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Comparative Immune-reactivity Patterns of Arginine Vasotocin (AVT) and Melatonin Receptors (Mel1a & Mel1b) in Hypothalamic Regions of Male Japanese Quail *Coturnix coturnix japonica*: Possible Role in Water-Electrolyte Balance

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Abstract

Photoperiod influences circulatory Melatonin (Mel) and hypothalamic functions *via* retino-hypothalamic tract. However, interrelation between Mel receptors and Arginine Vasotocin (AVT), a water-electrolyte balancing hormone receptor expression in hypothalamic regions of avian brain has never been explored. We noted the expression pattern of two Mel receptors (Mel1a & Mel1b) along with AVT, in terms of neuronal immuno-positivity, in hypothalamic region of Japanese quail under different photoperiodic conditions with/without melatonin treatment. This is interesting and equally important because both Mel and AVT levels are regulated by light/dark cycle. Confocal imaging revealed specific regional localization of Mel1a/Mel1b in Supra-Chiasmatic Nucleus (SCN), Supra-Optic Nucleus (SON) and Para-Ventricular Nucleus (PVN), the intensity of which was dependent on the photoperiodic condition (long day, LD or short day, SD) and melatonin treatment. Mel1a/Mel1b was mostly co-localized along with AVT. Mel1b was abundant in hypothalamic regions in contrast to the Mel1a as reported in mammals. Mel1a immune-positivity was detected in SCN and SON regions of brain. Compared to control birds, a high intensity of Mel1a immunoreactivity was found in hypothalamic regions of birds under short photoperiod (SD, 8h L: 16h D) after Mel treatment. Further, Mel1b immunopositivity was high only in birds exposed to long days (LD, 16h L: 8h D). In SCN, abundant Mel1a and AVT immunoreative cells were found in Mel pretreated and SD-exposed birds compared to LD-exposed ones. Mel1a and AVT immunoreative cells were less in PVN of both SD and LD exposed birds. Our data of co-localization of Mel receptor(s) along with AVT in hypothalamic regions (exposed to short or long days with/without melatonin administration) strongly suggest a role for Mel along with AVT in water-electrolyte balance of birds which is important during long duration nuptial migration/flight.

Keywords: Co-localization, Hypothalamus, Melatonin/AVT, Quail, Receptor, Water-Electrolyte Balance

1. Introduction

Arginine Vaso-Pression (AVP) and Arginine Vaso-Tocin (AVT) were originally identified as antidiuretic neurophyseal hormones produced in magnocelluar neurons of the SON and PVN of the hypothalamus of vertebrates. These nuclei extend their axon terminals to the neurohypophysis where they release AVT into blood to act on peripheral targets for antidiuretic, stress, oviposition, water-electrolyte balance and reproductive functions[1-6]. AVT is the mammalian counterpart of AVP, and shows a complete disappearance in rodent (hamster) if exposed to short photoperiod (SD) whereas long photoperiods (LD) increased its production, suggesting a photoperiodic influence on AVP synthesis and *vice versa*[7,8].

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In birds, melatonin, a neurohormone from the pineal gland, is regulated by day length and is concerned with various physiological functions such as reproduction and immunity[9-11]. Further, melatonin, a chronobiological molecule and a photoperiodic time indicator in plasma, has its receptors in the hypothalamus, pituitary, Pars Tuberalis (PT), Supra-Chiasmatic Nucleus (SCN), Para-Ventriculus Nucleus (PVN), etc. The circulating level of this neurohormone, on the other hand, is inversely related to the plasma level of testosterone in birds[8-11]. Many species of birds are highly photoperiodic and their circulating gonadal steroidal levels fall drastically when exposed to short photoperiod[12]. A number of targets/ sites of Central Nervous System (CNS) for the action of melatonin have been identified in a variety of animal species[12-14]. Melatonin and AVP receptors are separately localized in the dorsomedial Supra-Chiasmatic Nucleus (dmSCN) of mammals[7,8] but it has not been possible to determine whether AVP or other neuropeptides/ neurotransmitters are co-localized in the same nucleus area of the brain of any poultry bird[15]. There is ample evidence to suggest that melatonin could be implicated in regulation of reproduction, stress, body temperature, water-electrolyte balance and cognition, functions that are mediated by the hypothalamus[16-18].

Thus, literature suggests that AVT and melatonin individually have different roles/effects on hypothalamic centers, but their functional/physiological correlation, if any, under different photoperiodic (short/long) condition as well as with or without melatonin administration in any avian species have never been explored. Hence, herein we studied the existence and co-localization of AVT and Mel1a/b receptors at different hypothalamic regions (SCN, PVN and SON) of the brain of a poultry bird, the Japanese quail *Coturnix coturnix japonica*, to find their functional correlation, if any, with photoperiodically regulated circulatory melatonin and AVT levels and water-electrolyte balance.

2. Materials and Methods

2.1 Animal

Male Japanese quail (*Coturnix coturnix japonica*) at the age of about 8 weeks were obtained from the Institute of Animal Welfare and Animal Husbandry, Celle, Germany. At the age of 9 weeks the quails were exposed to a photoperiod of 16 h light each day (16L: 8D). The Japanese quail is a photoperiodic species that does not develop absolute refractoriness. The birds, therefore, if kept under 16L: 8D may remain in full breeding condition throughout the year. Quails received food and water *ad libitum* throughout the period of the experiment. All experimental plans and procedures were reviewed by the appropriate authorities and were in compliance with the relevant laws and regulations of Ethical Committee of the Institute in Germany.

2.2 Experimental Design

Adult male Japanese quail were included in this study at the age of 25 to 45 weeks. Birds were kept in individual cages on a commercial diet and water *ad libitum* under two different photoperiodic schedules (light:dark) i.e., 16L: 8D (LD) and 8L:16D (SD) for four weeks. There were 10 birds in eachgroup. Four weeks after acclimatization, 5 birds of each photoperiodic schedule were given normal ethanolic saline (0.01% ethanol) 0.1 mL/day (vehicle control) and the remaining 5 birds were injected with 25 µg melatonin in 0.1 mL/100 g body weight/ through *sc* route[16] (Mel, Sigma, St. Louis, USA), for two weeks.

2.3 Sample Collection

At the end of the experiment, 24 h after the last injection, the birds were anesthetized with pentobarbital sodium (Narcoren, Merial GmbH, Hallbergmoos, Germany) at 40 mg/kg dose through *iv* route before perfusion. Coronal blood was collected directly from the heart in a heparinized syringe before perfusion with Phosphate-Buffered Saline (PBS) and Zamboni's fixative[17]. After perfusion, the brain was dissected out and post-fixed for 4 – 6 h in Zamboni's fixative followed by equilibration in 25% sucrose in PBS as a cryo-protectant until the tissue sank to the bottom of jar. The tissue was then frozen and stored at –80°C untill further processing. Plasma was separated by centrifugation and stored at -20°C until analysis of AVT and melatonin levels as well as the osmolality.

2.4 Nomenclature of Anatomical Regions and Selection of Brain Sections

Identification and nomenclature of brain areas relied on the chicken brain atlas[21,22] and the quail brain atlas[23]. Sub-regions of the SON were defined as recommended by Bayle *et al*[23]. The L2 region, as mentioned in the atlas, i.e., starting from the ventral crossing of the TSM (A8.8)

was used for analysis. The rostral border of the SON was identified by AVT immunoreactivity of two to ten neurons at the ventral tip of the TSM. Two randomly selected sections from each animal were used for further analysis- the first section was approximately from 160 µm location from the beginning, and the second was from approximately 320 µm caudal to the rostral tip of the SON region. Both hemispheres of each section were included in the analysis. Thus, all data were recorded from the rostral part of the SON, where the highest concentrations of oxytocin neurons were reported[23].

