Neuronal Control of Gut Melatoninergic System in Carp

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Abstract

Introduction

Demonstration of the role of food and feeding time in determining daily rhythm profiles of gut derived melatonin in carp (Catla catla) led to present study on the mechanism of neural regulation of its gut melatoninergic system. In this in vitro study, different pharmacological agents were used to demonstrate the role of adrenergic, dopaminergic and cholinergic signals in the post-prandial regulation of AANAT and melatonin titres in the carp gut.

Methods

The gut tissues of carp, which were supplied daily with an equal amount of food at a fixed time point in the morning and sacrificed 2 hours thereafter, were used for in vitro study. The gut tissues were incubated initially in the culture medium for 2 hours and then separately with specific agonists and antagonists of adrenergic, dopaminergic and cholinergic receptors for 2 hours before using them for measuring their levels of AANAT and melatonin.

Results

Gut levels of AANAT and melatonin were significantly increased following incubation with nor-epinephrine or agonists of its α₁ and β₁ receptors, but decreased when incubated with their antagonists. Gut melatoninergic system was stimulated by dopamine or its D₁ agonist, but inhibited by antagonist of D₁ receptor. While acetylcholine or, agonist of its nicotinic receptor or, muscarinic receptor significantly reduced the levels of AANAT and melatonin, antagonists of respective receptors caused an opposite effect. AANAT and melatonin levels in gut were elevated by cAMP or adenylate cyclase activator, but reduced by its inhibitor.

Conclusion

This study provides the first evidence that different neuronal signals, by employing a signal transduction pathway where cAMP seems to act as an intracellular messenger, play important role in regulating post-prandial synthesis of AANAT and melatonin in any fish gut.

Keywords: AANAT; Adrenergic; Cholinergic; Dopaminergic; Carp; Gut Melatonin

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a lipophilic compound that in different tissues diffuses through biological membranes and performs endocrine, paracrine and autocrine actions [1]. This tryptophan derivative is synthesized in the pinealocytes of the pineal gland as well as in distinct cells of several extra-pineal tissues, like enterochromaffin (EC) cells in the gastrointestinal tract (GIT) in different vertebrates [2,3]. Gut melatonin is implicated in the control mechanisms of several gastrointestinal functions [4,5,6], of which regulation of food intake seems to be important especially in fish [7,8]. A recent study on carp depicted for the first time that availability of food and feeding time may act as external synchronizer for daily rhythm profiles of melatonin in the gut tissues of any fish [9]. Several lines of evidence suggest possible paracrine actions of endogenous gut melatonin on the intestinal muscles directly or indirectly via myenteric nervous system [10,11]. However, the neural regulatory mechanisms of gut melatoninergic system in fish are yet to be known.

The GIT in fish, as in other vertebrates, is a highly innervated organ. In response to various stimuli, different transmitters are released from the enteroendocrine cells, which not only perform paracrine actions on the neighbouring enterocytes, but activate specific receptors within the enteric nervous system as well [12]. Enteroendocrine cell products also activate receptors at afferent terminals in vagal and spinal pathways [13] and serve as a potent connecting link in the afferent transfer of information from the gut surface to the nervous system. The autonomic nerves are involved in involuntary reflex signalling and control a wide range of physiological functions of gut covering breakdown of complex food materials to transport of digested food products to the circulation, osmoregulation to barrier functions. The autonomic innervations include local nerves (enteric nervous system; ENS) as well as extrinsic sympathetic and parasympathetic nerves. The ENS contains an enormous number of nerve cell bodies located within the gut wall where majority of them are found in the myenteric plexus between the two layers of smooth muscles. Enteric nerves express a wide variety of transmitters, majority of them in gut are identical or similar in forms. Sensory extrinsic neurons respond to several stimuli related to the availability of food in the GIT and make up elaborate reflex pathways [14]. Most of these transmitters

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found in the mammalian GIT are also found in the majority of fish species [14].

