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An in vitro study on the role of protein kinase C, phosphodiesterase and Ser/Thr phosphatases in adrenergic stimulation of arylalkylamine-N-acetyltransferase (AA-NAT) activity in the pineal gland of *Clariasgariepinus*

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Abstract

The effects of inhibitors of PKC, PDE and ser/thr phosphatase 1, 2A and 2B on the activity of arylalklyamine N-acetyltransferase (AA-NAT) was studied in vitro in the pineal glands of the catfish, Clariasgariepinus.Pineals were pre-treated with specific inhibitors prior induction by norepinephrine then maintained in organ culture for 7 hr. In vitro treatment of the fish pineal organ with chelerythrine (PKC inhibitor) significantly decreased AA-NAT activity as compared to that of the control group. Theophylline (PDE inhibitor) treated at different incubation periods significantly increased AA-NAT activity. Okadaic acid (inhibitor of ser/thr phosphatase 1) and calyculin A (inhibitor of ser/thr phosphatase 2A) significantly inhibited basal AA-NAT activity. Cypermethrin (inhibitor of ser/thr phosphatase 1) show no significant effect neither on basal AA-NAT activity nor on NE-induced increased in the enzyme activity. These results suggest that PKC does not seem to be involved in adrenergic stimulation but necessary for maintaining basal activity of the enzyme. PDE also crucially regulate the activity of the AA-NAT enzyme. The normal as well as the adrenergic stimulation of AA-NAT by Ser/Thr phosphatase 1 and 2A are essential, but, Ser/Thr phosphatases 2B do not play any major part in adrenergic signal transduction in the fish pinealocytes.

Keywords: Pineal, AA-NAT, fish, inhibitors, PKC, PDE, ser/thr phosphatases.

Introduction

The regulation of the activity of the enzymearylalkylamine-N-acetyltransferase (AA-NAT) in the pineal gland of different species of homeotherms differs considerable involving the process of transcriptional, posttranscriptional and posttranslational control mechanisms[1]. In mammalian pineal, the rate by which melatonin is synthesised is regulated predominantly by the primary neurotransmitter norepinephrine (NE), which binds to α_1- and β-adrenergic receptors present on the membrane of pinealocytes and triggers the adrenergic signal transduction *via*cAMP and cGMP in the pineal[2-5]. Several fold increasesin cAMP and cGMP levels lead to phosphorylation and activation of protein kinase A (PKA) and PKG, respectively. The adrenergic signal transduction *via*cAMP on one hand leads to induction and activation of AA-NAT enzyme that accelerates the process of melatonin synthesis. NE-induced increase in cGMP levels transduce signal via cGMP-Protein kinase G (PKG)-mitogen-activated protein kinase (MAPK)pathway. Intracellular elevation of calcium ions (Ca^{2+}) and the enzyme protein kinase C (PKC) play critical roles in α_1-adrenergic potentiation of β-adrenergically stimulated cAMP accumulation[6, 7].Activation of calcium-dependent protein kinase C (PKC) in pineal of mammals has been reported to increase phosphorylation of signaling proteins involved in adrenergic signal transduction which involved Ca^{2+}-dependent α_1-adrenergic stimulation of pineal phospholipase A_2 activity [6]. Similarly, norepinephrine is well known to activate PKC [8].PKC also directly phosphorylated the rat recombinant AA-NAT *in vitro*[9].

Similarly, in the mammalian pineal, the enzyme cyclic nucleotide phosphodiesterase (PDE) stimulates breakdown of both cAMP and cGMP and thereby regulates cyclic nucleotide-dependent physiological processes [10]. In the pinealocytes of mammals the levels of cAMP and cGMPinside the cell are precisely controlled by NE through both α_1- and β_1-adrenergic receptors in a synergistic manner *via*cAMP generating pathway, which also play an important role in the regulation ofthe activityof the AA-NAT enzymeand melatonin synthesis[1, 11]. Phosphodiesterasesas reported in the mouse pineal gland are uncertain in suppressing AA-NAT induction because a phosphodiesterase inhibitor itself had no effect on the mRNA levels [12]. Expression and characterization of PDE 6 in the pineal gland of chicken has also been reported which indicated an inhibitory role of the enzyme on the activity of AA-NAT and also the synthesis of melatonin[13].