2.5 Cell Counts

Sections labeled by immunoreactivity were digitized with a Coho CCD camera connected to a Nikon Eclipse E600 light microscope and coupled with a Macintosh computer. Immunoreactive nuclei in the selected brain areas were digitized through 20x objective, and the images were then quantified by Image J software (NIH, USA). Samples for quantification (0.322 × 0.243 mm for all areas except hippocampus, and for the latter it was 0.153 × 0.115 mm) were taken from the center of each selected area. For each area quantified, the density histogram was adjusted with the gain to a standard value to normalize background levels. Cell counts were performed automatically by using the Image J software after standardized grey-level thresh holding based on the density histogram[24,25]. Counts were corrected with double counting. All quantifications were made by an experimenter blind to group assignments. Six brain areas, the Pre-Optic Margin (POM), brain striaterminalis (Bnst), Trans-nidopallium Area (TnA), parvocellular area ganglion PAG, hippocampus (Hp) and nidopallium (N) were selected for quantification[26] following the guidelines and detailed methods of Taziaux *et al*[27].

2.6 Plasma Analysis

Plasma samples were extracted and processed for Radio-Immuno-Assay (RIA) of ir-AVT by the method of Gray and Simon[28]. AVT was extracted from plasma with two volumes of acetone and two volumes of petroleum ether. The extract was dried under vacuum in a speed-vac concentrator (Savant Instruments Inc., New York, USA). The dried extract was dissolved in assay buffer (0.1 $molL^{-1}$Tris-HCl, pH 7.4, 2% BSA and 0.2% neomycin) and stored at -20°C until assayed. RIA was performed in duplicate using synthetic AVT as the standard (Sigma Chemical Co, St Louis, USA). RIA of melatonin was performed using anti-melatonin antibody (Stockgrand, UK) following the modified method of Rollag and Niswender[29]. Plasma osmolality was measured by vapor pressure osmometry (Wescor, model 5500; Logan, UT, USA)[30].

2.7 Immunohistochemistry

Frozen coronal sections of brains were cut at30 µm thickness in a Leica 2008E Frigocut (Reichert-Jung, Nussloch, Germany) and collected in 0.02 M PBS and were rinsed thoroughly in 0.02M PBS. Thereafter, sections were incubated in a blocking solution of 5% normal goat serum and 5% chicken serum (Sigma, USA) containing 0.2% Triton X-100 (Sigma, USA) for 30 min. Sections were then incubated for 21 h in 1:40000 guinea pig anti-AVP (Bachem, UK) and 1:2000 solution of rabbit anti-Mel1a or Mel1b in PBS containing 0.2% Triton X-100, 1% normal goat serum and 0.1% sodium azide. Sections were then thoroughly washed and incubated in goat anti-guinea pig FITC and goat-anti-rabbit A555 or goat anti-rabbit FITC and goat anti-guinea pig A555 in PBS containing 0.2% Triton X-100 (1:400 each in PBS), respectively, for 90 min at room temperature and then rinsed in PBS (3 x 15 min). Finally sections were covered with Vectashield H-100 (Vector Laboratories) and analyzed on a confocal laser scanning microscope (LSM 510, Zeiss, Gottingen, Germany) equipped with lasers emitting at 488 and 543 nm to excite FITC and Alexa 555, respectively[6].

2.8 Control of Immunoelabeling Specificity

Immunolabeling of the guinea pig anti-AVP antibody was tested on neighboring sections with the previously used rabbit anti-AVT for the sections containing the SON, SCN and PVN. No differences in the immunoreactivity pattern were observed between the two antibodies in all the three regions. Pre-absorption method was used to test the specificity of antibodies in brain tissues. The primary antibodies were replaced with a pre-absorbed mixture of Mel1a and Mel1b receptor antisera and their respective antigenic peptides (600 ng/100 µL of Mel1a receptor; sc-13186P & 600 ng/100 µL of Mel1b receptor; sc-13177P peptides; Santa Cruz, USA). For pre-absorption, the antigens were added to the same diluted antisera (Mel1a and Mel1b; 1:200) and incubated overnight at 4^0C, centrifuged and the supernatant was used (data not shown)[31,32]. The fluorescent labeling patterns were completely abolished when the primary antibodies were

omitted. Sections adjacent to the one used for dual labeling had been subjected to single labeling with any of the three antibodies. There were no differences in the immunoreactivity patterns observed. The number of immunoreactive cells, co-localization of receptor labels, AVT and cell size in each of the three regions were evaluated from two sections per bird for two birds of each treatment by a person who did not know the treatment of the samples. A relative classification was made in direct comparison of all evaluated sections per region.

2.9 Statistical Analysis

The data were presented as the mean + SEM. The data obtained from hormonal assay were analyzed by one-way analysis of variance (ANOVA by SPSS) followed by Tukey post-hoc test. Differences of mean were considered significant when $p< 0.05$.

3. Results

3.1 Plasma Level of Melatonin

Two-way ANOVA was conducted to examine the effect of photoperiod and melatonin treatment on plasma melatonin level. Significant interaction between the effects of photoperiod and melatonin treatment on plasma melatonin level [F (1, 12) = 4.718, $p< 0.001$] was found. The LD birds presented low plasma melatonin concentration [$p< 0.05$, F = 44.46], while significantly elevated concentrations of melatonin were found in the SD group of birds [$p< 0.001$, F = 35.71]. Short term melatonin administration to both LD- and SD-exposed birds had no significant ($p< 0.05$) effect on plasma melatonin level (Figure 1).

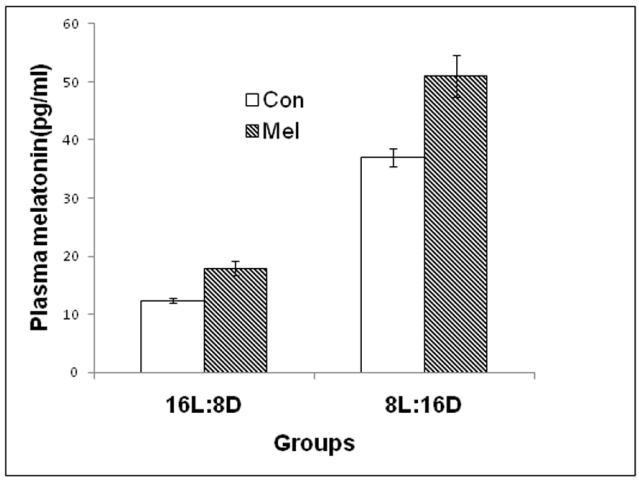

Figure 1. Levels of plasma melatonin (pg/mL) under different photoperiodic conditions (LD, 16L:8D and SD, 8L:16D) with and without melatonin treatment in Japanese quail. Histograms represent Mean + SE, n=5. $p< 0.05$.

3.2 Plasma Level of AVT

Two-way ANOVA was conducted to examine the effect of photoperiod and melatonin treatment on plasma AVT level. A significant interaction between the effects of photoperiod and melatonin treatment on AVT level [F (1, 12) = 116.060, $p<0.001$] was found. Plasma AVT was elevated in SD-exposed birds ($p<0.05$, F = 35.71), whereas it decreased to a significant level in LD-exposed birds ($p<0.05$, F = 42.47) when compared to control birds. Again, short-term melatonin administration had no significant ($p> 0.05$) effect on the peripheral level of AVT in birds under different photoperoid exposures (Figure 2).

