Differential role of cAMP, cGMP and Ca^{2+} and involvement of kinases and CBP-CREB-CRE pathway in regulation of arylalkylamine N-acetyltransferase 2 gene in the pineal organ of air-breathing catfish, *Clarias gariepinus*

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Research Article

Keywords: Aanat2 gene, Pineal organ, cAMP, cGMP, Ca^{2+} , CREB-CBP.

Posted Date: April 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-422474/v1>

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Abstract

Transcription of arylalkylamine N-acetyltransferase 2 (*aanat2*) gene leads to formation of AANAT2 - the rate-limiting enzyme in melatonin synthesis pathway in photosensitive fish pineal organ. However, unlike in avian and mammalian pineal gland, there is practically no information on signal transduction pathway(s) involved in regulation of *aanat2* gene transcription in the fish pineal organ. Therefore, we investigated the role of important molecular components of signalling via cAMP, cGMP, Ca²⁺ involving PKA, PKG, PKC, MeK and p38 MAP kinase as well as possible role of serine/threonine phosphatases, CREB and CBP using their specific inhibitors and/or activators in *aanat2* gene transcription in the fish pineal organ maintained under *in vitro* culture-conditions. db-cAMP and db-cGMP stimulated the expression of *aanat2* gene. db-cAMP- and cGMP-induced *aanat2* gene expression was significantly reduced in the presence of H-89 (specific inhibitor of PKA), KT5823 (specific inhibitor of PKG), chelerythrine chloride (specific inhibitor of PKC), U0126 ethanolate (specific inhibitor of MeK) and SB 202190 monohydrochloride hydrate (specific inhibitor of p38 MAP kinase). Inhibitors of PP1 and PP2A significantly increased *aanat2* gene expression as well as significantly reduced cAMP- and cGMP-induced gene transcription, while inhibitor of PP2B had no effect on *aanat2* gene expression. Inhibitors of both CREB and CBP-CREB interaction completely blocked cAMP-induced *aanat2* gene transcription. Based on these findings, we suggest that cAMP, cGMP and Ca²⁺ stimulate *aanat2* gene transcription via PKA, PKG and PKC, respectively. Further, protein phosphatases and CBP-CREB-CRE pathway are actively involved in regulation of on *aanat2* gene expression in the fish pineal organ.

Introduction

Arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) acts as the rate-limiting enzyme, and controls the rhythmic synthesis of melatonin in the pineal gland of vertebrates (Gupta et al. 2005; Iuvone et al. 2005; Klein et al. 2007). Unlike mammals and birds which possess a single *aanat* gene, teleosts express three isoforms of *aanat* gene, namely *aanat1a*, *aanat1b* which are primarily expressed in the retina, and *aanat2* which is specifically expressed in the pineal organ (Coon and Klein 2006; Falcón et al. 2007; Kulczykowska et al. 2017; Singh et al. 2017). In the mammalian pineal gland, activation of adrenergic receptors by norepinephrine stimulates transcription of *aanat* gene via cAMP-PKA-CREB-CRE pathway (Gupta et al. 2005; Ho and Chick 2010). In the pineal gland of rat, PKC elevates AANAT activity by increasing the protein stability and phosphorylation-mediated activation of AANAT via cAMP-independent signalling cascade that involves PKC activation through the α_1 -adrenergic receptor (Choi et al. 2004; Gupta et al. 2005). In rat pinealocytes, the effect of norepinephrine on MAPK phosphorylation is due to integration of multiple signalling mechanisms, and activation of PKA and PKG have been reported to produce opposite effects on phosphorylation of MAPK (Ho and Chick 2000). PKG is the main kinase involved in the activation of MAPK, which acts upstream of MeK in activation of MAPK, while activated PKA has been reported to inhibit MAPK (Ho and Chick 2000). Furthermore, activation of PKA effectively abolished MAPK phosphorylation stimulated by an activator of PKC or a Ca²⁺ elevating agent (Ho and Chick 2000). Thus, cAMP/PKA antagonise both the stimulatory effect of cGMP and Ca²⁺ activation on

MAPK phosphorylation. Thereby, cAMP/PKA pathway functions as a general inhibitory signal to activation of MAPK in the pineal gland of rat.

In rat pineal gland, NE-stimulated cascade mediated phosphorylation of p42/44MAPK and p38MAPK increase the rate and the amplitude of AANAT induction, respectively (Ho and Chik 2000; Chik et al. 2004; Ho et al. 2006; Ho and Chik 2010). Activation of p42/44MAPK is mediated mainly through PKG (Ho et al. 1999), whereas p38MAPK is mainly activated through PKA (Chik et al. 2004).