Protein phosphatase (PSPs) apparently controls the levels of AA-NAT protein and also melatonin biosynthesis in

mammalian pinealocytes [14, 15]showing a direct influence on the phosphorylation state of AA-NAT protein [16, 15]. Further, in the mammalian pineal glands, dephosphorylation of phosphorylated cAMP response element binding protein (pCREB) by PSPs seems to be an essential mechanism for the down-regulation and induction of AA-NAT enzyme activity and biosynthesis of melatonin[17,1]. Similarly, it has also been stated in the pineal of chicks that the expression of serine/threonine protein phosphatase 2A reveals a strong circadian manner under both *in vivo* and *in vitro*[18]. Pharmacological inhibition of the enzyme PSP-2A in chick pineal tissue brings aboutan elevation on the levels of phosphoCREB which also seems to be associated with melatonin secretion indicating that the enzyme may as well participate in the nocturnal control of pineal melatonin synthesis at some level[18].

Having stated the importance of calcium-dependent protein kinase C, phosphodiesterase and serine/threonine phosphatases in mammalian as well as in the avian pineal functions, there is essentially no reports on the different roles of played by PKC, PDE and serine/threonine phosphatases on the adrenergic induction of the activity of the enzyme arylalkylamine-N-acetyltransferaseand the synthesis of melatonin in the pineal gland of poikilothermic vertebrates especially in fishes.

A critical examination of the earlier information indicates that, unlike in mammalian and avian pineal, there is lack of information on the role of PKC, PDE and serine/threonine phosphatases in regulating the activity of AA-NAT and/or melatonin synthesis in the pineal gland of any fish species. Therefore, it was decided to investigate the role of,PKC, PDEs and serine/threonine phosphatases in adrenergic regulation on the activity of AA-NAT and also on the synthesis of melatonin in the pineal of catfish, *Clariasgariepinus.*

Methodology

Use of the experimental animal in the environmental endocrinology laboratory has been approved by the Institutional Ethics Committee.

Experimental animal: All the experiments were conducted on the air-breathing fish, *Clariasgariepinus*due to its easy availability, excellent survival under laboratory conditions and reasonable cost throughout the year at Shillong.

Clariasgariepinus is a teleost, which is widely distributed all over India and other tropical countries. In nature, it usually lives in shallow rivers, ponds and muddy places, and survives even in water with low oxygen content. It exhibits a bimodal (aquatic and atmospheric) breathing habit, and often comes to the surface of water to engulf atmospheric air. *Clariasgariepinus* breeds during monsoon[19]. The gonadal activity undergoes a cyclic change (both in morphology and histology) with the change in season so that spawning takes place in the most favorable time of the year ensuring better development and rapid growth of the young ones. At Shillong, activity of the gonadsis at the least level during the quiescent phasein the months of January and February, which steadily rises when reaching the month of March to May i.e. in the progressive phase. Breeding phase starts in when the fish breeds that is in the month of June to August. Subsequently in November and December i.e. during the regressive phasethe gonadal activity regresses. This cyclic change in the gonadal activity is possibly cued by the external factors (e.g. water temperature, photoperiod etc.).

The medium use for culturing the pineals is Dulbecco's Modified Eagle's Medium (DMEM) which was acquired from HyClone (Utah). Penicillin-Streptomycin, Norepinephrine, Isoproterenol, phenylephrine, propranolol, prazosin, clonidine, yohimbin and carbachol were obtained from Sigma-Aldrich, USA. ^{14}C-acetyl coenzyme A was purchased from Amersham-Pharmacia Biotech, U. K and Bovine Serum Albumin (BSA) was acquired from SRL, Mumbai.