3.3 Plasma Osmolality

Two-way ANOVA revealed a significant interaction between the effects of photoperiod and melatonin treatment on plasma osmolality [F (1, 12) = 25.116, $p< 0.001$]. Melatonin treatment suppressed plasma osmolality ($p< 0.05$, F = 66.66) to significant level in SD-exposed birds, while it had no significant effect on the plasma osmolality of LD-exposed birds ($p> 0.05$, F = 27.5) (Figure 3).

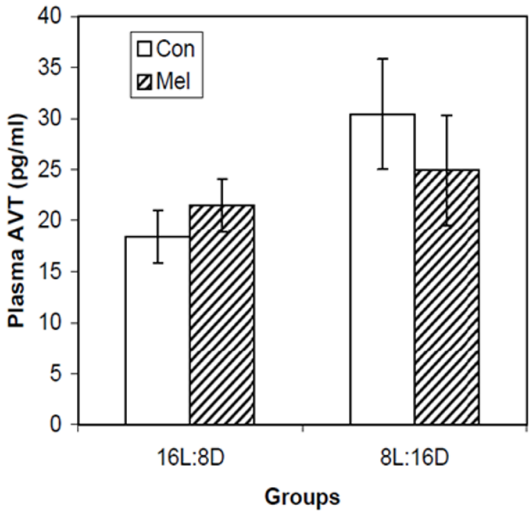

Figure 2. Levels of plasma AVT (pg/mL) of Japanese qail under different photoperiodic conditions (LD, 16L:8D and SD, 8L:16D) and following melatonin treatment. Histograms represent Mean + SE, n=5. $p< 0.05$.

3.4 Localization of AVT and Mel1a/Mel1b Receptors in the Different Hypothalamic Regions

3.4.1 Suprachiasmatic Nucleus (SCN): AVT and Mel1a

High AVT immunoreactivity was found in SCN region of birds under both the photoperiodic conditions (Figure 4B, H; Table 1). Melatonin treatment increased the AVT immunoreactivity in the hypothalamic regions under both photoperiodic conditions (Figure 4E and K). Further, Mel1aR immunoreactivity was more prominent in the magnocellular neurons of hypothalamus of SD-exposed birds as compared to LD-exposed birds (Figure 4C). In addition, melatonin treatment increased the prominent co-localization of Mel1a and AVT immunopositive cells in SD-exposed birds (Figure 4G) and in contrast decreased the same in LD-exposed birds (Figure 4J).

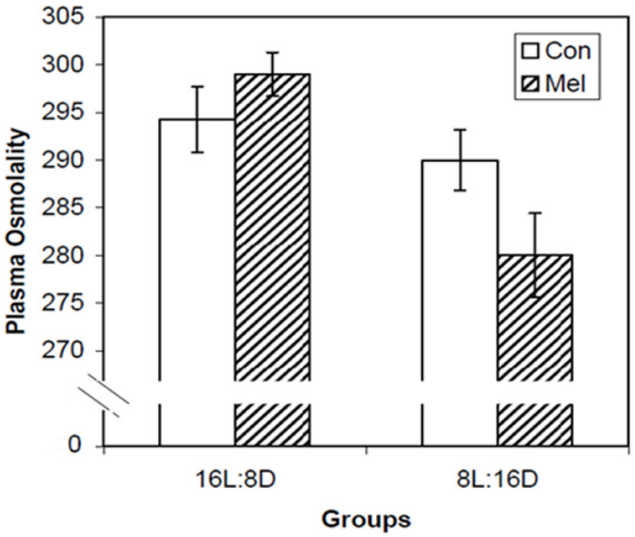

Figure 3. Blood osmolality of Japanese quail under different photoperiodic conditions (LD, 16L:8D and SD, 8L:16D) and following melatonin treatment. Histograms represent Mean + SE, n=5. $p< 0.05$.

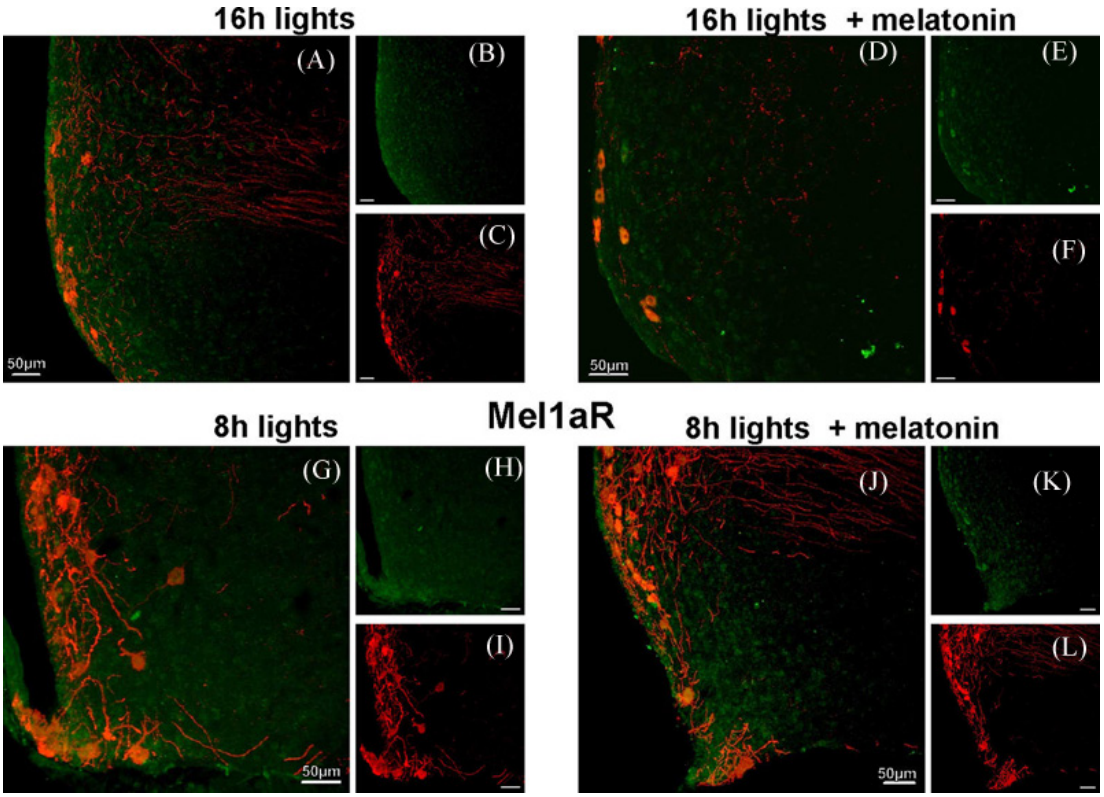

Figure 4. A-C, Demonstration of AVT and Mel1a in SCN under16L:8D without, and D-F with melatonin treatment. A and D represent co-localization of immunoreactivities of AVT (green) and Mel1aR (red) while B and E show immunoreactivity of AVT and C and E show immunoreactvity for Mel1aR. G-I, demonstration of AVT and Mel1aR in SCN under 8L:16D without and J-L with melatonin treatment. G and J represent co-localization of immunoreactivity of AVT and Mel1aR; H and K show immunoreactivity of AVT whereas I and L show immunoreactvity for Mel1aR. Note the high intensity of immunoreativities for AVT and Mel1aR under SD with or without melatonin treatment.