In mammalian pinealocytes, protein phosphatases have also been reported to control the activity of AANAT enzyme by dephosphorylating pCREB that down regulates the induction of *aanat* gene, and hence AANAT protein and melatonin synthesis (Spessert et al. 2001; Gupta et al. 2005). Dephosphorylation of pCREB by protein serine/threonine phosphatases (PSPs) is an essential mechanism for downregulating *aanat* gene expression in the rat pineal gland (Koch et al. 2003). Further, the inhibitors of serine/threonine phosphatases (calyculin A, microcystin-LR and okadaic acid) have been reported to attenuate dephosphorylation of pCREB, and to block the decline in *aanat* mRNA levels (Koch et al. 2003). Characterization of (PSPs) by immunocytochemistry and immunoblotting showed accumulation of PSP1-catalytic subunit (CSU) in pinealocyte nuclei by NE-stimulation for 8 hr without affecting the distribution of PSP2A-CSU (Koch et al. 2003). cAMP-dependent protein kinase has been reported to phosphorylate cAMP-responsive element (CRE)-binding protein (CREB) in rat pineal gland (Maronde et al. 1997). cAMP-dependent phosphorylation of CREB in trout pineal organ has been reported to be accompanied by increased melatonin synthesis (Kroeber et al. 2000). CREB binding protein (CBP) is also reportedly involved in *aanat* gene expression in mammalian pineal gland (Davies et al. 2011). Further, cAMP-induced activation of *aanat* gene requires a CRE.CCAAT complex, and a CRE and an inverted CCAAT box have been reported in the rat pineal (Baler et al. 1997). Thus, cAMP induces *aanat* gene via PKA-CREB.CBP-CRE pathway in the rat pineal (Gupta et al. 2005).

In the fish pineal organ, AANAT activity is reportedly regulated at the transcriptional level as well as at the post-translational level (Falcón et al. 1998, 2001; Zilverman-Peled et al. 2007; Saha et al. 2019). A prominent circadian rhythm has been reported in *aanat2* gene expression in the photosensitive pineal organ of gilthead seabream and *Clarias gariepinus* with maximum and minimum *aanat2* transcript levels during dark and light phase, respectively (Zilverman-Peled et al. 2007; Singh et al. 2017). However, there is practically no information on the role of molecular components of signalling pathway(s) involved in the transcription of *aanat2* gene in the pineal organ of any fish species. Therefore, we investigated the role of major second messengers (cAMP, cGMP and Ca^{2+}), protein kinases (PKA, PKG and PKC, MeK and MAPK), PSPs (PP1, PP2A and PP2B), and CBP-CREB complex in transcription of *aanat2* gene in the pineal organ of the catfish, *Clarias gariepinus*. Our findings suggest differential role of cyclic nucleotides, Ca^{2+} and protein kinases and involvement of CBP-CREB complex in *aanat2* gene transcription.

Materials And Methods

Ethics statement

All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals after obtaining institutional ethical clearance from the Institutional Ethics Committee, North-Eastern Hill University, Shillong, India.

Experimental Animals And Their Maintenance

All experiments were conducted on the pineal organ of sexually mature male *Clarias gariepinus*, which is readily available throughout the year in Shillong. For all experiments, *Clarias gariepinus* (length: 23–27 cm; body weight: 95–100 g) were procured from local fish suppliers, and acclimatized for 15 days in a fish room under 12 h light: 12 h dark cycle (lights 'on' at 06:00 h, lights 'off' at 18:00 h) and water temperature $25 \pm 2^\circ\text{C}$ at Shillong (Latitude $25^\circ30'$ N, Longitude $91^\circ52'$ E; Altitude 1450 ASL). During acclimatization, the fish were fed with commercial dry fish pellets and minced earthworms *ad libitum*. Water was changed every day to avoid infections.

Pineal organ culture and *in vitro* treatments

In order to collect the pineal organ, the acclimatized fish was anesthetized with MS-222 (Sigma-Aldrich, USA), decapitated, the pineal window was exposed, the pineal organ was rapidly removed, washed and placed in the culture medium in a multi-wells culture plate (Nunc™ Delta Surface, Thermo Scientific, USA). All dissections were carried out between 10:30 h – 11:30 h. Pineal organs were collected and pooled into three samples of three pineals each (9 in total), and were used for estimating the relative expression of *aanat2* gene. Sterile Dulbecco's Modified Eagle Medium (DMEM; Gibco Thermo Fisher Scientific, USA) supplemented with Bovine Serum Albumin (BSA, 1mg/ml), Ascorbic acid (0.1 mg/ml), Penicillin-Streptomycin (10 µl/ml), HEPES salt (4.77 mg/ml, Sigma, USA), and calcium carbonate (0.125 mg/ml, HIMEDIA, India) were used for the pineal culture. Pineals were pre-incubated at $25 \pm 2^\circ\text{C}$ for 1 h in an atmosphere of 85% O₂, 5% CO₂ and 95% relative humidity in a gas (O₂-CO₂) incubator (Heraeus: Cytoperm, Germany).