So far, the role of specific receptors of different neurons (adrenergic, dopaminergic and cholinergic) has been investigated chiefly in the pineal organ of different vertebrates including carp [15] defining several physiological functions, but comparable data on the neuronal regulatory mechanism of melatoninergic system in the gastrointestinal tissue of any fish or other vertebrates are unknown. Among different neuronal signals, nor-epinephrine (NE) is known as the possible key physiological regulator of melatonin synthesis in any cell where, under appropriate stimulation, NE acts through $\alpha_1$ and $\beta_1$ adrenergic receptors [16]. Activation of $\alpha_1$ adrenergic receptors increases Ca++ stimulating the synthesis of AANAT and melatonin, while activation of $\beta_1$ adrenergic receptors may result in increased intracellular cAMP level by stimulating adenylate cyclase [17]. cAMP in turn enhances the production of AANAT and melatonin [15,18,19,20]. Although a bulk of information on the neural signal transduction mechanism involving adrenergic, dopaminergic and cholinergic neurons are reported in the study on pineal organ in different vertebrates, including fish [14,21,22,23], the role of such signals in the regulation of gut melatoninergic system in any fish remained unknown. Though an involvement of nicotinic and muscarinic receptors of the cholinergic neurons in the regulation of several gastrointestinal functions has been investigated, the studies are limited mostly to mammals [24] and few fish species [25]. Indications are also available to show that melatonin synthesis in the mammalian GIT in greatly influenced by the local neuronal signals, but such information is not known for any piscine species. Application of melatonin MT$_{1}$-selective antagonist- luzindole almost abolished the rise in the secretion of HCO$_3^-$ induced by intra-cerebro-ventricular infusion of the adrenergic agonist phenylephrine, but did not affect the release of melatonin in the duodenal lumen [26]. These findings strongly suggest that a centrally elicited neural stimulus releases melatonin from EC cells in the duodenal mucosa, which thereafter acts on MT$_1$ receptors increasing electrolyte export of enterocytes [27]. However, the role of individual neuronal receptors and the exact signalling mechanisms involved in the regulation of melatoninergic system in the gut tissues of any fish are far from being resolved and thereby warrant careful study.

The purpose of currently undertaken in vitro study is to evaluate the role of specific neuronal (adrenergic, dopaminergic and cholinergic) signals in the regulation of feeding- induced expression of AANAT (arylalkylamine N-acetyltransferase) protein, which catalyses conversion of serotonin to N-acetylsertotonin in melatonin biosynthetic pathway [28], as well as melatonin concentrations in the carp gut with the use of specific agonists and antagonists of the adrenergic, dopaminergic and cholinergic receptors. On the basis of an earlier study on fish pineal showing a decrease in the intracellular cAMP levels due to photo-induced hyperpolarization [29], possible role of cAMP in mediating specific neuronal signals in the fish gut has also been evaluated by employing specific activators and inhibitors of intra-cellular cAMP.

**Materials and Methods**

**Chemicals and Reagents**

All pharmacological agents used in the present study were procured from Sigma-Aldrich Chemical Co. (USA), RPMI 1640 culture medium, bovine serum albumen (BSA), penicillin G, streptomycin sulfate, phenylmethylsulphonyl fluoride (PMSF), polyvinylidenefluoride (PVDF) membranes, $\beta$-actin primary antibody (mouse monoclonal anti-$\beta$-actin IgG, A3853), were also purchased from the same chemical company. The melatonin antiserum (sheep antimelanin antibody, AB/S/021) and tritiated melatonin (O-methyl-$^3$H melatonin, specific activity 84.0 Ci/mmol) were purchased from Stockgrand Ltd., UK, and GE Healthcare Life Sciences, UK, respectively. The primary antibody specific for AANAT [rabbit polyclonal anti-AANAT immunoglobulin G (IgG), AB5467] was supplied by Millipore Corporation, USA. The alkaline phosphatase- conjugated secondary antibodies and BCIP/ NBT (5-bromo-4-chloro-3 indole phosphate/nitro blue tetrazolium) were purchased from Bangalore Geneti Pvt. Ltd. (Bangalore, India). All other chemicals and reagents of the highest available purity grade were purchased from Sigma-Aldrich Chemical Co. (USA).

**Collection and Care of Experimental Animals**

Juvenile carp *Catla catla* (Cyprinidae, Cypriniformes), weighing in between 250-300 g, were captured by local fishermen using large dragnets from the water bodies in and around Santiniketan (Lat. 23° 39’N, Long. 87° 42’E) in India and quickly transported to the laboratory, where they were held in a large open air cement tank, measuring ~ 4m (L) x 2m (W) x 1m (D) to make them acclimatized for about a week under ambient 24 h light-dark cycle. All the fish were fed with artificially prepared balanced diet food comprising 35% fish meal, 28% mustard oil cake, 28% rice bran, 2% each sunflower and cod liver oils, 5% carboxy methyl cellulose, and multivitamin tablets (“Becoyme Forte” Glaxo India Ltd., 25 tabs/kg foods) as employed elsewhere [5,6]. In order to maintain hygienic conditions throughout the investigation, water was replaced regularly to remove unwanted food wastes and faecal matters, after which adequate amount of food (200 g/tank/d equivalent to 6% body weight) was scattered in the tank once daily in the morning (10:00 h). Laboratory care of fish as well as adopted study schedules were in agreement with international standards [30].