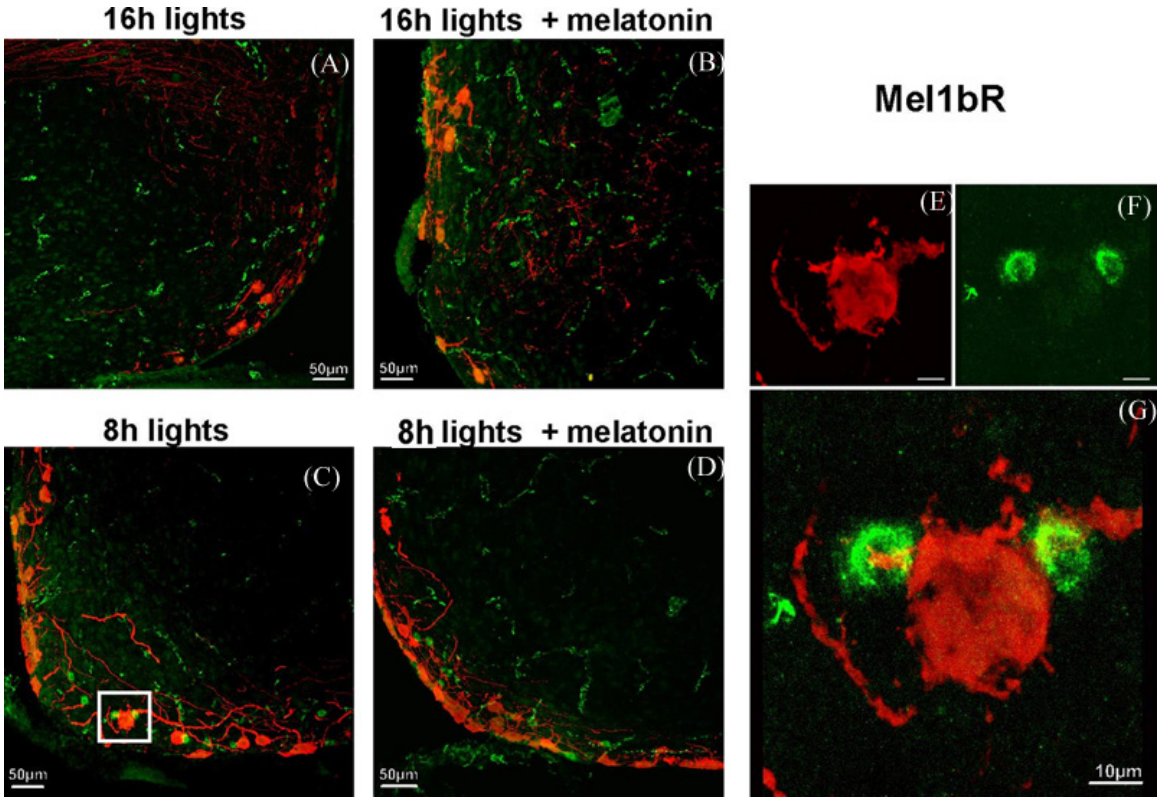

Figure 5. A, Demonstration of AVT and Mel1bR in SCN under 16L:8D without, and B with, melatonin treatment representing co-localization of immunoreactivities of AVT and Mel1bR; C and D show immunoreactivity of AVT and Mel1bR co-localization under 8L:16D. E-G, show higher magnification of C from a selected area (in white square). Note the high intensity of both the immunoreactivities for AVT and Mel1aR under SD with or without melatonin treatment.

3.4.2 Suprachiasmatic Nucleus (SCN): AVT and Mel1b

High co-localization of both AVT and Mel1b was found in LD-exposed quails as compared to SD-exposed quails (Figure 5B, D). Further, melatonin treatment increased the co-localization of AVT and Mel1b in LD exposed quails as compared to SD-exposed quails (Figure 5B, D).

*3.4.3 Supraoptic Nucleus (SON): **AVT and Mel1a***

AVT magnocellular neurons showed high immunoreactivity in LD-exposed quails as compared to SD-exposed quails (Table 1). Further, Mel1a immunoreactivity was more in SON of SD-exposed quails as compared to LD-exposed ones. Interestingly, melatonin treatment increased the co-localization of AVT and Mel1a immunoreactivity more in SD-birds than LD birds (Figure 6A, B, C, D).

3.4.4 Supraoptic Nucleus (SON): AVT and Mel1a

Strong immunoreactivity was observed for AVT as compared to Mel1bR in SON regions in both photoperiod exposed birds (Table1). In addition, the Mel1b immunoreactive cells were clearly observed between the SON ventral and SON external regions of SD-exposed birds as compared to LD exposed birds. Further, melatonin treatment increased the co-localization in SD birds as compared to LD birds (Figure 7A, B, C, D).

3.4.5 Paraventricular Nucleus (PVN): AVT and Mel1a

The PVN was the only region where mild Mel1a immunoreactivity was detected under both the photoperiod exposed birds (Figure 8A, G; Table 1). Mel1a immunoreactivity was localized more in both

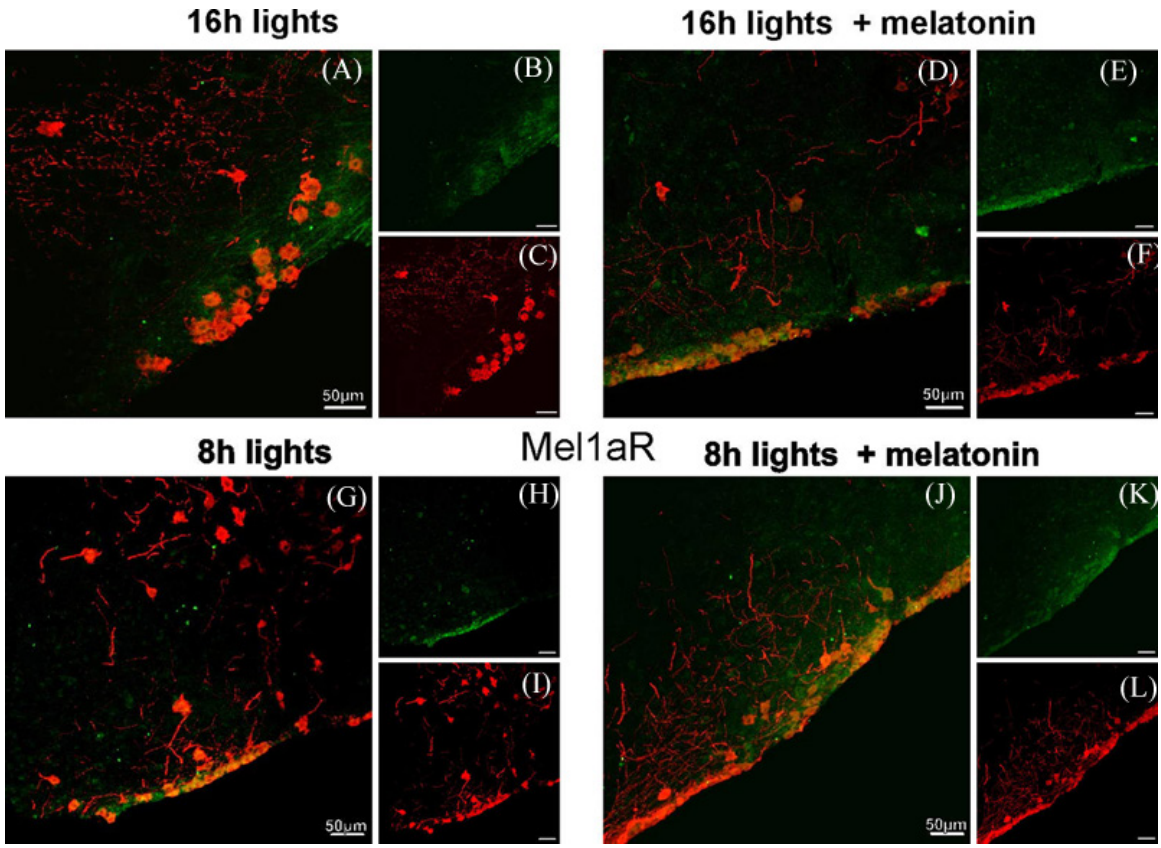

Figure 6. A-C, Demonstration of AVT and Mel1aR in SON under 16L:8D without, and D-F, with melatonin treatment. A and D represent co-localization of immunoreactivities of AVT (green) and Mel1aR (red) while B and E show immunoreactivity of AVT and C and E show immunoreactivity for Mel1aR. G-I, demonstration of AVT and Mel1aR in SON under 8L:16D without, and J-L with melatonin treatment. G and J represent co-localization of immunoreactivity of AVT and Mel1aR; H and K show immunoreactivity of AVT and I and L show immunoreactivity for Mel1a. Note the high intensity of both the immunoreactivities for AVT and Mel1aR under SD with or without melatonin treatment.