***In vitro* treatment of the pineal organ with cAMP, cGMP and selective inhibitors:**

In order to study the role of cAMP-dependent protein kinase (PKA) in *aanat2* gene regulation, 36 pineal organs were divided into 12 samples of 3 pineal organ each, then 12 samples were divided into 4 treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), H-89 (10^{-6} M; selective inhibitor of db-cAMP-dependent protein kinase), H-89 (10^{-6} M) + db-cAMP (10^{-6} M) with a suitable control group.

In order to study the role of cGMP-dependent protein kinase (PKG) in *aanat2* gene regulation, 36 pineal organs were divided into 12 samples of 3 pineal organs each, then 12 samples were divided into 4 treatment groups of 3 samples each, and treated with db-cGMP (10^{-6} M), KT5823 (10^{-6} M; selective inhibitor of cGMP-dependent protein kinase), KT5823 (10^{-6} M) + cGMP (10^{-6} M) with a suitable control group.

In order to study the role of calcium-dependent protein kinase (PKC) in *aanat2* gene regulation, 72 pineal organs were divided into 24 samples of 3 pineal organs each, then 24 samples were divided into 8

treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), db-cGMP (10^{-6} M), db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M), chelerythrine chloride (Che, 10^{-6} M; specific inhibitor of calcium-dependent protein kinase), Che (10^{-6} M) + db-cAMP (10^{-6} M), Che (10^{-6} M) + db-cGMP (10^{-6} M), and Che (10^{-6} M) + db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M) with a suitable control group.

In order to study the role of MeK in *aanat2* gene regulation, 72 pineal organs were divided into 24 samples of 3 pineal organs each, then 24 samples were divided into 8 treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), db-cGMP (10^{-6} M), db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M), U0126 ethanolate (UE, 10^{-6} M; specific inhibitor of MeK), UE (10^{-6} M) + db-cAMP (10^{-6} M), UE (10^{-6} M) + db-cGMP (10^{-6} M), and UE (10^{-6} M) + db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M) with a suitable control group.

In order to study the role of p38 MAP kinase (p38 MAPK) in *aanat2* gene regulation, 72 pineal organs were divided into 24 samples of 3 pineal organs each, then 24 samples were divided into 8 treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), db-cGMP (10^{-6} M), db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M), SB 202190 monohydrochloride hydrate (SB, 10^{-6} M; specific inhibitor of p38 MAP kinase), SB (10^{-6} M) + db-cAMP (10^{-6} M), SB (10^{-6} M) + db-cGMP (10^{-6} M), and SB (10^{-6} M) + db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M) with a suitable control group.

In order to study the role of serine/threonine phosphatase type 1 and 2A in *aanat2* gene regulation, 72 pineal organs were divided into 24 samples of 3 pineal organs each, then 24 samples were divided into 8 treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), db-cGMP (10^{-6} M), db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M), calyculin A (CA, 10^{-6} M; specific inhibitor of serine/threonine phosphatase type 1 and type 2A), CA (10^{-6} M) + db-cAMP (10^{-6} M), CA (10^{-6} M) + db-cGMP (10^{-6} M), and CA (10^{-6} M) + db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M) with a suitable control group.

In order to study the role of serine/threonine phosphatase type 2B in *aanat2* gene regulation, 72 pineal organs were divided into 24 samples of 3 pineal organs each, then 24 samples were divided into 8 treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), db-cGMP (10^{-6} M), db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M), cypermethrin (Cyp, 10^{-6} M; specific inhibitor of serine/threonine phosphatase type 2B), Cyp (10^{-6} M) + db-cAMP (10^{-6} M), Cyp (10^{-6} M) + db-cGMP (10^{-6} M), and Cyp (10^{-6} M) + db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M) with a suitable control group.

In order to study the role of CREB and CBP-CREB interaction in *aanat2* gene regulation, 54 pineal organs were divided into 18 samples of 3 pineal organs each, and then 18 samples were divided into 6 treatment groups of 3 samples each, and treated with db-cAMP (10^{-6} M), CREB inhibitor (CI, 10^{-6} M), CBP-CREB interaction inhibitor (CCII, 10^{-6} M), CI (10^{-6} M) + db-cAMP (10^{-6} M), and CC11 (10^{-6} M) + db-cAMP (10^{-6} M) with a suitable control group.

