IN VITRO STUDIES ON ADRENERGIC AND CHOLINERGIC REGULATION OF ARYLALKYLAMINE N-ACETYLTRANSFERASE (AA-NAT) ACTIVITY IN THE PINEAL OF CATFISH, *Clarias gariepinus*

BY

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I, Mrs. Khamtilin Kharshiing, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

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PREFACE

The pineal organ is the major site for biosynthesis of melatonin, an internal 'zeitgeber' which transmits information about environmental lighting to various parts of the body. The pineal is present in all groups of vertebrates (Vollrath, 1981; Tonsini and Menaker, 1996), and produces its hormone melatonin in a rhythmic manner. The diurnal rhythm of melatonin is primarily regulated by the light-dark cycle (Klein, 2006; Falcon, 2007). The rate of melatonin synthesis and the melatonin levels are minimum during the daytime (light phase) and maximum during the night time (dark phase). The nocturnal rise in the melatonin secretion is due to an increase in the activity of the rate-limiting enzyme arylalkylamine-N-acetyltransferase (AA-NAT; EC 2.1.3.87), which play a unique role in vertebrate biology by controlling the rhythmic production of melatonin in the pineal gland (Arendt, 1995). AA-NAT catalyzes N-acetylation of serotonin to N-acetylserotonin (Drijfhout *et al.*, 1996; Ganguly *et al.*, 2002).

The rate of AA-NAT synthesis depends on the activity of the *aa-nat* gene. Transcription of the *aa-nat* gene is stimulated during darkness by increased adrenergic inputs to the pineal from the suprachiasmatic nucleus (SCN) (Fukuhara *et al.*, 2005). The expression of the AA-NAT gene in the rat pineal gland is essentially turned off during the day and turned on at night, resulting in a more than 150-fold rhythm (Roseboom *et al.*, 1996). Turning 'off' AA-NAT expression appears to involve *de novo* synthesis of a protein that attenuates transcription (Roseboom *et al.*, 1996).

The circadian rhythm of melatonin involves three components, i.e., a photodetector, a circadian clock and the melatonin synthesizing machinery (Arendt, 1995). The anatomical organization of these three components has evolved during the course of evolution. In mammals, retina acts as a photodetector and synchronizes a circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, while the melatonin synthesizing machinery is located in pinealocytes (Klein, *et al.*, 1998; Iuvone *et al.*, 2005). The stimulatory and/or inhibitory circadian signals from the suprachiasmatic nucleus together with the photic information from the retina are conveyed by a multi-synaptic pathway from the SCN through brain stem, spinal cord, superior cervical ganglia and postganglionic sympathetic nerve fibres to the pineal gland to drive the circadian rhythms of AA-NAT activity and melatonin (Pevet *et al.*, 1997; van Esseveldt *et al.*, 2000; Hirota and Fukada, 2004). Unlike in mammals, the non-mammalian pineal organ itself contains all the three components (i.e., photodetector, circadian clock and melatonin forming system) essential for melatonin rhythm (Falcon *et al.*, 1991; Cahill, 1996; Bolliet *et al.*, 1997; Gupta and Premabati, 2002). As a result, the circadian rhythm of melatonin synthesis continues in non-mammalian pineals under *in vitro* conditions, and light acts directly on these photoreceptive pineals to reset the internal clock and switches 'off' melatonin synthesis (Zachmann *et al.*, 1992b; Bolliet *et al.*, 1996; Cahill, 1996)