magnocellular and parvocellular neurons of ventral and lateral regions of the PVN (Figure 8C, I). In SD-exposed birds, the Mel1a was with AVT and the size of AVT neurons were reduced when compared to LD-exposed birds (Figure 8C, I, F, L). Melatonin treatment reduced the Mel1a immunoreactivity and increased the AVT immunoreactivity, but it reduced the co-localizationin SD-exposed birds as compared to LD-exposed birds (Figure 8A, D, G, J).

3.4.6 Paraventicular Nucleus (PVN): AVT and Mel1b

A strong Mel1b immunoreactivity and mild AVT immunoreactivity was observed in magnocellular and parvocellular neurons of PVN regions of both the photoperoid- exposed birds (Figure 9B, C, H, I; Table 1). Melatonin treatment reduced the Mel1b immunoreactivity in LD quails as compared to SD quails in the PVN regions. Moreover, magnocellular AVT neurons were well co-localized with Mel1b immunoreactive cells (Figure 9D, G). Interestingly, melatonin treatment decreased the Mel1b immunoreactivity, while intensity of AVT immunoreactivity was not affected in the PVN hypothalamic region of SD-exposed birds as compared to LD-exposed birds (Figure 9A, G, D, J).

The relative localization of immunopositive cells in the different regions of the PVN, SON and SCN of Japanese quails under different photoperiodic conditions (LD and SD) is presented in Table 1.

Table 1. Relative abundance of immunopositive cells in PVN, SON and SCN regions of the hypothalamus of Japanese quail under different photoperiodic conditions (LD, 16L: 8D and SD, 8L:16D) with and without melatonin treatment

PVN

	Endoth	MT1b	MT1b/ AVT	AVT-mc	F	AVTges	McAVT/AVT	Mc MTR1b
LC	# / #	++	++/+++	++	##	++	++/+++	-
LM	### / ##	+	+	+++	##/###	+++	+++	-/++
SC	#? / #	+++	+++	++	#/##	++/+++	++	+
SM	# / #	++	++/+++	+/++	#/##	++/+++	++/+++	-/+

SON

	Endoth	MR1b	MT1b/ AVT	AVT-mc	F	AVTges	McAVT/AVT	Mc MR1b
LC	# /#,###	++	++	+++	#/##	+++	+++	-
LM	# / #	++	++	++	#/##	++	++/+++	-
SC		+++	++++	++/+++	##	++	++/+++	-
SM	# / ##	++	+	++/+++	##/###	+++	++	-

SCN

	Endoth	MR1b	MR1b/ AVT	AVT-mc	F	AVTges	McAVT/AVT	Mc MR1b
LC	# / #	++	+++	+	##	++	+/++	-
LM	# / ##	+	++/+++	+	##/###	+	++/+++	-
SC	## / ##	+++	++/+++	+++	###	++/+++	++/+++	-
SM	## / ##	+	+	++	###	+	++	-

LC - long day controls

LM – long day melatonin treated

SC – short day controls

F – fiber structures with AVT labels

Mc – magnocellular neurons (diameter > 20µm)

SM – short day melatonin treated

MR1b/AVT – proportion of receptor positive cells to the number of AVT cells of the same field
msAVT/AVT – proportion of magnocellular AVT neurons to the total number of AVT neurons

(### =50 to 80% expression; ## =20 to 50% expression; # = less than 20%;
+++ =50 to 80% expression; ++ =20 to 50% expression; + = less than 20%; - = Absent).

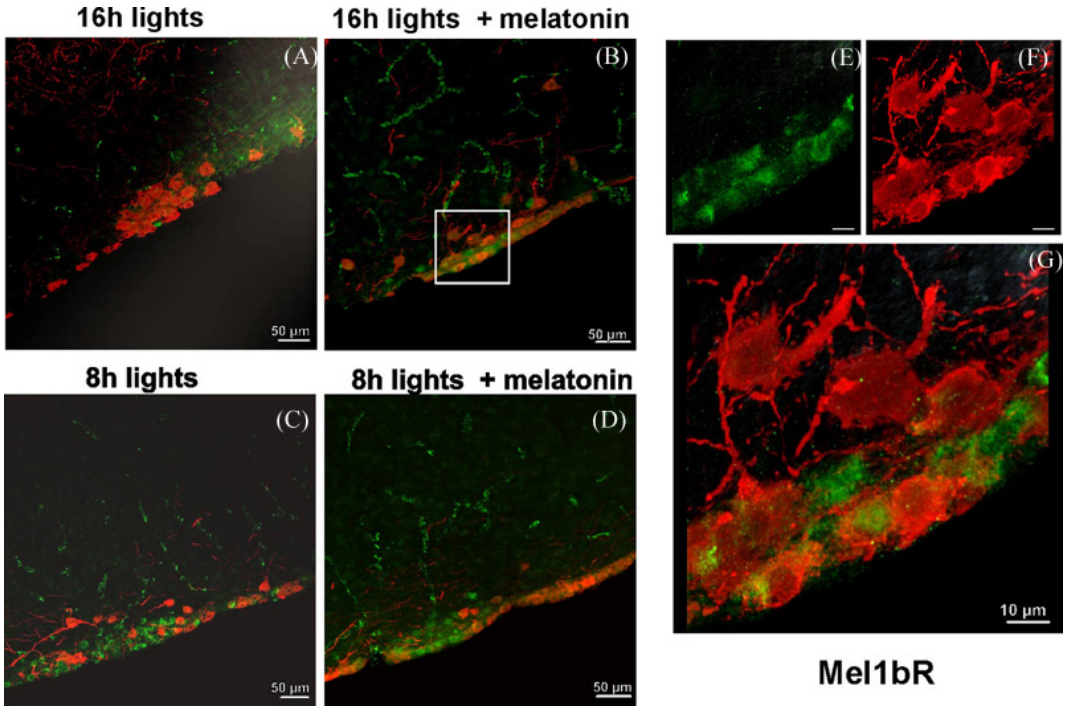

Figure 7. A, Demonstration of AVT and Mel1bR in SON under 16L:8D without and B, with melatonin treatment representing co-localization of immunoreactivities of AVT and Mel1bR; C and D show immunoreactivity of AVT and Mel1bR co-localization under 8L:16D. E-G, higher magnification of B from a selected area (in white square).