Collection of pineal: It has been found in our laboratory that the activity of AA-NAT in the photoreceptive pineal gland of *Clariasgariepinus* do not seem to respond to stimulatory adrenergic agonists (e.g., norepinephrine, isoproterenol etc.) during the daytime (photophase), therefore, all the *in vitro* studies were conducted on the pineals collected instantaneously after the sunset. Collection of thepineals was done after decapitating the fish. This was done under dim red light in which the pineal window was rapidly exposed using a sterilized surgical blade. The pineal was then removed quickly, washed in the culture medium and then kept in a well of multi-well culture plate (Corning cell wells-25820) containing the culture medium.

Pineal gland culture: Dulbecco's Modified Eagles Medium (DMEM) with supplemented amounts of Calcium carbonate ($CaCo_3$), Bovine Serum Albumin (BSA), Ascorbic acid and Penicillin-Streptomycin was used for culturing the pineals using multi-well culture plate. The pineals were pre-incubated in the culture medium for one hour after which the medium was change with a fresh medium containing desired concentration of the specific agonists/antagonists. The pineals were maintained at 25^0 C in an O_2-CO_2 Gas Incubator (Heraeus:Cytoperm; Germany)in an atmosphere of 85% O_2, 5% CO_2 and 95% relative humidity. Pineals incubated in the medium without any drugs was taken as control. The pinealsafter incubating for a duration of 6 hours were thentaken and kept in Eppendorf tubes which has been carefully numbered they were immediately frozed in liquid nitrogen for the measuringthe activity of AA-NAT enzyme.

Pineal AA-NAT activity measurement: Radio-enzymatic assay described by [20]was used for measuring the activity of arylalkylamine N-acetyltransferase (AA-NAT) with minor modifications. For measuring the activity of AA-NAT, 75 µl of homogenization buffer (phosphate buffer: pH 6.5 with 6 nM acetyl Co-enzyme A) was taken and the pineals were sonicated with it. Further, from the sonicatedpineals, 15 µl samples was taken in duplicatealong with 5 µl of reaction mixture (i.e. tryptamine hydrochloride solution, phosphate buffer and ^{14}C-

acetyl Co-A), incubated at 25˚ C for about 20 minutes. Additions of 100 µl borate buffer to the tubes stop the reaction. A mixture of isoamyl alcohol and toluene in the ratio 3:97 was added to extract the acetylated tryptamine from the tubes. Thenthe tubes were fitted to a rotary mixer (Stuart Scientific, U. K) and rotated for 15 minutes, followed by centrifugation for 5 minutes at 3000 rpm. Then, from the upper phase of the mixture two ml was taken and transferred to a scintillation vial containing 5 ml of scintillation fluid. Then the radioactivity of each sample were counted using a liquid scintillation counter (Wallace-1409) and the activity was represented in terms of disintegration per minute (DPM). Vials (in duplicates) containing only the homogenization buffer, reaction mixture, extraction solution, and scintillation fluid served as 'Blanks'. The activity of AA-NATaftercalculations were expressed in terms of nmol/pineal/hour.

Data analysis: All data were statistically performed using One-way ANOVA. Values of P <0.05 were considered statistically significant.

Results and Discussion

The *in vitro* effect of chelerythrine on NE-induced activity of AA-NAT: The data were presented as mean ± SEM on Table-1 and Figure-1. The pineals were pre-treated *in vitro* with chelerythrine (specific inhibitor of PKC) for 15 minutes prior to stimulation by norepinephrine. The treatment with chelerythrine decreased activity of AA-NAT enzyme when compared to the control group. However, the chelerythrine chloride did not block the NE-induced increase in the enzyme activity.

***In vitro* effects of norepinephrine and theophylline (Incubation period-dependent)on the activity of AA-NAT:** The data werepresentedas mean ± SEM on Table-2 and Figure-2. When the fish pinealswere incubation with NE and theophylline (specific inhibitor of PDE) *in vitro*, the activity of AA-NAT was significantly increased. Similarly, the stimulatory effect of theophylline on the enzyme activity was found to increase with the increase in the incubation time.