In all experiments, after pre-incubation the culture medium was replaced with sterile culture medium containing H-89 dihydrochloride hydrate (selective inhibitor of cAMP-dependent protein kinase, 10^{-6} M, Sigma, USA), KT5823 (specific inhibitor of cGMP-dependent protein kinase, 10^{-6} M, Sigma, USA), chelerythrine chloride (specific inhibitor of calcium-dependent protein kinase, 10^{-6} M, Sigma, USA), U0126 ethanolate (MeK inhibitor, 10^{-6} M, Sigma, USA), SB 190202 monohydrochloride hydrate (selective inhibitor of p38 MAP kinase, 10^{-6} M) (Sigma, USA), calyculin A (specific inhibitor of serine/threonine phosphatase type 1 and 2A, 10^{-6} M, Sigma, USA), cypermethrin (specific inhibitor of serine/threonine phosphatase and 2B, 10^{-6} M, Sigma- Aldrich, USA), CREB inhibitor (10^{-6} M, 666-15-Calbiochem®; Merck, Germany), and CBP-CREB interaction inhibitor (10^{-6} M, CAS 92-78-4-Calbiochem, Calbiochem®; Merck, Germany) for 30 min. The timing of pre-treatment was kept in such a way that each group treated only with H-89, KT5823, chelerythrine chloride, cypermethrin, CREB inhibitor CBP-CREB interaction inhibitor, U0126 ethanolate, SB 190202, dibutyryl-cAMP (db-cAMP, an analogue of cAMP, 10^{-6} M) (Enzo, USA), dibutyryl-cGMP (db-cGMP, an analogue of cGMP, 10^{-6} M) (Enzo, USA), and db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M) for 30 min and treatment of pre-treated pineals with db-cAMP (10^{-6} M), db-cGMP (10^{-6} M) and db-cAMP (10^{-6} M) + db-cGMP (10^{-6} M), respectively for 30 min were kept the same. Pineal organs incubated in DMEM supplemented with BSA (1mg/ml), CaCO_3 (0.125 mg/ml), Ascorbic acid (0.1 mg/ml), Penicillin-Streptomycin (10 $\mu\text{l/ml}$) and HEPES salt (4.77 mg/ml) without any drugs were treated as control. After treatment with desired drugs for 30 min, the pineal organs were removed and collected in numbered micro-centrifuge tubes containing TRI reagent (500 μL) and processed for isolating total RNA.

Total RNA extraction, cDNA synthesis, and analysis by Quantitative Real-Time PCR

Total RNA was extracted from the pineal organs using TRI reagent (Sigma, USA) by following the manufacturer's protocol. Then quantity and quality of extracted RNA were checked by using QIAxpert (QIAGEN, Germany). In order to maintain good quality of the samples, RNA samples with a ratio of sample absorbance at 260 and 280 nm between 1.8 and 2.1 were used for all experiments. Synthesis of cDNA was performed using 300 ng of total RNA using Verso cDNA Synthesis Kit (Thermo Scientific, USA) in a total reaction volume of 20 μL containing 4 μL of 5X cDNA synthesis buffer (1X final concentration), 2 μL of dNTP mix (500 μM each), 1 μL of anchored oligo-dt (500 ng/ μL), 1 μL of RT Enhancer, 1 μL of Verso Enzyme mix, template RNA and nuclease free water was added to make the volume of reaction mixture 20 μL and then incubated at 42°C for 60 min and then at 95°C for 2 min. qPCR was performed in triplicate reactions along with non-template control and negative control in each plate using a 7500 Fast Real-Time PCR system (Applied Biosystems, USA). The reactions were carried out in a total volume of 10 μL containing power SYBR® Green Master Mix, gene-specific primers, RNase-free water, and cDNA. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was used as the reference gene. Following primers were used:- *aanat2* gene-specific primers: CGNAT-F; 5'-ACT GGA CGA GGT GCT GAA CT-3', CGNAT-R; 5'-CGT GGA TGT GGA CAG TAG GA-3', and *gapdh* gene-specific primers: CGGAP-F; 5'-ACC GGA GTC TTC CTC AGC A-3', CGGAP-R; 5'-TCA TGT TGG AGG GGT CGT A-3' (Singh et al. 2017). Real-Time RT-PCR was performed using the following protocol: 8 minutes at 95°C to activate the enzyme, then 40 cycles of

denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute. Relative expressions of *aanat2* mRNA of all samples were calculated using the $2^{-\Delta\Delta C_T}$ (threshold cycle) method (Livak and Schmittgen, 2001).

Statistical analysis

All data were presented as the mean of three independent experimental results $\pm$ standard error of the mean. The data for *aanat2* gene expression was analysed using One-way ANOVA followed by Tukey's Multiple Comparison test. A $p < 0.05$ was considered as significant for checking the levels of significance between control and treated groups or between treated groups. All the data were analysed by using GraphPad Prism V4.03.

Results

***In vitro* effect of H-89 (specific inhibitor of PKA) on db-cAMP- induced *aanat2* gene expression**

The data are presented in [Fig. 1 (a)]. *In vitro* treatment of the pineal organ with db-cAMP significantly increased *aanat2* transcript levels ($F_{3,11} = 268.8$, $p < 0.001$). *In vitro* pre-treatment of the pineal organ with H-89 had no significant effect on the transcript levels of *aanat2* gene, but the pre-treatment of the pineal organ with H-89 significantly reduced the db-cAMP-induced increase in *aanat2* transcript levels ($F_{3,11} = 268.8$, $p < 0.001$) suggesting a role of PKA in *aanat2* gene expression [Fig. 1 (a)].