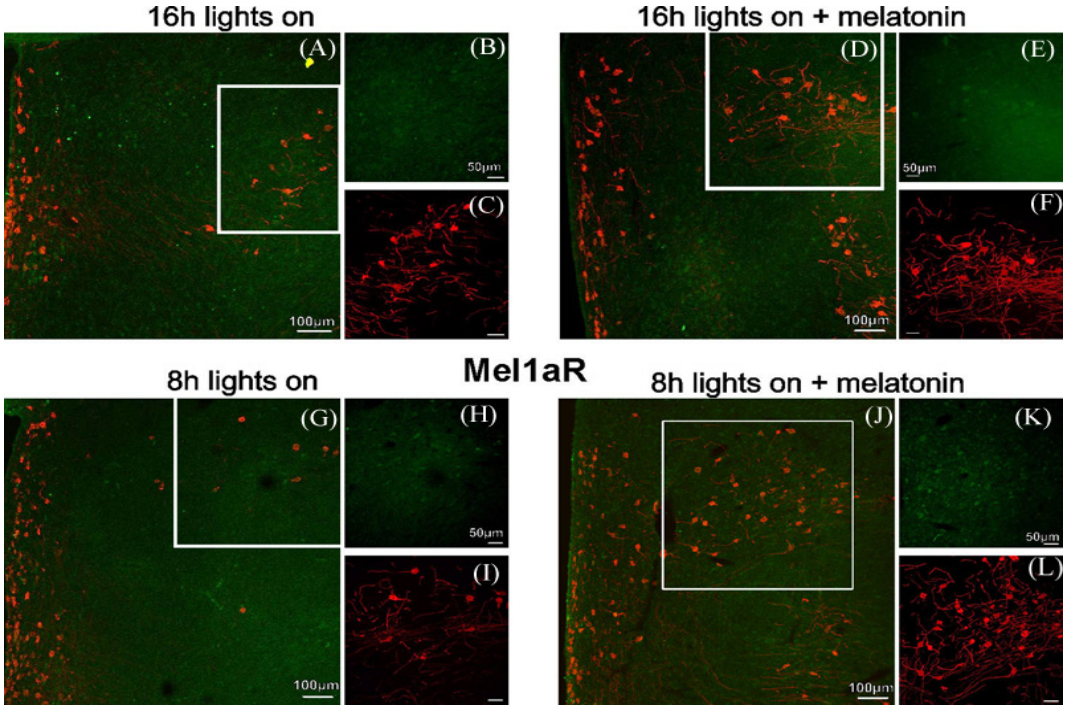

Figure 8. A-C, Demonstration of AVT and Mel1aR in PVN under 16L:8D without and D-F with melatonin treatment. A and D represent co-localization of immunoreactivities of AVT (green) and Mel1aR (red); B and E show immunoreactivity of AVT and C, and E shows immunoreactivity of Mel1aR. G-I demonstrate AVT and Mel1aR in PVN under 8L:16D without, and J-L, with melatonin treatment. G and J represent co-localization of immunoreactivity of AVT and Mel1aR while H and K show immunoreactivity of AVT and I and L show immunoreactivity for Mel1aR.

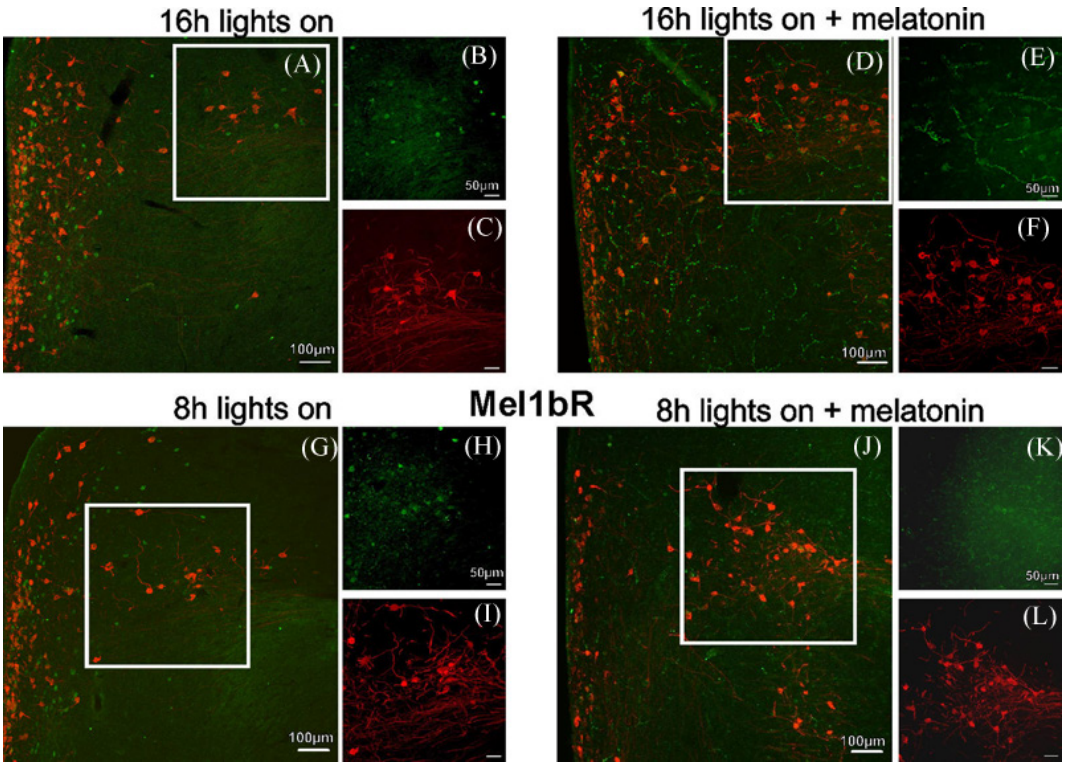

Figure 9. A-C, Demonstration of AVT and Mel1aR in PVN under 16L:8D without, and D-F with melatonin treatment. A and D represent co-localization of immunoreactivities of AVT (green) and Mel1aR (red); B and E show immunoreactivity of AVT and C and E show immunoreactivity for Mel1aR. G-I, demonstration of AVT and Mel1aR in SCN under 8L:16D without, and J-L with melatonin treatment. G and J represent co-localization of immunoreactivity of AVT and Mel1aR while H and K show immunoreactivity of AVT and I and L show immunoreactivity for Mel1aR.

4. Discussion

Avian group has widely been used for the study of photoperiodic responses of hypothalamic regions associated with reproductive function with special reference to arginine-vasotocin[5]. On the other hand regulation of various seasonal functions including reproduction and immunity was reported to be associated with the neurohormone melatonin[9-13]. Further, it has been reported that circulatory AVT and melatonin are both regulated by photoperiod in a rhythmic manner[5,8,18,33,34]. The individual functional role of AVT and melatonin in circulation and their receptors on target site and on hypothalamic regions have been well identified and reported[1-7]. But, the co-localization of expression pattern of melatonin (Mel1a & Mel1b) and AVT receptors in hyphothalamic regions was never reported earlier, and is of great interest in order to delineate their functional coordination in reproduction and water-electrolyte balance (required under extreme conditions of long nuptial migration, etc.) of the birds[34].

Differential localization of melatonin receptor subtype(s) in avian hyphothalamic regions and its correlation with AVT under different photoperiodic condition have not been reported earlier in any poultry bird. Our results suggest an interrelation of AVT and melatonin as reflected by co-localization in certain hypothalamic centers (SON, PVN) and also on the fact that their expressions are influenced by photoperiod (SD/LD) and exogenous melatonin administration as it influenced the immunoreactivity of AVT and thereby water-osmolality of the bird. Exogenous melatonin administration was partially able to restore the effect of photoperiods (LD) on AVT expression, suggesting a direct effect of melatonin on AVT neuron *via* Mel1a/b receptors as both the receptors were found co-localized with AVT. This effect was prominent in the SON and PVN regions of the hypothalamus suggesting them as major areas of origin.