***In vitro* effects of okadaic acid, calyculin A and cypermethrin on NE-induce activity of AA-NAT:** The data were presented as mean ± SEMon Table-3 and Figure-3. The pineals were pre-treated *in vitro* with the specific inhibitors for 15 minutes prior to stimulation by norepinephrine. Treatment of the fish pineals with 10^{-4} M solution of okadaic acid (OA; serine/threonine phosphatase 1specific inhibitor) and calyculin A (CA; serine/threonine phosphatise 2A specific inhibitor) significantly inhibited basal AA-NAT activity. However, NE treatment reversed the inhibitory effects of both OA and CA on the enzyme activity partially but significantly as compared with the OA and CA treated groups, respectively. The treatment with 10^{-4} solution of cypermethrin (specific inhibitor of serine/threonine phosphatise 2B), however, neither has significant effect on the basal activity of AA-NAT nor on NE-induced increase in the enzyme activity.

Discussion: Thus, as far as our knowledge is concern, this is the first report showing thedifferential role of Ca^{2+}-dependent Protein kinase (PKC), phosphodiesterase and serine/threonine phosphatase (type-1, 2A and 2B) in regulating the activity of AA-NATenzyme in photoreceptive pineal gland of a fish species. The interpretations from the current study indicated that Ca^{2+}-dependent Protein kinase (PKC) act as an important component in regulating the activity of the AA-NATenzyme adrenergically, and hereafter in the synthesisof melatonin in the pineal gland of fish (Table-1, Figure-1). The activity of the AA-NAT enzyme in the fish pineal was significantly decreased following blocking of the PKC activity by chelerythrine (Table-1 and Figure-1). Thus, it appears that PKC seem to be involved in maintaining the basal activity of AA-NAT, but might not play any role in stimulating the activity of AA-NATadrenergically since the PKC inhibitor,chelerythrine was unable to block the NE-induced increase in the enzyme activity (Table-1 and Figure-1).

As in the case of mammalian and avian pineal, PKC has been reported to be play an essential role in adrenergically stimulating the pineal cAMP accumulation[6, 7], cGMP[6]and the activity of the enzyme arylalkylamine--N-acetyltransferase[9]. The present findings indicate theinvolvement of Ca^{2+}-dependent PKC in the regulatingthe activity of AA-NAT enzyme in the fish pineal organ which is comparable to that seen in the pineal gland of mammals[9].

In the current investigation, the activity of AA-NAT in the pineal glandof fish was significantly stimulated following treatment with norepinephrine and theophylline (PDE inhibitor). The effect of the PDE inhibitor in stimulating the activity of AA-NAT appears to increase with increase in incubation time. These observations indicate that inhibition of PDE activity in the fish pineal organ stimulates AA-NAT activity in a time-dependent manner probably due to gradual increase in the cAMP levels (Table 2 & Fig. 2. It is noteworthy that in the mammalian pineal, administration of theophylline during the dark phase causes an upsurge in pineal cAMP[21]and both the activity AA-NAT and increase in the level of melatonin following treatment with PDE inhibitors (IBMX or aminophylline) irrespective of the time of the day[22]. It, thus, seems that as in mammals, in the fish pineal also the cyclic nucleotide-dependent phosphodiesterase plays a crucial role in regulating the activity of AA-NAT and also synthesis of melatonin even without adrenergic stimulation.