***In vitro* effect of KT5823 (specific inhibitor of PKG) on db-cGMP-induced *aanat2* gene expression**

The data are presented in [Fig. 1 (b)]. *In vitro* treatment of the pineal organ with db-cGMP significantly increased the transcript levels of *aanat2* gene ($F_{3,11} = 15.09$, $p < 0.001$). *In vitro* pre-treatment of the pineal organ with KT5823 had no effect on transcript levels of *aanat2* gene, but the pre-treatment of the pineal organ with KT5823 significantly reduced the stimulatory effect of db-cGMP on *aanat2* transcript levels ($F_{3,11} = 15.09$, $p < 0.01$) [Fig. 1 (b)].

***In vitro* effect of chelerythrine chloride (specific inhibitor of PKC) on cyclic nucleotide-induced *aanat2* gene expression**

The data are presented in [Fig. 1 (c)]. *In vitro* treatment of the pineal organ with db-cAMP and cGMP significantly increased the transcript levels of *aanat2* gene ($F_{7,23} = 29.85$, $p < 0.001$). *In vitro* pre-treatment of the pineal organ with chelerythrine chloride had no effect on *aanat2* transcript levels, but completely blocked the stimulatory effect of both cAMP and cGMP, separately as well as in combination on *aanat2* transcript levels [Fig. 1 (c)].

***In vitro* effect of U0126 ethanolate (specific inhibitor of Mek) on cyclic nucleotide-induced *aanat2* gene expression**

The data are presented in [Fig. 1 (d)]. *Aanat2* transcript levels were significantly increased following *in vitro* treatment of the pineal organ with db-cAMP ($F_{7,23} = 12.32$, $p < 0.001$) and db-cGMP ($F_{7,23} = 12.32$, $p < 0.001$). *In vitro* treatment of the pineal organ with U0126 ethanolate had no effect on *aanat2* transcript levels. But the pre-treatment of the pineal organ with U0126 ethanolate completely blocked the stimulatory effect of db-cAMP ($F_{7,23} = 12.32$, $p < 0.001$) and db-cGMP on *aanat2* transcript levels when administered separately, but resulted in significant increase in the *aanat2* transcript levels by the combined treatment with db-cAMP + db-cGMP ($F_{7,23} = 12.32$, $p < 0.05$) (Fig. 1d).

***In vitro* effect of SB 202190 monohydrochloride hydrate (specific inhibitor of p38 MAPK) on cyclic nucleotide-induced *aanat2* gene expression**

The data are presented in [Fig. 1 (e)]. *In vitro* treatment of the pineal organ with SB 202190 monohydrochloride hydrate had no effect on *aanat2* transcript levels. Pre-incubation of the pineal organ with SB 202190 monohydrochloride hydrate completely blocked the stimulatory effect of db-cAMP ($F_{7,23} = 18.93$, $p < 0.001$), db-cGMP ($F_{7,23} = 18.93$, $p < 0.001$) as well as combined effect of db-cAMP and db-cGMP on *aanat2* gene expression [(Fig. 1 (e))].

***In vitro* effect of calyculin A (specific inhibitor of PSPs 1 and 2A) and cypermethrine (specific inhibitor of PSP 2B) on cyclic nucleotide-induced *aanat2* gene expression**

The data are presented in [Fig. 2 (a) & (b)]. Transcript levels of *aanat2* gene were significantly increased following *in vitro* treatment of the pineal organ with db-cAMP ($F_{7,23} = 44.03$, $p < 0.001$), db-cGMP ($F_{7,23} = 44.03$, $p < 0.001$), and calyculin A ($F_{7,23} = 44.03$, $p < 0.001$). Pre-incubation of the pineal organ with calyculin A completely blocked the stimulatory effect of db-cAMP ($F_{7,23} = 44.03$, $p < 0.001$), db-cGMP ($F_{7,23} = 44.03$, $p < 0.001$) and cAMP + db-cGMP ($F_{7,23} = 44.03$, $p < 0.001$) [Fig. 2 (a)]. Transcript levels of *aanat2* gene were significantly increased following *in vitro* treatment of the pineal organ with db-cAMP ($F_{7,23} = 29.63$, $p < 0.001$), db-cGMP ($F_{7,23} = 29.63$, $p < 0.001$), and db-cAMP + db-cGMP ($F_{7,23} = 29.63$, $p < 0.05$). Pre-incubation of the pineal organ with cypermethrin had no effect on *aanat2* transcript levels, and it also did not block the stimulatory effect of db-cAMP and db-cGMP [Fig. 2 (b)].