**Incubation of Gut Tissue and Experimental Paradigm**

All the fish (n=20) were provided with the same food at the same time schedule as followed during acclimatization period. After 2 h (in between local time: 12.00 h - 13.00 h) of food supply, each fish was individually anaesthetized with 2-phenoxyethanol and then sacrificed by decapitation. Quick dissection was followed for collection and processing of gut tissue samples for in vitro study. The gut tissues corresponding to apical part of small intestine were thoroughly washed in ice cold phosphate-buffered saline (PBS) for complete removal of food/fecal matters and then transferred to

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sterilised pectri plates containing RPMI 1640 culture medium in which they were cut into small pieces (each piece weighing 25 mg). One ml of RPMI 1640 culture medium (pH 7.5) was supplemented with BSA (1 mg/ml), penicillin G (100 U/ml), streptomycin (100 µg/ml) and ascorbic acid (0.1 mg/ml). The isolated tissue samples were distributed equally in individual wells of 24-well culture plates. Each well contained 100 mg gut tissue immersed in 1 ml of RPMI 1640 culture medium. The samples were incubated at 20±1°C in presence of 95% O2 and 5% CO2 initially in the culture medium for 2 h and then with different pharmacological agents separately for 2 h. Each set replicated for 5 times. The dose of each drug, used in the present study with known specific neuro-modulatory or intracellular signal-modulatory effects, is depicted below.

**Adrenergic Neuromodulatory Drugs:**
Each of the following drugs was used separately at the dose of 10 µM [32]:

i. **Adrenergic receptor agonists**
   1. D,L-Norepinephrine hydrochloride (NE)
   2. (R)(-)-Phenylephrine hydrochloride (α1-receptor agonist; α1-Ag)
   3. Clonidine hydrochloride (α2-receptor agonist; α2-Ag)
   4. (±) Isoproterenol hydrochloride (β1-receptor agonist; β1-Ag)

ii. **Adrenergic receptor antagonists**
   1. Prazosin hydrochloride (α1-receptor antagonist; α1-Ant)
   2. Yohimbine hydrochloride (α2-receptor antagonist; α2-Ant)
   3. (±) Propanolol hydrochloride (β1-receptor antagonist; β1-Ant)

**Dopaminergic Neuromodulatory Drugs:**
Each of the following drugs was used separately at the dose of 10 µM [33]:

i. **Dopaminergic receptor agonists**
   1. Dopamine hydrochloride (DA)
   2. (±)-SKF 38393 hydrochloride (D1-receptor agonist; D1-Ag)

**Dopaminergic Receptor Antagonists**
(R)(+)-SCH23390 hydrochloride (D1-receptor antagonist; D1-Ant)

**Cholinergic Neuromodulatory Drugs:**
Each of the following drugs was used separately at the dose of 100 µM [34]:

i. **Cholinergic Receptor Agonists**
   1. Acetylcholine chloride (Ach)
   2. (±)-Nicotine (nicotinic receptor agonist; Nic-Ag)
   3. Oxotremorine M (muscarinic receptor agonist; Mus-Ag)

ii. **Cholinergic receptor antagonists**
   1. (±)-Tubocurarine chloride hydrate (nicotinic receptor antagonist; Nic-Ag)

2. Atropine (muscarinic receptor antagonist; Mus-Ant)

**Intracellular cAMP Regulatory Agents:**

i. Adenosine 3’5’-cyclic monophosphate (cAMP) – 1 mM [35]
ii. Adenylate cyclase activator: Forskolin (Fsk) – 100 µM [35]
iii. Adenylate cyclase inhibitor: 9-Cyclopentyladenine (9CPA) – 100 µM [36]

The employed dose of each drug was the same as used erstwhile for in vitro study on isolated carp pineal tissues [15,37].

**Collection and Preparation of Samples**
Gut tissues from each incubation medium were collected in separate micro-centrifuge tubes and washed thrice with ice cold extraction buffer (EB; 80 mM sodium β-glycerophosphate, 20 mM HEPES, 15 mM MgCl2, 5 mM EGTA, 0.5 mM PMSE, 1 mM DTT, leupeptin, 10 µg/ml aprotinin, pH 7.5). The washed gut tissues were separately used to prepare 10% tissue homogenate following homogenization at 4°C in a buffer containing 100 mM Tris–HCl buffer, pH 7.4, 250 mM sucrose, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1% leupeptin hemisulphate. After centrifugation, clear supernatant of each tissue homogenate was stored at -80°C until used further for analytical studies.

**Measurement of Gut Melatonin Concentrations**
The supernatant (500 µl in duplicate) of each gut tissue homogenate from individual fish was collected and extracted separately with chloroform (2 ml) following the method described elsewhere [5,6,38]. Each sample containing the extracted residue was re-suspended in 300 µl of PBS–gelatin buffer and a specific well-calibrated radioimmunoassay technique employing [1H] melatonin (specific activity 84.0 Ci/mmol) as the tracer (100 µl equivalent to ~4000 cpm) and melatonin antiserum (sheep anti-melatonin antibody) at a final dilution of 1:4000 [39] was used to measure the concentrations of melatonin. The assay was validated by both parallelism tests and recovery tests. The parallel displacement curve was studied between the serial dilutions (2.5-500 pg/ml) of melatonin standard and the serial dilutions of carp gut homogenate samples (containing 33.0 pg/300 µl of re-suspended homogenate). Moreover, to check the assay accuracy, prior to extraction the gut homogenates (with known concentration of 33.0 pg/300 µl melatonin) were spiked with increasing quantities (5.0- or, 10.0- or, 25.0- or, -50.0 pg) of pure standard melatonin. Gut melatonin concentration was expressed as pg/g of tissue and the recovery rate varied between 96 and 98%. The sensitivity of assay was 10 pg/g gut tissues, and the intra- and inter-assay co-efficient of variations for concentrations of gut melatonin were 6.3 and 8.5% respectively.