Both Ser/Thr phosphatase 1 and 2A (PSP 1 and PSP 2A) significantly inhibited basal activity of AA-NATenzyme in the fish pineals organ (Table-3; Figure-3). However, the influence ofcalyculin A was more in suppressing the activity of AA-NATwhen compared to that of okadaic acid (Table-4, Figure-4). But, the inhibitory effect of the two PSPs inhibitors on AA-NAT activity was reversed by NE partially but significantly as compared with the OA and CA treated groups, respectively. On the other hand, serine/threonine phosphatase 2B do not show

any significant effect either on basal activity of AA-NAT or on the NE-induced increases in the enzyme activity (Table-3 and Figure-3). It is important to mention that protein phosphatase in the mammalian pinealocytes control AA-NAT protein levels and melatonin biosynthesis[14,15,17,1]. Further, PSP-2A has also been reported to control the nocturnal pineal melatonin synthesis in the chicken pineal[18].Thus, based on theresults of the current findings, it can be proposed that Ser/Thr phosphatase 1 and 2A in the fish pineal organ are essential for the basal as well as for adrenergic stimulation of the activity of the AA-NAT enzyme. Similarly, in the fish pineal gland the presence of PSP-1 and PSP-2A might serve as a key regulatory component in regulatingthe AA-NAT protein. There is also a prospect that the two phosphatases are essential to keep c-Jun and c-Fos pathway functional, and possibly by dephosphorylating pCREB these phosphatases may also be involved in the regulating the activity of AA-NAT in the pineal organ of fish.

Table-1
The *in vitro* effect of chelerythrine(a specific inhibitor of PKC) on NE-induced activity of arylalkylamine-N-acetyltransfarase(AA-NAT) in the pineal of *Clariasgariepinus.*

Treatment	AA-NAT activity (nmol/pineal/h)
Control	1.94 ± 0.22 *
Norepinephrine (NE; 10^{-4} M)	2.34 ± 0.03[a]
Chelerethrine (Chel; 10^{-4} M)	1.35 ± 0.15[a]
Chel (10^{-4} M) + NE (10^{-4} M)	2.13 ± 0.24[a, d]

*All values are expressed as Mean ± Standard Error (S. E.); N = 3. [a]Differs significantly from the control group: $p < 0.05$. [d]Differs significantly from the chelerythrine treated group: $p < 0.05$.

Table-2
In vitro* effects of norepinephrine and theophylline (Incubation period-dependent)on the activity of AA-NAT in the pineal of *Clariasgariepinus

Treatment	AA-NAT activity (nmol/pineal/h)			
	← Duration of incubation →			
	30 Mins	1 Hour	4 Hours	6 Hours
Control	0.51 ± 0.05	0.85 ± 0.06	2.17 ± 0.19	3.44 ± 0.18
Norepinephrine (10^{-4} M)	1.93 ± 0.08[b]	2.61 ± 0.21[b]	3.33 ± 0.04[b]	4.35 ± 0.02[a]
Theophylline (10^{-4} M)	1.22 ± 0.08[b]	1.53 ± 0.05[b]	3.49 ± 0.01[b]	4.58 ± 0.06[b]

*All values are expressed as Mean ± Standard Error (S. E.); N = 3. [a, b] Differ significantly from the control group: $p < 0.02$ and 0.001, respectively.

Table-3
In vitro* effects of norepinephrine, okadaic acid (specific inhibitor of Ser/Thr phosphatase 1), calyculin A (specific inhibitor of Ser/Thr phosphatase 2A) and cypermethrin (a specific inhibitor of ser/Thr phosphatase 2B) on arylalkylamine-N-acetyltransferase (AA-NAT) in the photoreceptive pineal of male *Clariasgariepinus

Treatment	AA-NAT activity (nmol/pineal/h)
Control	0.30 ± 0.01*
Norepinephrine (NE; 10^{-4} M)	0.44 ± 0.04
Okadaic Acid (OA; 10^{-4} M)	0.15 ± 0.02 [a]
OA (10^{-4} M) + NE (10^{-4} M)	0.29 ± 0.01 [e]
Calyculin A (CA; 10^{-4} M)	0.09 ± 0.01 [b]
CA (10^{-4} M) + NE (10^{-4} M)	0.20 ± 0.02 [a, i]
Cypermethrin (Cyp; 10^{-4} M)	0.31 ± 0.04
Cyp (10^{-4} M) + NE (10^{-4} M)	0.38 ± 0.03

*All values are expressed as Mean ± Standard Error (S. E); N = 3. [a, b] Differ significantly from the control group: $p < 0.02$ and 0.01, respectively. [e]Differs significantly from okadaic acid treated group: $p < 0.05$. [i]Differs significantly from calyculin A treated group: $p < 0.02$.