Signal transduction cascades have been investigated most intensely in mammalian pineal (Klein *et al.*, 1997; Korf *et al.*, 1998). AA-NAT activity in mammals is primarily controlled by norepinephrine (NE), which regulates the activation of *aa-nat* gene and AA-NAT induction through α_1- and β_1-adrenergic receptors *via* NE-adrenergic receptors-G-proteins-adenylyl cyclase (AC)-cAMP-protein kinase A (PkA)-cAMP response element (CREB)-cAMP response element (CRE) pathway (Gupta *et al.*, 2005). Activation of β_1-adrenergic receptors leads to increased production of cAMP and AA-NAT activity, while activation of α_1-adrenergic receptors does not have any measurable effects on cAMP nor influence AA-NAT induction in mammalian pineal (Vanecek *et al.*, 1985). Stimulation of α_1-adrenergic receptors alone has no effect on cAMP accumulation, but causes a dramatic increase in the intracellular concentration of free calcium ions and potentiates the β_1-adrenergic receptors effects on cAMP levels so that simultaneous activation of α_1- and β_1-adrenergic receptors results in a 100-fold increase in cAMP (Maronde *et al.*, 1997) and AA-NAT activity in mammalian pineal (Roseboom *et al.*, 1996; Klein *et al.*, 1997; Klein, 1999). Nonetheless, a 3-fold daily rhythm in mRNA-encoding α1B- adrenergic receptors with a peak at midnight has been reported in the mammalian pineal (Coon *et al.*, 1997). Dephosphorylation of pCREB by protien serine/threonine phosphatase (PSPs) acts as an essential mechanism for down regulation of aa-nat gene transcription (Koch *et al.*, 2003). Inducible cAMP early repressor (ICER) is believed to play a negative role in pineal *aa-nat* gene transcription (Maronde *et al.*, 1999b). While cAMP formation stimulates AA-NAT activity *via* cAMP-PKA-CREB-CRE-AA-NAT pathway in mammalian pineal, the role of adrenergic signal transduction *via* the cGMP generating pathway in the regulation of pineal physiology is not well established (Klein, 1999; Man *et al.*, 2004). Adrenergic stimulation of mammalian pineal has also been reported to switch 'on' melatonin synthesis by additional mechanisms such as direct cAMP-induced activation of AA-NAT, inhibition of proteasomal proteolysis of AA-NAT, activation and protection of AA-NAT due to complex formation with 14-3-3, and also direct and indirect actions of Ca^{2+} on AA-NAT, which do not involve formation of AA-NAT mRNA in the pineal of some mammals like ungulates and primates (Gupta *et al.*, 2005). In mammals, in addition to transcriptional mechanism which controls AA-NAT protein and activity, there is a post-transcriptional regulation of AA-NAT protein levels *via* cAMP-dependent inhibition of proteosomal proteolysis of AA-NAT protein in all vertebrates studied so far (Gastel *et al.*, 1998; Zatz *et al.*, 2000; Schomerus *et al.*, 2000; Falcon *et al.*, 2001; Klein *et al.*, 2002; Gupta *et al.*, 2005). This mechanism involves phosphorylation-dependent binding of AA-NAT to 14-3-3 protein and thereby protection from proteolysis (Ganguly *et al.*, 2001). NE induced activation of PKC *via* cAMP has been reported to phosphorylate endogenous AA-NAT in pineal gland (Choi *et al*, 2004). Phospholipase A_2 also plays a role in AA-NAT induction and melatonin synthesis (Gupta *et al.*, 2001). However, unlike in mammals, AA-NAT activity in the avian pineal has been found to be decreased by noradrenergic agonists that act *via* α_2-adrenergic receptor (Rudeen *et al.*, 1990).

The regulation of AA-NAT activity in the fish pineal seems to be more complex. Unlike in mammals and birds where a single *aa-nat* gene has been found, two *aa-nat* genes have been reported in teleosts (Coon *et al.*, 1999). The AA-NAT preferentially expressed in retina (AA-NAT-1) has broad arylalkylamine substrate specificity and differs in kinetics from AA-NAT which is preferentially expressed in the pineal gland (AA-NAT-2) that has narrower substrate specificity (Falcon *et al.*, 1996; Coon *et al.*, 1999; Benyassi *et al.*, 2000; Zilberman-Peled *et al.*, 2004). AA-NAT-1 acetylates indole- and phenyl-ethylamines equally well, while AA-NAT-2 strongly prefers indole-ethylamines (Falcon *et al.*, 1996; Benyassi *et al.*, 2000). The kinetic differences between AA-NAT-1 and AA-NAT-2 appear to be defining characteristics of these gene sub-families, and are not species-specific (Benyassi *et al.*, 2000).

