424 intense labelling are indicated. Abbreviations: BO= Bulbus olfactorius; E = Entopallium; H = Hyperpallium;
425 HI = Hyperpallium intercalatum; HVC = HVC; L = Area L pallii 2; LMAN = N. lateralis magnocellularis
426 nidopallii anterioris; MD = Mesopallium dorsale; MV = Mesopallium ventrale; N = Nidopallium; nXII = N.
427 nervi hypoglossi; P = pallidum; St = Striatum; T = Thalamic region; TO = Tectum Opticum; X = Area X.

428

429 Figure 3. Schematic drawings of parasagittal sections of the songbird brain, from later (A) to medial (C). The
430 distribution of melatonin receptors subtypes is shown by red triangules for MelIa, blue dots for MelIb, and
431 green squares for MelIc. To facilitate visualization of receptor distribution, we show only major anatomical
432 landmarks, areas expressing melatonin receptors, and songbird-specific regions (i.e. song nuclei) were no
433 receptor expression was found. Density of markers for each receptor subtype reflects density of labelling
434 reported in Table 1. Abbreviations: A = Archistriatum; Arc = N. arcuatus; AVT = Area ventralis tegmenti;
435 BO = Bulbus olfactorius; BSh = Shell region of the N. basorostralis pallii; BSs = N. basorostralis pallii; CA
436 = Commissura anterioris; CMM = Mesopallium caudomediale; CO = Chiasma opticum; DLA = N.
437 dorsolateralis anterior thalami; DLM = N. dorsolateralis anterior, pars medialis; DLP = N. dorsolateralis
438 posterior thalami; E = Entopallium; EW = N. Edinger-Westphal; GL = Corpus geniculatus lateralis; H =
439 Hyperpallium; HI = Hyperpallium intercalatum; HP = Area parahippocampalis; HVC = HVC; ICo = N.
440 intercollicularis; Imc = N. isthmus magnocellularis; Ipc = N. isthmus parvocellularis; LGr = Lamina

441 granularis; LMAN = N. lateralis magnocellularis nidopallii anterioris; LMD = Lamina mesopallialis dorsalis;
442 LMI = Lamina mesopallialis intermedia; LMi = Lamina mitralis; LMV = Lamina mesopallialis ventralis;
443 LPS = Lamina Pallio-subpallialis; LSt = Lateral striatum; M = Mesopallium; MD = Mesopallium dorsale;
444 MV = Mesopallium ventrale; MLD = N. mesencephalicus lateralis, pars dorsalis; MMAN = N. medialis
445 magnocellularis nidopallii anterioris; NC = Nidopallium caudale; NCM = Nidopallium caudomediale; nIII =
446 N.oculomotorius; Nio = N. isthmo-opticus; NSC = N. suprachiasmaticus; NSTL = N. striae terminalis
447 lateralis; NSTM = N. striae terminalis medialis; nXII = N. nervi hypoglossi; OS = N. olivaris superior; N.
448 ovoidalis; Pj = Purkinje cell layer; MPOA = Area praeoptica medialis; RA = N. robustus arcopallialis; Rt =
449 N. rotundus; StM = Striatum mediale; TeO = Tectum Opticum; v = ventriculus; Ve = N. vestibularis; VMH
450 = N. ventromedialis hypothalami; X = Area X.
451
452 Figure 4. Schematic drawings based on adjacent sections stained for MelIa, MelIb, and MelIc, showing the
453 pattern of expression of melatonin receptors in the bulbus olfactorius (left) and the tectum opticum (right).
454 Red dots indicate MelIa expression; blue dots, MelIb; green dots, MelIc. Right is frontal, top is dorsal.
455
456 Figure 5. Brightfield photomicrographs of Nissl-counterstained autoradiograms of parasagittal sections or
457 blackcap (A) and zebra finch (B) brains. Few scattered Mel1c expressing cells occur in the HVC of
458 blackcaps (A) but no such cells are found in the HVC of zebra finches (B). V indicates the lateral ventricle
459 that is the dorsal border of HVC. Clearly labelled cells (arrows) have several silvergrains over their somata.
460 Scale bar is 50 um.