**Electrophoresis and Immunoblot Analysis of AANAT Protein in Gut**
Gut tissue homogenates were subjected to Western blot analysis of the AANAT protein [5]. Pre-absorption test was performed to determine the specificity of AANAT antibody. Briefly, each diluted primary antibody was incubated with the immuno-reactive antigen.
(10 µg/ml) and pre-absorbed for 12 h at 4°C before incubation of the immunoblot [39]. Relative abundance of the AANAT protein on each blot was normalized by the intensity of β-actin (internal control) and further quantified by densitometric analysis using ImageJ software [5,38], and expressed in relative densitometric units (ratio of the band intensities of AANAT to β-actin for individual gut sample).

Statistical Analysis
Mean ± SEM (standard error of means) of the densitometric data of AANAT immunoblot and melatonin concentration in the gut tissue homogenates were calculated and employed separately for statistical analysis. Shapiro-Wilks test was employed to determine whether the data sets were normally distributed or not. As all data sets passed the normality test (p< 0.01), analysis of variance (ANOVA) was followed. One-way ANOVA was employed for analysis of the data on each variable in between different treatment groups. When F values indicated significance, means were compared by a post-hoc Duncan’s multiple range tests considering p< 0.05 as threshold of significance [41]. Statistical analysis and data presentation were carried out using SPSS and GraphPad Prism 6.03 software.

Results
Effects of Adrenergic Neuromodulatory Drugs on Gut Levels of AANAT and Melatonin
Incubation of gut tissues with nor-epinephrine (NE), or agonists of its α₁ and β₁ receptors resulted in significant increase in the band intensity of AANAT (F= 26.20; p<0.001) and the concentration of melatonin (F= 44.50; p<0.001) relative to the values of respective variables in the samples incubated with antagonists of α₁ (α₁-Ant) and β₁ (β₁-Ant) receptors. In presence of agonist or antagonist of α₁ receptor, the band intensity of AANAT and also the gut melatonin level remained almost identical to that noted in the control sample (Fig. 1).

Effects of Dopaminergic Neuromodulatory Drugs on Gut Levels of AANAT and Melatonin
Gut tissues incubated with dopamine (DA), or agonist of D₁ receptor (D₁-Ag) revealed significant increase in the band intensity of AANAT (F= 36.53; p<0.001) as well as in the level of melatonin (F= 42.04; p<0.001) compared to respective values in the sample incubated with D₁ receptor antagonist (D₁-Ant) (Fig. 2).
Effects of Cholinergic Neuromodulatory Drugs on Gut Levels of AANAT and Melatonin:

Incubation with acetylcholine (Ach), or agonists of its nicotinic (Nic-Ag), or muscarinic (Mus-Ag) receptors resulted in significant decrease in both the band intensity of AANAT (F= 40.60; p<0.001) and the level of melatonin (F= 53.00; p<0.001) in the gut compared to respective values in the gut tissues incubated with antagonist of nicotinic (Nic-Ant), or muscarinic (Mus-Ant) receptors (Fig. 3).

Effects of Intracellular cAMP Regulatory Agents on Gut Levels of AANAT and Melatonin:

The expression of AANAT (F= 33.14; p<0.001) as well as the level of melatonin (F= 46.18; p<0.001) were significantly increased in the gut tissue samples following incubation with cAMP, or adenylate cyclase activator- forskolin (Fsk), compared to those noted in the adenylate cyclase inhibitor (9CPA) treated samples (Fig. 4).

Discussion

In fish, gut melatonin concentrations undergo daily variations with a peak at midday [38]. Extensive experimental studies on gut melatonnergic system in carp held under different photoschedules revealed that the daily profiles of both melatonin and AANAT protein in the gut are not dependent on the light-dark conditions under which the fish are held [42]. Thus it is evident that daily temporal pattern of melatonnergic system in gut, unlike pineal [15,37], is not under the direct control of lighting conditions and, in all probability, non-photic cue(s) may play the role of its synchronizer. The conjecture earned support from a recent study, which provided clear indications that availability of food and feeding time may serve as most important external cue in determining daily periodicity of gut derived melatonin in carp [9]. Thus, feeding induced stimulus seemed appropriate for investigating the neuronal regulatory mechanism involved in determining profiles of AANAT and thereby melatonin in carp gut.