Melatonin synthesis in the photosensitive fish pineal is regulated by a complex mechanism involving cAMP-dependent and cAMP-independent pathways (Thibault *et al.*, 1993; Decressac *et al.*, 2002). Pineal cyclic nucleotide gated channel mediate calcium entry into the pineal photoreceptors. It is most probably a key element in the signaling pathways that control the rhythms production of melatonin (Decressac *et al.*, 2002). In darkness, the influx of Ca^{2+} seems to switch "ON" the melatonin synthesis. Ca^{2+}-calciprotein complexes might be involved in the activation of adenylyl cyclase and increased formation of cAMP in photoreceptor cells of the fish pineal (Falcon, 1999; Begay *et al.*, 1994a, b). The increased cAMP concentration has been reported to be responsible for activation of protein kinase A (PKA), which phosphorylates cAMP response element binding protein (CREB). The phosphorylated CREB (pCREB) has been reported to stimulate *aa-nat* gene to form AA-NAT mRNA, which is translated to AA-NAT protein (Stehle, 1995; Coon *et al.*, 1999; Mizusawa *et al.*, 2000; Besseau *et al.*, 2006).

The presence of AA-NAT activity has been reported in the pineal of a number of temperate zone piscine species (Falcon *et al.*, 1996, 1998; Begay *et al.*, 1998; Coon *et al.*, 1999; Kroeber *et al.*, 2000; Benyassi *et al.*, 2000, 2001). Fish pineal of several species has also been reported to possess another crucial enzyme HIOMT, which exhibits a prominent diurnal rhythm in its activity and converts N-acetylserotonin to melatonin

(Falcon *et al.*, 1994; Vuilleumier *et al.*, 2007). The aa-nat gene activity, AA-NAT activity and melatonin synthesis are increased during darkness, and light inhibits AA-NAT activity and melatonin synthesis (Begay *et al.*, 1998; Coon *et al.*, 1999; Falcon *et al.*, 2003b; Iigo *et al.*, 2003). It is noteworthy that the cAMP content of pineal is increased during darkness and decreased in the presence of light, and the diurnal rhythm of cAMP in fish pineal is influenced by light-dark cycle (Falcon and Gaildrat, 1997). However, unlike in mammalian and avian pineals, there is scarcity of information on adrenergic signal transduction pathway (s) involved in the regulation of AA-NAT activity in the pineal of any poikilothermic vertebrates in general and in the fish pineal in particular. There is a single report that activation of β-adrenergic receptor increases cAMP formation and AA-NAT induction in fish pineal, while activation of α-adrenergic receptors inhibits AA-NAT activity (Falcon *et al.*, 1991). There is practically no information on the effects of simultaneous activation of both α- and β-adrenergic receptors on AA-NAT activity of the fish pineal. The agonists of cholinergic M1 receptors have been reported to modulate pineal physiology in mammals (Gupta *et al.*, 1991, 1992). The fish pineal also receives parasympathetic nerve fibres (Gupta and Premabati, 2002), but there is no information on effects of cholinergic stimulation separately or in combination with adrenergic stimulation on AA-NAT activity on the fish pineal. In mammalian pineal, stimulatory sulphydryl G-proteins and phospholipase A_2-associated G-proteins have been reported to be involved in adrenergic signal transduction (Gupta *et al.*, 2001). However, the nature of G-proteins involved in adrenergic signal transduction and subsequent AA-NAT induction in the fish pineal remains unknown.