***In vitro* effect of CREB inhibitor and CBP-CREB interaction inhibitor on cAMP-induced *aanat2* gene expression**

The data are presented in [Fig. 2 (c)]. *In vitro* treatment of the pineal organ with CREB inhibitor (CI) and CBP-CREB interaction inhibitor (CCII) had no effect on *aanat2* transcript levels, but both CI and CCII significantly inhibited db-cAMP-induced increase in *aanat2* transcript levels ($F_{5,17} = 23.79$, $p < 0.001$) [Fig. 2 (c)].

Discussion

Expression of *aanat2* gene was significantly increased following *in vitro* treatment of the fish pineal organ with db-cAMP, and db-cAMP-induced increase in *aanat2* transcript levels was significantly decreased following pre-treatment of the pineal organ with H-89 (specific inhibitor of PKA) suggesting that the stimulatory effect of cAMP on *aanat2* gene transcription is mediated by PKA [Fig. 1 (a)]. A direct relationship has been reported between cAMP-dependent AA-NAT inducibility and the pineal PKA activity in the rat pineal (Engel et al., 2004).

Partial but significant reduction in cAMP-induced increase in *aanat2* transcript levels by H-89 seems to suggest that besides its action via PKA, cAMP might also be stimulating *aanat2* gene transcription via additional pathway(s). Similar to cAMP, *in vitro* treatment of the pineal organ with db-cGMP also significantly increased *aanat2* transcript levels suggesting direct involvement of cGMP in *aanat2* gene transcription. Further, it is important to mention that *in vitro* treatment with KT5823 (specific inhibitor of PKG) significantly increased basal *aanat2* gene transcription in the fish pineal, and completely blocked cGMP-induced transcription of *aanat2* gene in the fish pineal suggesting that cGMP stimulates *aanat2* gene transcription only via PKG [Fig. 1 (b)]. However, simultaneous treatment of the pineal organ with db-cAMP and cGMP had no effect on *aanat2* gene transcripts. In the mammalian pineal organ cAMP and cGMP produce stimulatory and inhibitory effect on *aanat* gene transcription, respectively (Gupta et al., 2005). These findings seem to suggest that, in contrast to the mammalian pineal, both cAMP and cGMP are capable in stimulating *aanat2* gene transcription alone but in the absence of each other [Fig. 1 (c)]. Thus, simultaneous activations of both cAMP- and cGMP-producing pathways in the fish pineal might be opposing the stimulatory effect of each other on *aanat2* gene transcription in the photoreceptors of the fish pineal organ. Based on our present findings, we suggest that both cAMP and cGMP generating pathways are separately involved in stimulation of *aanat2* gene transcription as simultaneous treatment of the fish pineal organ with db-cAMP + db-cGMP had no effect on *aanat2* gene transcription. There is a possibility that simultaneous increase in cAMP and cGMP levels might be activating cyclic nucleotide-dependent phosphodiesterase enzyme, which inactivates cAMP and cGMP to AMP and GMP, respectively, and switches 'off' downstream signalling for *aanat2* gene regulation (Gupta et al. 2005). These findings also suggest that the stimulatory role of cAMP and cGMP in *aanat2* gene transcription in the fish pineal organ is in contrast to the reported opposing effects of cAMP and cGMP on *aanat* gene transcription in the mammalian pineal suggesting evolution of the role of cyclic nucleotides in regulation of *aanat* gene and melatonin synthesis.

Complete inhibition of db-cAMP- and db-cGMP-induced *aanat2* gene transcription following pre-treatment of the pineal organ with chelerythrine chloride (specific inhibitor of PKC) indicates that PKC is a prerequisite for stimulatory action of both cAMP and cGMP on *aanat2* gene expression [Fig. 1 (c)]. Since increased intracellular levels of Ca^{2+} are essential for activation of PKC (Newton, 2018), both the cyclic nucleotides seem to stimulate *aanat2* gene transcription by increasing intracellular Ca^{2+} followed by PKC activation. In trout pineal organ, voltage-gated L-type calcium channels have been reported to play a major role in the regulation of intracellular Ca^{2+} in both oscillating and non-oscillating trout photoreceptors (Korf et al. 1997). Further, in the chick pineal photoreceptor cells, increase in cAMP levels

has been reported to cause mobilization of internal Ca^{2+} stores (D'Souza and Dryer 1994). Therefore, there is a possibility that cAMP stimulates *aanat2* gene transcription via both PKA and Ca^{2+} . In the mammalian pineal organ, binding of cAMP to PKA activates the enzyme, and the catalytic subunit of PKA phosphorylates CREB to pCREB that binds to CRE resulting in increased transcription of *aanat* gene (Gupta et al. 2005; Karolczak et al. 2005).