It has also been reported that in mammals plasma melatonin level and AVP present a circadian rhythm,

being high at night and low during day time[24,31]. This could be one of the reasons why we found co-localization of their receptor in the hypothalamic region. We observed the presence of Mel1a/b immunoreactivity in the SON region which suggests that the circadian control of diurnal pattern of the AVT secretion may be under the influence of melatonin. The mechanism of melatonin action involves its membrane receptors, G-protein coupled Mel1a/b receptors, residing predominantly in neuronal regions of the brain especially cerebellum, hippocampus, SCN, hypothalamus, thalamus, preoptic area, plexiform layer of retina and of cerebral cortex of vertebrates[30,33,35]. We report Mel1a/Mel1b immunoreactivity pattern in the hypothalamic region of a photoperiodic poultry bird *Perdicula asiatica,* in which breeding may be induced by photoperiodically, for the first time. Higher intensity of AVT immunoreactivity was observed in the SON of LD-exposed birds. Mel1a immunoreactivity was high in PVN, SCN and SON regions of SD-exposed birds as compared to LD-exposed birds. Further, co-localization of AVT with the melatonin receptor subtypes (Mel1a and Mel1b) in the SON, SCN, and PVN regions suggest that melatonin, the chemical code of scotophase (SD), plays a crucial role in fine-tuning of a number of physiological processes including water-electrolyte balance[31].

We observed that long day (16L:8D) exposures reduced the plasma AVT level when compared with short day (8L:16D) exposed birds, while evening injection of melatonin for a very short durations (2 consecutive days) could not affect the level of AVT in the birds under both long and short days. At first we conceived that very short duration (2 days) of melatonin administration may promote the "flash-light"-like effect on hypothalamus-pituitary-pineal axis. But, a very short duration of melatonin administration used by us had less or no influence on peripheral AVT level and/or its associated function, the water-electrolyte balance. Interestingly, the results of melatonin treatment under SD showed significant decrease in osmolality suggesting that short exogenous melatonin administration along with SD increased peripheral melatonin level leading to change in hypothalamic immunoreactivity and also decrease in osmolality[34]. Further, melatonin treatment increased Mel1a expression in PVN of LD-exposed birds, whereas it was more expressed in SON in SD-exposed birds while Mel1b expression was more in lateral magnocellular area and less in PVN region. It was less or the least affected by short melatonin treatment to SD-exposed birds that had already high circulatory melatonin level.

Previous studies on mammals have suggested co-localization of AVP and Mel1a in SCN neuronal subpopulation, indicating an action of melatonin on the SCN and its rhythmic correlation with AVP[35]. Our present observation in the photosensitive avian model suggests for the first time that Mel1b receptors are widely expressed throughout the hypothalamus but Mel1a receptors are localized mostly in the PVN region of the brain and is being regulated by different photoperiodic schedules. However, it may be up-regulated in the hypothalamic region (PON) following short (SD) photoperiodic exposure suggesting that Mel1a might be involved in regulation of circadian rhythm in functions of AVT expression and thereby hydromineral balances[6].

Therefore, from our finding, a potential cross-talk between the Mel1a/b receptors in avian hypothalamic areas along with neuropeptide AVT system can be suggested for regulation of reproductive (seasonal/egg laying) and electrolyte balance (kidney/salt gland). The differences of receptor immunoreactivity and photoperiodic sensitivity of those centers reflect fine-tuning of different neural connectivities and function(s) of the magnocellular neurons of the preoptic, SON and PVN regions of birds[34,35]. Partial results (MT1R mRNA expression pattern in SCN) are available in respect of rodents, birds, amphibians and fish which also support our finding[33-37].

In conclusion, melatonin receptor Mel1a/1b along with AVT expression in various regions of the hypothalamic areas of avian species may indicate not only a direct role of melatonin in regulation/control of rhythmic functions such as reproduction and nuptial migration[38] but also the photosensitive nature and coordination of water-mineral-electrolyte balance that are essential during egg laying (shell gland) and long nuptial/breeding migration to keep them hydrated. The combined receptor expression of AVT/Mel1a/b in neurons being a photoperiod-dependent phenomenon led us to propose that osmoregulation in birds might be a photoperiod-/season-dependant phenomenon as also noted in fish and amphibians[34,36].

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6. References

1. Sasaki T, Shimada K, Saito N. Changes of AVT in plasma, neurohypophysis and hypothalamus in relation to oviposition in the laying hen. Comp Biochem Physiol. 1990; A121:149-53. https://doi.org/10.1016/S1095-6433(98)10116-2.
2. Panzica GC, Plumar L, Garcia-Ojeda E, Deviche P. Central vasotocin immunoreactive system in male passarine birds (*Junco hyemalis*). J Comp Neurol. 1999; 409:105-117. https://doi.org/10.1002/(SICI)1096-9861(19990621)409:1<105::AID-CNE8>3.0.CO;2-8.
3. Chaturvedi CM, Chaudhary A, Wall PT, Cornett LE. A sexual dimorphism in hypothalamus Arginine Vaso-Tocin (AVT) gene expression and AVT plasma levels in the Japanese quail (*Coturinx coturnix* japonica) in response to water deprivation. Gen Comp Endocrinol. 2000; 117:129-137. https://doi.org/10.1006/gcen.1999.7400. PMid:10620429.
4. Deviche P, Gracia-Ojeda E, Plumari L, Panzica GC. Vasotocinergic innervations in a male passerine bird (*Junco hyemylis*): Effect of photoperiodic condition Soc Neurosci Abs. 1996; 22:1551.
5. Grossman R, Jukevich A, Kohler A. Sex dimorphism in the avian arginin vasopressin system with special emphasis on the bed nucleus of the *Stria terminalis*. Comp Biochem Physiol. A. 2002; 131:833-837. https://doi.org/10.1016/S1095-6433(02)00021-1.
6. Buijs RM, Pevet P, Masson-Pevet M, *et al*. Seasonal variation in vasopressin innervations of the European hamster (*Cricetus cricetus*). Brain Res. 1986; 371:193-196. https://doi.org/10.1016/0006-8993(86)90829-2.
7. David JT, Cervantes MC, Trosky KA, Salinas JA, Delville Y. A neural network underlying individual differences in emotion and aggression in male golden hamsters. Neuroscience. 2004; 126:567-578. https://doi.org/10.1016/j.neuroscience.2004.04.031. PMid:15183506.
8. Singh SS, Haldar C, Peripheral melatonin modulates seasonal immunity and reproduction of Indian tropical male bird *Perdicula asiatica*. Comp Biochem Physiol. A. 2007; 146:446-450. https://doi.org/10.1016/j.cbpa.2006.12.024. PMid:17257874.
9. Yadav SK, Haldar C. Singh SS. Variation in melatonin receptor types, Mel1a and Mel1b and androgen receptor AR expression in spleen of a seasonally breeding bird, *Perdicula asiatica*. J Reprod Immunol. 2011; 92:54-61. https://doi.org/10.1016/j.jri.2011.08.003. PMid:21963392.
10. Fraley GS, Steiner RA, Lent KL, Brenowitz EA. Seasonal changes in androgen receptor mRNA in the brain of the white-crowned sparrow. Gen Comp Endocrinol. 2010; 166:66-71. https://doi.org/10.1111/j.1439-0310.1986.tb00581.x.
11. Maitra SK, Dey M. Castration and testosterone induced changes in the pinealocytes of roseringed parakeet, *Psittacula krameri*, during different phases of the annual testicular cycle. Ann Anat.1994; 176(4):363-368. https://doi.org/10.1016/S0940-9602(11)80520-0.
12. Skotonica E, Alyneak AJ. Melatonin and its possible role in regulation of water and electrolyte metabolism. Med Water. 2001; 57(5):299-303.
13. Singh SS, Haldar C, Melatonin prevents testosterone-induced suppression of Immune parameters and splenocyte proliferation in Indian tropical jungle bush quail, *Perdicula asiatica*. Gen Comp Endocrinol. 2005; 141:226-232. https://doi.org/10.1016/j.ygcen.2005.01.005. PMid:15804509.
14. Reghunandanan V, Reghunandanan R. Neurotransmitters of the suprachiasmatic nuclei. J Circadian Rhythms. 2006; 4:2. doi:10.1186/1740-3391-4-2. https://doi.org/10.1186/1740-3391-4-2. PMid:16480518 PMCid:PMC1402333.
15. Howard CM, Lutterschmidt DI. The Effects of Melatonin on Brain Arginine Vasotocin: Relationship with Sex and Seasonal Differences in Melatonin Receptor Type 1 in Green Treefrogs (Hylacinerea). J Neuroendocrinol. 2015; 27(8):670-679. https://doi.org/10.1111/jne.12292. PMid:25967351.
16. Lutterschmidt DI, Wilczynski W. Sexually dimorphic effects of melatonin on brain arginine vasotocin immunoreactivity in green tree frogs (*Hyla cinerea*). Brain Behav Evol. 2012; 80(3):222-232. https://doi.org/10.1159/000341238. PMid:22906877 PMCid:PMC3506391.
17. Mühlbauer E, Hammann D, Xu B, *et al*. Arginine vasotocine gene expression during osmotic challanges in chicken. J. Neuroendocrinol. 1992; 4:347-351. https://doi.org/10.1111/j.1365-2826.1992.tb00178.x. PMid:21554616.
18. Prusik M, Lewczuk B. Roles of direct photoreception and the internal circadian oscillator in the regulation of melatonin secretion in the pineal organ of the domestic turkey. A novel in vitro clock and calendar model. Int J Mol Sci. 2019; 20(16):4022. https://doi.org/10.3390/ijms20164022. PMid:31426535 PMCid:PMC6721154.