Role of Neuronal Signals in Determining Gut Levels of AANAT and Melatonin

The findings that the fish gut is supplied with abundant adrenergic nerves and their dense innervations [43] provided the basis of present study on the role of adrenergic neurons in regulating gut melatonnergic system in carp. The results of this study demonstrate for the first time that the expression of AANAT and the levels of melatonin in the gut tissues in any fish are significantly enhanced by NE, or agonists of its $\alpha_1$ and $\beta_1$ receptors, but decreased following application of antagonists of $\alpha_1$ and $\beta_1$ receptors. Notably, an earlier study on pike indicated that melatonin synthesis in the pineal organ was enhanced by $\beta$-adrenergic agonist (isoproterenol), but decreased by $\alpha$-adrenergic agonist (clonidine and phenylephrine) [44]. On the contrary, melatonin synthesis in the pineal organ of zebrafish was unaltered following cyclic exposure to adrenergic agonists, like NE and selective agonists of $\alpha_1$ (phenylephrine), $\alpha_2$ (clonidine) and $\beta_1$ (isoproterenol) receptors [45]. Likewise, NE had
Fig. 3. (A) Western blot of AANAT in the homogenates of carp gut tissues following incubation with either Ach, or Nic-Ag, or Nic-Ant, or Mus-Ag, or Mus-Ant, or no pharmacological agent (Con). Band intensity of the protein is compared to that of β-actin (in lower panel). (B) Histogram represents the results of the densitometric analysis of the immunoblot of AANAT in the corresponding treatment groups of gut tissues as shown in (A). (C) Diagrammatic presentation of melatonin concentration in the same control and treatment groups of gut tissues as shown in (A). Each bar and vertical line in each histogram represent mean ± SEM of five (n=5) independent observations. Different alphabets in small scripts on the bars indicate significant (p<0.001) differences between different experimental groups in the values of AANAT intensities (Fig. 3B) and melatonin levels (Fig. 3C) in gut tissues, while same alphabets in small scripts on the bars indicate no statistically significant differences between different experimental groups in the values of respective variables following one-way ANOVA and a post-hoc Duncan’s multiple range test.

Fig. 4. (A) Western blot of AANAT in the homogenates of carp gut tissues following incubation with either Fsk, or 9CPA, or cAMP, or no pharmacological agent (Con). Band intensity of the protein is compared to that of β-actin (in lower panel). (B) Histogram represents the results of the densitometric analysis of the immunoblot of AANAT in the corresponding treatment groups of gut tissues as shown in (A). (C) Diagrammatic presentation of melatonin concentration in the same control and treatment groups of gut tissues as shown in (A). Each bar and vertical line in each histogram represent mean ± SEM of five (n=5) independent observations. Different alphabets in small scripts on the bars indicate significant (p<0.001) differences between different experimental groups in the values of AANAT intensities (Fig. 4B) and melatonin levels (Fig. 4C) in gut tissues, while same alphabets in small scripts on the bars indicate no statistically significant differences between different experimental groups in the values of respective variables following one-way ANOVA and a post-hoc Duncan’s multiple range test.
no detectable effects on the intracellular cAMP level of the trout pineal organ [46,47]. The adrenoceptor agonist phenylephrine was found to act through \(\alpha\)-adrenoceptors [27,48,49]. Thus, the results of present study argue that NE in the carp gut, as in the pike pineal [44], possibly acts through \(\alpha\) and \(\beta\) receptors to trigger and stimulate the synthesis of AANAT which ultimately leads to augmented melatonin levels. An earlier in vitro study demonstrated identical effects of specific agonists and antagonists of adrenergic receptors on the carp pineal organs which were cultured under complete darkness [15]. Nonetheless, underlying molecular events in such neural signalling mechanisms involved in the regulation of melatonin synthesis in fish gut remained obscure and require further study.

Dopamine (DA) is an important neurotransmitter in many parts of the central nervous system and appears to play a role in a wide range of physiological processes. It represents the major catecholamine in teleostean pineal organ with relatively higher levels than those for norepinephrine (NE) and epinephrine (E). The results of present study depict that incubation of gut tissues with DA or its \(D_1\)-agonist leads to significant increase in the levels of AANAT as well as melatonin, while use of antagonist of \(D_1\)-receptor exerts an inhibitory effect on the gut melatoninergic system indicating an important role of \(D_1\) receptor in determining melatonin levels in the carp gut. The study on zebrafish showed that melatonin production in the pineal organ was partially decreased when treated with quinpirole, an agonist of \(D_1\) like dopamine receptors, but remained unaffected following application of either DA or SKF 38393, an agonist of \(D_2\) receptors [45] in the culture medium. Incubation of carp pineal organ with DA or \(D_1\)-Ag also triggered the melatoninergic system, while \(D_1\)-Ant restricted such response [15]. Thus, it seems possible that DA acts through its \(D_1\) receptor to regulate the synthesis of AANAT and melatonin in the gut tissues as well as in the pineal organ of carp. However, further in vitro studies would be required to provide more insight into the intracellular signalling mechanisms involved in catecholamines (such as NE and DA)–mediated postprandial neuronal regulation of the levels of melatonin and its key regulatory protein AANAT in the carp gut. DA is reported to be involved in the regulation of cAMP levels in the gut of fish [46,50].