In vitro pre-treatment of the pineal organ with U0126 ethanolate (specific inhibitor of MeK) blocked the stimulatory effect of cAMP completely, and of cGMP partially but significantly on *aanat2* transcripts. Further, pre-treatment of the fish pineal organ with SB 202190 (specific inhibitor of p38 MAPK) completely blocked the stimulatory effect of both the cyclic nucleotides on the gene transcription. These findings seem to suggest that while MeK differentially mediates the stimulatory effect of cAMP- and cGMP-induced pathways on *aanat2* gene expression, normal MAPK activity is essential for the stimulatory effect of both cAMP and cGMP on *aanat2* gene transcription [Figs. 1 (d) and (e)]. In the rat pineal gland, cAMP, PKA and intracellular Ca^{2+} signalling pathways have been reported to be involved in norepinephrine-induced phosphorylation of p38 MAPK (Chick et al. 2004; Gupta et al. 2005). In the rat pinealocytes, MAPK phosphorylation has been reported to integrate PKA- and PKG-mediated two separate signalling pathways, which have an opposite effect on MAPK phosphorylation (Ho and Chik 2000). It has also been reported in the rat pineal gland that p38 MAPK (but not p42/44MAPK) activation is tightly coupled to protein synthesis and degradation (Ho et al. 2006). Our findings also suggest that p38 MAPK plays an important role in cAMP- and cGMP-forming signalling pathways in *aanat2* gene transcription in the photoreceptive pineal organ of the fish.

Significant increase in *aanat2* gene transcription in the fish pineal organ treated with calyculin A [specific inhibitor of serine/threonine phosphatase 1 and 2A] indicate that both Ser/Thr phosphatase 1 and 2A are also involved in *aanat2* gene activation via cAMP- and cGMP-forming pathways in the fish pineal organ [Fig. 2 (b)]. Unlike calyculin A, treatment of the fish pineal organ with cypermethrin (specific inhibitor of serine/threonine phosphatase 2B) neither had any effect on *aanat2* gene transcription nor on stimulatory effect of db-cAMP and db.cGMP on *aanat2* gene transcription suggesting no involvement of serine/threonine phosphatase 2B in the fish pineal organ (Fig. 2b). It is important to mention that cAMP and calcium signals regulate cAMP-responsive element (CRE)-binding protein (CREB) target genes by stimulating the nuclear translocation of a cAMP-regulated transcriptional co-activator (CRTC). In the absence of signals, CRTC remains phosphorylated and inactive in the cytoplasm. However, both cAMP and Ca^{2+} promote CRTC dephosphorylation and activation of CRTC followed by its translocation to the nucleus, where it binds to CREB and stimulates its activity (Altarejos and Montminy 2011). In mammalian retinal photoreceptors, CRTCs (p300 and CBP) have been reported to play essential roles in maintaining photoreceptor-specific structure, function and gene expression (Hennig et al. 2013). Therefore, there is a possibility that PP-1 and PP-2A are actively involved in *aanat2* transcription by increasing dephosphorylation and activation of CRTC followed by its binding to phosphorylated CREB in the photoreceptors fish pineal organ. Besides their role in dephosphorylation of kinases, CRTC and pCREB, PSPs have also been reported to modulate sub-nuclear distribution of pre-mRNA splicing factors

(Ceulemans and Bollen 2004), to play a prominent role in the regulation of specific signal transduction cascades (Janssens and Goris 2001), and to dephosphorylate and stimulate translocation of the transcription factor NFAT, which binds DNA and stimulates gene transcription (Dodge and Scott 2003). Therefore, our findings suggest that PSPs play a complex role in regulation of *aanat2* gene in the photosensitive fish pineal organ.

Significant inhibition of the stimulatory effect of db-cAMP on the levels of *aanat2* gene transcripts in the fish pineal organ pre-treatment with CREB inhibitor (CI) and CBP-CREB interaction inhibitor (CCII) separately suggest that CREB and CREB-CBP interaction seem to be essential for stimulating *aanat2* gene expression by cAMP-mediated pathway [Fig. 2 (c)]. CREB and CBP have been reported to play a critical role in *aanat* gene transcription in the mammalian and avian pineal gland (Gupta et al. 2005; Davies et al. 2011).

Conclusions

On the basis of our findings, we suggest that *aanat2* gene expression in the photosensitive fish pineal organ is regulated by cAMP, cGMP and Ca^{2+} as second messengers via a complex intracellular signalling machinery involving cAMP-PKA-CREB/CBP-CRE, cGMP-PKG/MeK-MAPK and Ca^{2+} -PKC pathway. Further, serine/threonine phosphatases also play a complex role in up- and down regulation of *aanat2* gene expression in the fish pineal organ.

Declarations

Funding

This work was financially supported by Science and Engineering Research Board (SERB), New Delhi (No. SB/SO/AS-21/2014) and the University Grants Commission (UGC-SAP DSA-Phase II), New Delhi (No.F.4-1/2011(SAP-II)).

Conflicts of interest

The authors have declared no conflict of interest.