19. Kuenzel WJ, Masson MA. Stereotaxic Atlas of the Brain of the Chick (*Gallus domesticus*). Johns Hopkins University Press; 1988.
20. Shipley MT, Luna J, McLean JH. Processing and Analysis of Neuroanatomical Images. In: Heimer L, Zaborsky L. editors. Neuroanatomical Tract Tracing Methods 2. Plenum Press; New York; 1989. p. 331-390. https://doi.org/10.1007/978-1-4757-2055-6.
21. Bayle JD, Ramade F, Oliver J. Stereotaxic topography of the brain of the quail (*Coturnix coturnix japonica*).J Physiol Paris.1974; 682:219-241.
22. Foidart A, Harada N, Balthazart J. Effects of steroidal and non-steroidal aromatase inhibitors on sexual behavior and aromatase-immunoreactive cells and fibers in the quail brain. Brain Res. 1994; 657(1-2):105-123. https://doi.org/10.1016/0006-8993(94)90958-X.
23. Taziaux M, Cornil CA, Dejace C, *et al*. Neuroanatomical specificity in the expression of the immediate early gene c-fos following expression of appetitive and consummatory male sexual behaviour in Japanese quail. Eur J Neurosci. 2006; 237:1869-1887. https://doi.org/10.1111/j.1460-9568.2006.04719.x. PMid:16623844.
24. Gray DA, Simon E. Mammalian and avian antidiuretic hormone: Studies related to possible species variation in osmoregulatory systems. J Comp Physiol. A. 1993; 151:2416.
25. Rollag MD, Niswender GD. Radioimmunoassay of melatonin in sheep exposed to different light regimes. Endocrinology. 1976; 98:482-488. https://doi.org/10.1210/endo-98-2-482. PMid:1248456.
26. Brydon L, Roka F, Petit L, *et al*. Dual signalling of human Mel1a melatonin receptors via G(i2), G(i3) and G(q/11) proteins. Mol Endocrinol. 1999; 13:2025-2038.
27. Angeloni D, Longhi R, Fraschini E. Production and characterization of antibodies directed against the human melatonin receptors Mel-1a (mt1) and Mel-1b (MT2). Eur J Histochem. 2000; 44:199-204.
28. Savaskan E, Ayoub MA, Ravid R, *et al*. Reduced hippocampal MT2 melatonin receptor expression in Alzheimer's disease. J Pineal Res. 2005; 38(1):10-16. https://doi.org/10.1111/j.1600-079X.2004.00169.x. PMid:15617532.
29. Wu YH, Zhou JN, Balesar R, *et al*. Distribution of MT1 melatonin receptor immunoreactivity in human hypothalamus and pituitary gland: Co-localization of MT1 with vasopressin, oxytocin and corticotrophin-releasing hormone. J Comp Neurol. 2006; 499:897-910. https://doi.org/10.1002/cne.21152. PMid:17072839.
30. Panzica GC, Fraschini F, Aste N, *et al*. The density of melatonin receptor is dependent upon the prevailing photoperiod in Japanese quails. Neurosci Lett. 1994; 173:111-114. https://doi.org/10.1016/0304-3940(94)90161-9.
31. Sweta A, Pratap D, Haldar C. Stunning facts of bird migration: Mini-review. J Endocrinol Reprod. 2019; 23(1):44-47. ISSN (Online): 0976-5131.
32. Prusik M, Lewczuk B. Roles of direct photoreception and the internal circadian oscillator in the regulation of melatonin secretion in the pineal organ of the domestic turkey. A novel in vitro clock and calendar model. Int J Mol Sci. 2019; 20:4022. https://doi.org/10.3390/ijms20164022. PMid:31426535 PMCid:PMC6721154.
33. Petrusewicz-Kosińska M, Przybylska-Gornowicz B, Ziółkowska N, *et al*. Developmental morphology of the turkey pineal organ. Immunocytochemical and ultrastructural studies. Micron. 2019; 122:8-20. https://doi.org/10.1016/j.micron.2019.04.002. PMid:31026727.
34. Nascimento ES Jr, Souza AP, Duarte RB, *et al*. The suprachiasmatic nucleus and the intergeniculate leaflet in the rock cavy (*Kerodon rupestris*): Retinal projections and immunohistochemical characterization. Brain Res. 2010; 1320:34-46. https://doi.org/10.1016/j.brainres.2010.01.034. PMid:20096673.
35. Reppert SM, Weaver DR, Cassone VM, *et al*. Melatonin receptors are for the birds: Molecular analysis of two receptor subtypes differentially expressed in chick brain. Neuron.1995; 15:1003-1015. https://doi.org/10.1016/0896-6273(95)90090-X.
36. Ziółkowska N. Melatonin Biosynthesis and Mechanisms of Its Regulation in The Domestic Goose Pineal Organ. Doctoral Thesis. University of Warmia and Mazury, Olsztyn, Poland; 2015.
37. Morin LP, Shivers KY, Blanchard JH, Muscat L. Complex organization of mouse and rat suprachiasmatic nucleus. Neuroscience. 2006; 137:1285-1297. https://doi.org/10.1016/j.neuroscience.2005.10.030. PMid:16338081.
38. Gubrij KI, Chaturvedi CM, Ali N, *et al*. Molecular cloning of an oxytocin like receptor expressed in the chicken shell gland. Comp Biochem Physiol. B. 2005; 142:37-45. https://doi.org/10.1016/j.cbpc.2005.05.011. PMid:16005652.