Acetylcholine (Ach) is the neurotransmitter which is triggered by cholinergic neurons to perform actions through both nicotinic and muscarinic receptors [51]. These muscarinic and nicotinic Ach receptors are well recognized in the gastrointestinal tract of fish [24,52]. Administration of Ach endorsed gut mobility by stimulating excitatory synaptic transmission through the nicotinic cholinergic receptors [53]. Prior to this carp study, there was no report on the role of acetylcholine in the regulation of AANAT and melatonin synthesis in the gut of any animal. Current study demonstrates that application of Ach or Nic-Ag or Mus-Ag significantly inhibits expression of AANAT and reduces the level of melatonin in respective gut tissues. On the other hand, relative abundance of AANAT protein and melatonin levels in the gut tissue samples are increased when specific Ach receptor antagonists are used in the culture medium. Thus it seems logical to argue that stimulation of the muscarinic cholinergic receptors could result in a decrease in melatonin synthesis by inhibiting the norepinephrine output from the sympathetic nerve fibres [16]. Additionally, acetylcholine by binding with its nicotinic receptors may lead to release of an inhibitory neurotransmitter glutamate to inhibit transcriptional activation of AANAT and melatonin synthesis [54]. These findings essentially argue in favour an involvement of cholinergic neurons during postprandial signalling in gut. More specifically, cholinergic neurons seem to play an inhibitory role through its muscarinic as well as nicotinic receptors to regulate the synthesis of AANAT and thereby melatonin titres in the gut. Taken together, present study provides the first experimental data to suggest a stimulatory role of catecholaminergic (both adrenergic and dopaminergic) system, and an inhibitory role of cholinergic signal on postprandial synthesis of melatonin and its rate limiting enzyme AANAT in the carp gut.

**Role of Intracellular cAMP in the Regulation of Melatoninergic System in the Carp Gut**

The studies on isolated pineal organ documented clearly that \(\beta_1\) adrenergic receptors and \(D_1\) dopaminergic receptors are closely associated with the activity of adenylyl cyclase to trigger the intracellular level of cAMP [34,55]. Selective stimulation of \(\beta_1\) adrenergic receptors and \(D_1\) dopaminergic receptors in cultured bovine pinealocytes resulted in an increased synthesis of AANAT and enhanced melatonin concentrations through elevation of intracellular cAMP [33,34,56]. It is generally agreed that cAMP, primarily by acting at the level of transcription or translation or post-translation, stimulate the synthesis/activity of AANAT in the pineal organ. cAMP response element may regulate the expression of AANAT gene [57]. Likewise, occurrence of cAMP dependent phosphorylation of transcription factors suggests in favour of E-box (an enhancer element present in AANAT mRNA promoter) driven increase of AANAT mRNA and protein. cAMP dependent protein kinase A is also known to phosphorylate AANAT at highly conserved N- and C-terminal phosphorylation sites. As a result of stabilization of AANAT to an active state through an interaction with 14-3-3 proteins and protecting it from proteosomal proteolysis, ultimately melatonin synthesis is increased [20,29]. cAMP is also known to increase the concentration of intracellular acetyl CoA to result in an elevation of intracellular acetylation of 5-methoxytryptamine (5-MT) to melatonin [58]. However, prior to this study, there was no report on the functional role of cAMP in mediating neuronal signals in the regulation of AANAT and melatonin profiles of gastrointestinal tract in any animal. Thus the results of currently undertaken in vitro study support the contention that cAMP may serve as an important component of the regulatory mechanism in the expression of AANAT and finally synthesis of melatonin in the carp gut. Notably, incubation of carp gut tissues with cAMP, or adenylyl cyclase activator- forskolin resulted in significant increase in both the expression of AANAT and the level of melatonin. These observations are consistent with the earlier studies on the pinealocytes of pike and trout, where the cAMP production and AANAT activity were enhanced several times.
folds over control values after application of forskolin [31,35,58]. Moreover, our study on cultured gut tissues reveals that incubation with cAMP or, adenylylcyclase activator forskolin mimicked stimulatory response of NE, or β1-Ag, or DA, or D1-Ag. These stimulatory effects are antagonized by adenylylcyclase inhibitor (9CPA), while it imitated the inhibitory influence of Ach, or Mus-Ag on the levels of AANAT and melatonin in the gut tissues of carp.