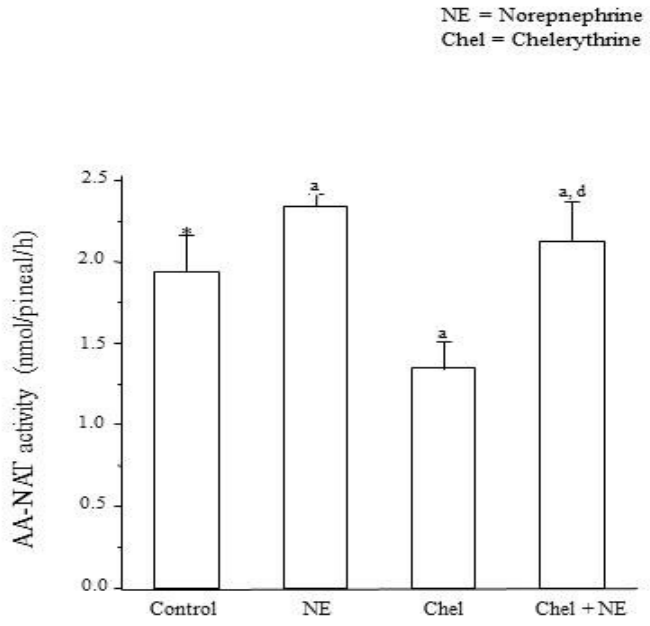

Figure-1
The *in vitro* effect of chelerythrine(a specific inhibitor of PKC) on NE-induced activity of arylalkylamine-N-acetyltransfarase(AA-NAT) in the pineal of *Clariasgariepinus*

Pineals were pre incubated without any treatment and then transfered to fresh medium containing the desired concentration of the inhibitors/agonists. The pineal glands were pre-treated with 10^{-4} M of the inhibitor (chelerythrine) 15 minutes prior treatment with 10^{-4} M norepinephrine. AA-NAT activity was measured in each pineal gland immediately at the end of the experiment. *All values are expressed as mean ± standard error (S. E) of three animals.[a]Differs significantly from the control group: $p < 0.05$. [d]Differs significantly from the chelerythrine treated group: $p < 0.05$.