A careful analysis of the available information on the pineal of fish clearly indicates that, as compared to mammalian and avian pineal, there is scarcity of information on adrenergic signal transduction pathway involved in the induction of the rate-limiting enzyme AA-NAT and melatonin synthesis in fish pineal. Since melatonin influences a wide spectrum of fish physiology (e. g., breeding, migration, intermediary and oxidative metabolism, pigmentation, electrolyte balance, growth and development etc.) (Gupta and Premabati, 2002), a sound knowledge of the role adrenergic and cholinergic signal transduction pathway involved in the regulation of AA-NAT activity is essential. During the course of evolution, the fishes were the first group of vertebrates where the pineal complex developed, probably as a phototransducer neuroendocrine organ in order to use photoperiod as an environmental synchronizer. Therefore, keeping in mind the phylogenetic position of the fish as well as the importance of melatonin as a 'zeitgeber' and an important regulator/modulator of fish physiology, it was thought worthwhile to investigate adrenergic and cholinergic signal transduction pathways involved in regulation of AA-NAT activity in the photoreceptive pineal of the fish *Clarias gariepinus* as a model.

The present Ph. D. thesis has been divided into five chapters. An introduction of the chapters is given in the following sections.

Chapter - I: Materials and Methods

This chapter deals with the details of materials and methods used for this Ph. D. dissertation. It incorporates description of the experimental animals, maintenance of the experimental fishes, tissue culture of the fish pineal, mode of treatment, methods used for measuring the pineal arylalkylamine-N-acetyltransferase (AA-NAT) activity and biostatistical methods for analyzing the data.

Chapter - II: *In vitro* studies on differential role of α and β adrenergic receptors and cholinergic receptors in adrenergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

This chapter deals with the study of *in vitro* effects of different concentrations of norepinephrine (potent adrenergic sitmulator), carbachol (a specific muscarinic M1 receptor agonist) and different agonists and antagonists of both α- and β-adrenergic receptors, i.e, phenylephrine (a specific α_1 adrenergic receptor agonist), isoproterenol (a specific β_1-adrenergic receptor agonists), clonidine (a specific α_2-adrenergic receptor agonist), prazosin (a specific α_1 adrenergic receptor antagonist), propranolol (a specific β_1-adrenergic receptor antagonists) and yohimbin (a specific α_2-adrenergic receptor antagonist) on pineal AA-NAT activity in the fish *Clarias gariepinus*, maintained under natural climatic conditions.

Chapter - III: *In vitro* studies on the nature of G-protein (s) involved in adrenergic and cholinergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

This chapter deals with the study of *in vitro* effects of norepinephrine (adrenergic agonist), carbachol (specific M1 receptors agonist), G7637 (Guanosine 5'-[β-thio] diphosphate trilithium salt; a specific inhibitor of stimulatory G-proteins), N-ethylmaleimide (a specific inhibitor of sulfhydryl G-proteins) and pertussis toxin (a specific inhibitor of inhibitory G-protiens) on pineal AA-NAT activity in the fish *Clarias gariepinus*, maintained under natural climatic conditions.

Chapter - IV: *In vitro* studies on the role of Ca^{2+}, protein kinase C, phosphodiesterase and serine/threonine phosphatase in adrenergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

This chapter deals with the study of *in vitro* effects of norepinephrine (adrenergic agonist), nitrendipine (specific L-type voltage calcium channel blocker), chelerethrin chloride (specific inhibitor of protein kinase C), theophylline (specific inhibitor of phosphodiesterase), okadaic acid (specific inhibitor of ser/thr phosphatase 1), calyculin A (specific inhibitor of ser/thr phosphatase 2A)and cypermethrine (specific inhibitor of ser/thr phosphatase 2B) on pineal AA-NAT activity in the fish maintained under natural climatic conditions.

Chapter - V: Summary and Conclusions

This chapter incorporates the summary of major findings of Chapter II, Chapter III and Chapter IV as well as major conclusions derived from the findings of the Ph. D. dissertation.