Conclusion

The information gathered from the current investigation collectively promoted the idea that postprandial neuronal response of the melatonin synthesizing cells in the carp gut might be initiated by binding of NE with α1 and β2 adrenergic receptors and DA to D1 dopaminergic receptors. Activation of α1 adrenergic receptors could be associated with the activation of β2 adrenergic and D1 dopaminergic receptors, which by activation of adenylylcyclase could enhance the intracellular cAMP level to trigger the synthesis of AANAT leading to enhanced synthesis of melatonin. This is also not unlikely that Ach stimulated nicotinic and muscarinic receptors play a role in decreasing intracellular cAMP level by an inhibition of adenylylcyclase. The suppressed level of cAMP in turn could inhibit the expression of AANAT and production of melatonin in the gut tissues. Role of muscarinic and nicotinic cholinergic receptors in the regulation of cAMP and adenylylcyclase were well characterized in earlier study on cultured pineal organ of the same fish species [15]. Collectively, the results of this study present hitherto unknown information on the role of cAMP in the neuronal signal-mediated regulation of feeding induced synthesis of AANAT and melatonin in the carp gut. Nonetheless, further studies would be required to elucidate the cascade of intracellular events in the mechanism involved in the expression of AANAT genes for the synthesis of melatonin in the gut. Determination of possible role of L-type Ca2+ channels in mediating such neuronal signal would be an interesting area of future research.

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References

1. Chowdhury I, Maitra SK (2012) Melatonin in the promotion of health. In: Watson RR (ed) Melatonin time line: from discovery to therapy. Taylor and Francis Boca Raton FL 1-60.

2. Huether G (1993) The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. Experientia 49: 665-670.

3. Kvetnoy IM, Ingel IE, Kvetnaia TV, Malinovskaya NK, et al. (2002) Gastrointestinal melatonin: cellular identification and biological role. Neuro Endocrinol Lett 23: 121-132.

4. Mukherjee S, Maitra SK (2015) Gut melatonin in vertebrates: chronobiology and physiology. Front Endocrinol 6: 112. doi: 10.3389/fendo.2015.00112.

5. Pal PK, Hasan NK, Maitra SK (2016) Gut melatonin response to microbial infection in carp Catla catla. Fish Physiol Biochem 42: 579-592.

6. Pal PK, Hasan NK, Maitra SK (2016) Temporal relationship between the daily profiles of gut melatonin, oxidative status and major digestive enzymes in carp Catla catla. Biol Rhythm Res 47: 755-771.

7. Pinillos ML, De Pedro N, Alonso-Gómez AL, Alonso-Bedate M, Delgado MJ, et al. (2001) Food intake inhibition by melatonin in goldfish (Carassius auratus). Physiol Behav 72: 629-634.

8. Vera LM, De Pedro N, Gómez-Milán E, Delgado MJ , Sánchez-Muros MJ, et al. (2007) Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiol Behav 90: 518-524.

9. Mukherjee S, Maitra SK (2015) Effects of starvation, re-feeding and timing of food supply on daily rhythm features of gut melatonin in carp (Catla catla). Chronobio Int 32: 1264-1277.

10. Bubenik GA (2002) Gastrointestinal melatonin: localization, function, and clinical relevance. Dig Dis Sci 47: 2336-2348.

11. Bubenik GA (2008) Thirty four years since the discovery of gastrointestinal melatonin. J Physiol Pharmacol 2: 33-51.

12. Li Y, Wu XY, Owyang C (2004) Serotonin and cholecystokinin synergistically stimulate rat vagal primary afferent neurones. J Physiol 559: 651-662.

13. Kirkup AJ, Brunsden AM, Grundy D (2001) Receptors and transmission in the brain-gut axis: Potential for novel therapies. I. Receptors on visceral afferents. Am J Physiol Gastrointest Liver Physiol 280: G787-G794.

14. Olsson C (2011) Gut anatomy and morphology. In: Farrell AP (ed) Encyclopedia of fish physiology: From genome to environment. Academic Press, San Diego.

15. Seth M, Maitra SK (2011) Neural regulation of dark-induced abundance of arylalkylamine-N-acetyltransferase (AANAT) and melatonin in the carp (Catla catla) pineal: an in vitro study. Chronobio Int 28: 572-585.

16. Drijfhout WA, van der Linde S, Kooi C, Grol B, Westerink BHC, et al. (1996) Norepinephrine release in the rat pineal gland: the input from the biological clock measured by in vivo microdialysis. J Neurochem 66: 748-755.

17. Simonneaux V, Rodeau JL, Calgari C, Pevet P (1999) Neuropeptide Y increases intracellular calcium in rat pinealocytes. Eur J Pharmacol 11: 725-728.

18. Borjigin J, Wang MM, Snyder SH (1995) Diurnal variation in mRNA encoding serotonin N-acetyltransferase in pineal gland. Nature 378: 783-785.