461 Table 1. The distribution of 2-^{125}iodomelatonin (IMEL) binding and of MelIa, MelIb, and MelIc mRNA
462 expression in the songbird brain. The intensity of the binding and staining is reported on a scale from no
463 binding/labelling (-) to strong binding/labelling (+++). The (B) inside the table indicates areas of expression
464 found only in blackcaps.

465

System/Region	Nucleus/Subregion	Abbr.	IMEL	Mel1A	Mel1B	Mel1C
Visual: Tectofugal system	Stratum griseum fibrosum	SGF	+++	+++	-	+
	Stratum griseum centrale	SGC	+++	+++	+	+
	N. rotundus	Rt	+++	+++	-	-
	N. isthmo-opticus	Nio	+++	++	-	-
	N. isthmus parvocellularis	Ipc	+	-	-	-
	N. isthmus magnocellularis	Imc	+	-	-	-
	Entopallium	E	+++	+++	-	+(B)
Visual: Thalamofugal system	Corpus geniculatus lateralis, pars dorsalis	GLdp	++	++	-	+
	N. ventralis corporis geniculati laterali	GLv	+++	+++	-	+
	N. dorsolateralis anterior thalami	DLA	+++	+++	-	+
	Hyperpallium	H	+	+	+	++
	Hyperpallium intercalatum	HI	+++	-	+	+++
	Mesopallium dorsale	MD	++	-	+	++
	Mesopallium ventrale	MV	++	+	+	-
Visual: Acessory optic pathway	N. striae terminalis medialis	NSTM	++	++	-	+
	N. Edinger-Westphal	EW	+	+	-	+
	N.oculomotorius	nIII	++	+++	-	+++
	N. vestibularis superior	VeS	+	-	-	+
Retinohypothalamic	N. suprachiasmaticus	NSC	++	-	++	-
Auditory system	N. magnocellularis cochlearis	MCC	-	-	-	-
	N. angularis cochlearis	An	-	-	-	-
	N. olivaris superior	OS	-	-	-	-
	N. mesencephalicus lateralis, pars dorsalis	MLD	-	-	-	-
	N. ovoidalis	Ov	-	-	-	-
	Area L pallii 2	L	-	-	-	-
	Nidopallium caudomediale	NCM	+	++	++	++
	Mesopallium caudomediale	CMM	++	-	++	-
Olfactory system	Lamina granularis	LGr	++	-	++	+++
	Lamina mitralis	LMi	++	++	-	-
Vestibular system	N. vestibularis	Ve	++	++	-	+
Somatosensory system	N. basorostralis pallii	BSs	++	-	-	++
	Shell region of the N. basorostralis pallii	BSh	+	+	++	-
Other thalamic areas	N. dorsolateralis posterior thalami	DLP	+++	++	+	-
Other cortical areas	Area parahippocampalis	HP	++	++	+	+
Basal ganglia	Striatum mediale	StM	++	-	++	-
	N. striae terminalis lateralis	NSTL	+	-	++	-
Hypothalamus	Area praeoptica medialis	POA	+++	++	+++	-
	N. ventromedialis hypothalami	VMH	++	++	-	-
	N. arcuatus	Arc	++	++	++	+
Vocal control system	HVC	HVC	++	-	++	+(B)
	N. robustus arcopallialis	RA	+	-	+++	-
	N. lateralis magnocellularis nidopallii anterioris	LMAN	-	-	-	-
	N. medialis magnocellularis nidopallii anterioris	MMAN	-	-	-	-
	Area X	X	-/+	-	-/+	-
	N. dorsolateralis anterior, pars medialis	DLM	+	+	-	-
	Area ventralis tegmenti	AVT	+	-	-	+
	N. intercollicularis	ICo	+	-	+	+
	N. nervi hypoglossi	nXII	++	-	-	++
Cerebellum	Purkinje cell-layer	Pj	+	-	-	+

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