Ethics approval

All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals after obtaining institutional ethical clearance from the Institutional Ethics Committee, North-Eastern Hill University, Shillong, India.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material/ Data availability

All data generated or analysed during this study are included in this published article.

Code availability (software application or custom code)

Not applicable.

Authors' contributions

Dr. Braj Bansh Prasad Gupta prepared the project, planned the experiments, provided financial support and scientific infrastructure, supervised the execution of the experiments and preparation of the manuscript. Ms. Hijam Nonibala executed the experiments, collected the experimental data, analysed, plotted and interpreted the data, and prepared the manuscript.

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Figures

Figure 1

In vitro effect of (a) H-89 (selective inhibitor of cAMP-dependent protein kinase) on transcript levels of *aanat2* gene in the fish pineal organ. Each histogram represents Mean $\pm$ Standard Error; N = 3. c Represents level of significance as compared with control group: $p < 0.001$. f Represents level of significance as compared with db-cAMP treated group: $p < 0.001$. i Represents level of significance as compared with H-89 treated group: $p < 0.001$; (b) KT5823 (specific inhibitor of cGMP-dependent protein kinase) on transcript levels of *aanat2* gene in the fish pineal organ. Each histogram represents Mean $\pm$ Standard Error; N = 3. c Represents level of significance as compared with control group: $p < 0.001$. d and e Represent different levels of significance as compared with db-cGMP treated group: $p < 0.05$ and $p < 0.01$, respectively; (c) db-cAMP and db-cGMP on transcript levels of *aanat2* gene in the presence and absence of chelerythrine chloride (specific agonist of PKC) in the fish pineal organ. Each histogram represents Mean $\pm$ Standard Error; N = 3. c Represents level of significance as compared with control group: $p < 0.001$. f Represents level of significance as compared with db-cAMP treated group: $p < 0.001$. i Represents level of significance as compared with db-cGMP treated group: $p < 0.001$. j Represents level of significance as compared with chelerythrine chloride treated group: $p < 0.05$; (d) db-cAMP and db-cGMP on transcript levels of *aanat2* gene in the presence and absence of U0126 ethanolate (specific inhibitor of MeK) in the fish pineal organ. Each histogram represents mean $\pm$ standard error (S.E); N = 3. a and c Represent different levels of significance as compared with control group: $p < 0.05$ and $p < 0.001$, respectively. d and f Represent different levels of significance as compared with db-cAMP treated group: $p < 0.05$ and $p < 0.001$, respectively. g Represents level of significance as compared with db-cGMP treated group: $p < 0.05$; and (e) SB 202190 monohydrochloride hydrate (specific inhibitor of p38 MAP kinase) on transcript levels of *aanat2* gene in the fish pineal organ. Each histogram represents mean $\pm$ standard error (S.E); N = 3. c Represents level of significance as compared with control group: $p < 0.001$. f Represents level of significance as compared with db-cAMP treated group: $p < 0.001$. i Represents level of significance as compared with db-cGMP treated group: $p < 0.001$

Figure 2

In vitro effect of (a) db-cAMP and db-cGMP on transcript levels of *aanat2* gene in the presence or absence of calyculin A (specific inhibitor of serine/threonine phosphatase type 1 and type 2A) in the fish pineal organ. Each histogram represents mean $\pm$ standard error (S.E); N = 3. c Represents level of

significance as compared with control group: $p < 0.001$. f Represents level of significance as compared with db-cAMP treated group: $p < 0.001$. i Represents level of significance as compared with db-cGMP treated group: $p < 0.001$. k and l Represent different levels of significance as compared with db-cAMP + db-cGMP treated group: $p < 0.01$ and $p < 0.001$. o Represents level of significance as compared with calyculin A treated group: $p < 0.001$; (b) db-cAMP and db-cGMP on transcript levels of *aanat2* gene in the presence or absence of cypermethrin (specific inhibitor of serine/threonine phosphatase type 2B) in the fish pineal organ. Each histogram represents mean $\pm$ standard error (S.E); $N = 3$. a and c Represent different levels of significance as compared with control group: $p < 0.05$ and $p < 0.001$, respectively. d and e Represent different levels of significance as compared with db-cAMP group: $p < 0.05$ and $p < 0.01$, respectively. h Represents level of significance as compared with db-cGMP treated group: $p < 0.01$. k and l Represent different levels of significance as compared with cypermethrin group: $p < 0.01$ and $p < 0.001$, respectively. n Represents level of significance as compared with cypermethrin + db-cAMP treated group: $p < 0.01$; and (c) db-cAMP on transcript levels of *aanat2* gene in the presence and absence of a CREB inhibitor (CI) and a CBP-CREB interaction inhibitor (CCII) in the fish pineal organ. Each histogram represents Mean $\pm$ Standard Error (S.E); $N = 3$. c Represents level of significance as compared with control group: $p < 0.001$. f Represents level of significance as compared with db-cAMP treated group: $p < 0.001$