19. Ganguly S, Gastel JA, Weller JL, Schwartz C, Jaffe H, et al. (2001) Role of a pineal cAMP-operated arylalkylamine N-acetyltransferase/14-3-3-binding switch in melatonin synthesis. Proc Natl Acad Sci USA 98: 8083-8088.

20. Ganguly S, Coon SL, Klein DC (2002) Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. Cell Tissue Res 309: 127-137.
21. Martin C, Meissl H (1992) Effects of dopaminergic and noradrenergic mechanisms on the neuronal activity of the isolated pineal organ of the trout, *Oncorhynchus mykiss*. J Neural Transm (Gen Sect) 88: 37-51.

22. Brandstatter R, Hermann A (1996) Modulation of ganglion cell activity in the pineal gland of the rainbow trout: Effects of cholinergic, catecholaminergic, and GABAAergic receptor agonists. J Pineal Res 21: 59-72.

23. Ekström P, Meissl H (1997) The pineal organ of teleost fishes. Rev Fish Biol Fisheries 7: 199-284.

24. Stevenson SV, Grove DJ (1977) The extrinsic innervations of the stomach of the plaice, *Pleuronectes platessa* L. I. The vagal nerve supply. Comp Biochem Physiol 58: 143-151.

25. Kitazawa T, Temma K, Kondo H (1986) Presynaptic alpha-adrenoceptor mediated response to catecholamines in smooth muscle strips isolated from rainbow trout stomach (Salmogaiderni). B J Pharmacol 89: 259-266.

26. Sjöblom M, Flemström G (2004) Central nervous alpha1-adrenoceptor stimulation induces duodenal luminal release of melatonin. J Pineal Res 36: 103-108.

27. Flemström G, Sjöblom M (2005) Epithelial cells and their neighbors. II. New perspectives on efferent signaling between brain, neuroendocrine cells, and gut epithelial cells. Am J Physiol Gastrointest Liver Physiol 289: G377-G380.

28. Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, et al. (1997) The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res 52: 307-357.

29. Iuvone PM, Tosini G, Pozdeyev N, Haque R, Klein DC, et al. (2005) Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. Prog Retin Eye Res 24: 433-456.

30. Portaluppi F, Smolensky MH, Toutou Y (2010) Ethics and methods for biological rhythm research on animals and human beings. Chronobiol Int 27: 1911-1929.

31. Falcón J, Galarneau KM, Weller JL, Ron B, Chen G, et al. (2001) Regulation of melatonin production by pineal photoreceptor proteins in carp *Catla catla*. J Neurochem 114: 1049-1062.

32. Mukherjee S, Moniruzzaman M, Maitra SK (2014) Daily and seasonal profiles of gut melatonin and their temporal relationship with pineal and serum melatonin in carp *Catla catla* under natural photo-thermal conditions. Biol Rhythm Res 45: 301-315.

33. Maitra SK, Chattoraj A, Bhattacharyya S (2005) Implication of melatonin in oocyte maturation in Indian major carp *Catla catla*. Fish Physiol Biochem 31: 201-207.

34. Huang H, Lee SC, Yang XL (2005) Modulation by melatonin of glutamatergic synaptic transmission in the carp retina. J Physiol 569: 857-871.

35. Zar JH (1999) Biostatistical analysis. (4th edn) Upper Saddle River (NJ), Prentice Hall.

36. Mukherjee S, Moniruzzaman M, Maitra SK (2014) Impact of artificial lighting conditions on the diurnal profiles of gut melatonin in a surface dwelling carp (*Catla catla*). Biol Rhythm Res 45: 831-841.

37. Seth M, Maitra (2010) Neuronal regulation of photo-induced pineal photoreceptor proteins in carp *Catla catla*. J Neurochem 114: 1049-1062.
53. Schneider DA, Galligan JJ (2000) Nicotinic acetylcholine receptors on nerve terminals releasing slow synaptic transmitters in the myenteric plexus of guinea pig ileum. Am J Physiol 279: G528-G535.

54. Hernandez SC, Vicini S, Xiao Y, Davila-Garcia MI, Yasuda RP, et al. (2004) The nicotinic receptor in the rat pineal gland is an α3β4 subtype. Mol Pharmacol 66: 978-987.

55. Simonneaux V, Ribelayga C (2003) Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev 55: 325-395.

56. Santanavanich C, Chetsawang B, Ebadi M, Govitrapong P (2003) Effects of D1- and D2- dopamine receptor activation on melatonin synthesis in bovine pinealocytes. J Pineal Res 35: 169-176.

57. Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, et al. (1997) Avian melatonin synthesis: photic and circadian regulation of serotonin N-acetyltransferase mRNA in the chicken pineal gland and retina. J Neurochem 68: 213-224.

58. Coon SL, Weller JL, Korf HW, Namboodiri MAA, et al. (2000) Camp regulation of arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87). J Biol Chem 275: 24097-24107.