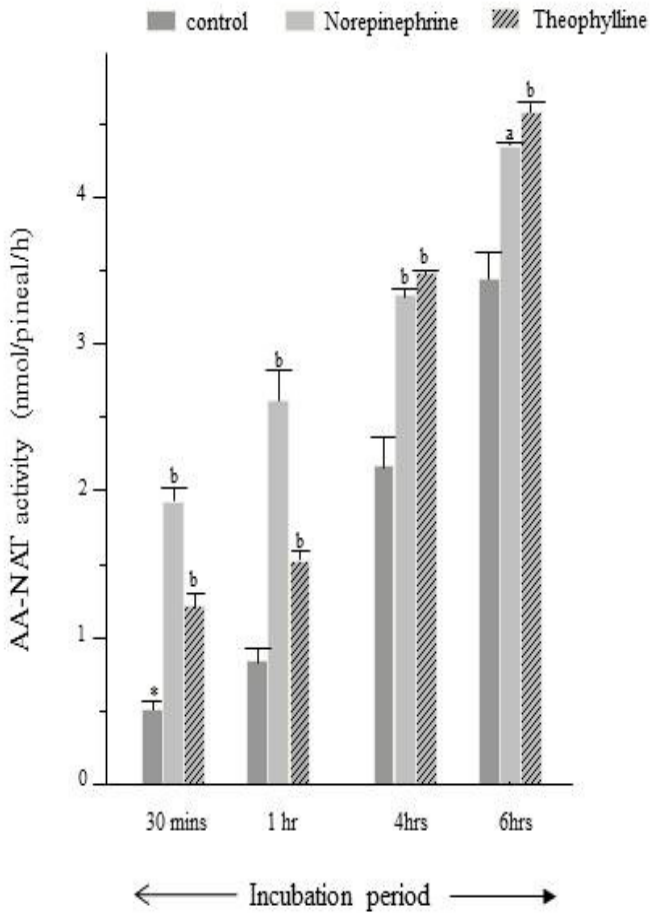

Figure-2

In vitro* effects of norepinephrine and theophylline (a specific inhibitor of phosphodiestesterase) on the arylalkylamine-N-acetyltransferase (AA-NAT) activity in the pineal of *Clariasgariepinus

Pineals were pre incubated without any treatment and then transfered to fresh medium containing the desired concentration of the agonists. The pineal glands were treated separately with 10^{-4} M norepinephrine and theophyline for 30 Mins, 1 Hour, 4 Hours and 6 Hours. AA-NAT activity was measured in each pineal gland immediately at the end of the experiment.All values are expressed as mean ± standard error (S. E) of three animals. [a, b] Differ significantly from the control group: $p < 0.02$ and 0.001, respectively.

NE = Norepinephrine
CA = Calyculin A
OA = Okadaic Acid
Cyp = Cypermetnrin

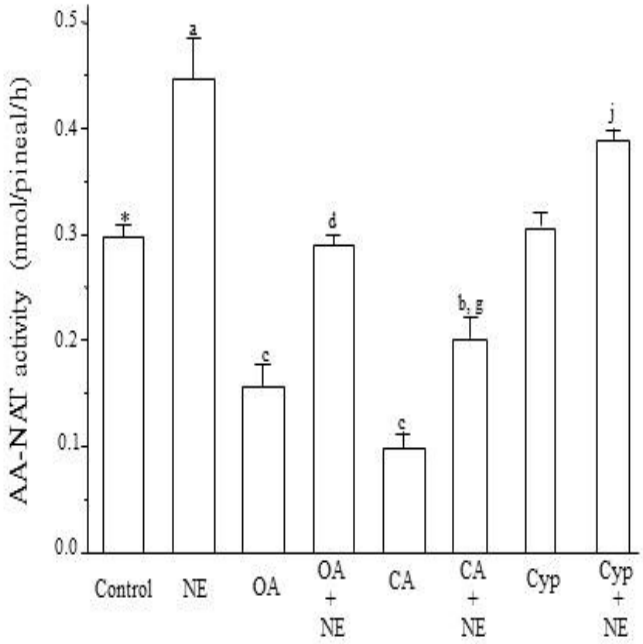

Figure-3

***In vitro* effects of norepinephrine, okadaic acid (specific inhibitor of Ser/Thr Phosphatase 1), calyculinA (specific inhibitor of Ser/Thr phosphatase 2A) and cypermethrin (a specific inhibitor of ser/Thr phosphatase 2B) on arylalkylamine-N-acetyltransferase (AA-NAT) in the photoreceptive pineal of male *Clariasgariepinus*.**

Pineals were pre incubated without any treatment for 1 hour and then transferred to fresh medium. The pineal glands were pre-treated with 10^{-4} M of the different inhibitor 5 minutes prior treatment with 10^{-4} M norepinephrine. AA-NAT activity was measured in each pineal gland immediately at the end of the experiment. *All values are expressed as mean ± standard error (S. E) of three animals.[a, b] Differ significantly from the control group: $p < 0.02$ and 0.01, respectively. [e]Differs significantly from okadaic acid treated group: $p < 0.05$. [i]Differs significantly from calyculin A treated group: $p < 0.02$.

Conclusion

Thus, basedon the current findings, we put forward that calcium-dependent PKC, PDE and serine/threonine phosphatase 1 and 2A are involved in regulating the activity of AA-NAT and, as wellas on the synthesis of melatonin in the photoreceptive pineal gland of fish.

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