To the best of our knowledge the present findings for the first time seem to provide a better insight and a complete picture on the adrenergic and cholinergic signal transduction pathway in the photoreceptive pineal organ of a fish species.

troduction

The pineal gland (epiphysis) plays a central role in transducing photoperiodic information into diurnal and annual physiological changes through rhythmic production and secretion of its hormone melatonin, which is an essential component of the photo-neuroendocrine system that allows vertebrates to measure and keep the time (Filadelfi and Castrucci, 1996). In mammals, the pineal gland is no longer photosensitive but serves as an endocrine gland in which melatonin synthesis is regulated by adrenergic inputs from the sympathetic nerves (Rekasi and Czompoly, 2002). Unlike mammalian pineal, the pineal organ of other vertebrates (e.g., lampreys, fishes, amphibians, reptiles and birds) generally retains photosensitivity, and plays an active role as a photosensory organ (Ekstrom and Meissl, 2003). The physiological role of the pineal gland has changed from a photosensory and photoendocrinal organ in fishes and amphibians to a neuroendocrinal organ in mammals (Kusmic and Gualtieri, 2000). In sub-mammalian vertebrates, the pineal organ responds to light directly (Gupta and Premabati, 2002).

This hormone is typically produced during the night at two major sites: the pineal gland and the retinal photoreceptor cells (Klein *et al.*, 1997; Susko *et al.*, 2004). Melatonin synthesised in the pineal is responsible for the circulating levels of melatonin, while retinal melatonin probably acts as a paracrine hormone (Bayarri *et al.*, 2004; Besseau *et al.*, 2006) which does not seem to contribute to the large rhythms of circulating melatonin (Cahill and Hasegawa 1997). Melatonin plays a major endocrine role in the regulation of a variety of daily and annual physiological rhythms (Altun and Ugur-Altun, 2007).

Melatonin acts as a pineal hormone and regulates/modulates a wide range of vertebrate physiology. The original function of melatonin was to serve as an antioxidant to protect organisms from ubiquitous oxidative stresses, while other receptor-related functions of melatonin were acquired during evolution (Vanecek, 1998). Melatonin contributes to physiologic timekeeping in a wide variety of organisms, including humans (Reppert *et al.*, 1995a, b). In mammals, melatonin production is regulated by the suprachiasmatic nuclei (SCN), the site of a biological clock that regulates numerous circadian rhythms also describe as the Mind's Clock (Klein *et al.*, 1991). The stimulatory and/or inhibitory circadian signals from the suprachiasmatic nucleus together with the photic information from the retina are conveyed by a multisynaptic pathway from the SCN through brain stem, spinal cord, superior cervical ganglia and postganglionic sympathetic nerve fibres to the pineal gland to drive the circadian rhythms of AA-NAT activity and melatonin (van Esseveldt *et al.*, 2000). The SCN controls the circadian rhythm of melatonin synthesis in the pineal gland by a multisynaptic pathway including successively preautonomic neurons of the paraventricular nucleus (PVN), sympathetic preganglionic neurons in the spinal cord and noradrenergic neurons of the superior cervical ganglion (SCG) (Perreau-Lenz *et al.*, 2003). Several studies in mammals have now demonstrated that many of the physiological, cellular and molecular rhythms that are present within the retina are under the control of a retinal circadian clock which is the first extra-SCN circadian oscillator or more likely a network of hierarchically organized circadian clocks that are present within this tissue (Tosini *et al.*, 2008).

Functions of melatonin in vertebrates

Melatonin is a common output signal of the vertebrate circadian clock which is produced at two major sites - the pineal organ and the retina. Despite the fact that the photoperiodic and circadian controls of melatonin production have been profoundly modified during evolution, the melatonin signal released into the blood is the same from fish to mammals (Falcon *et al.*, 2007). In almost all vertebrates, the melatonin increase occurs during the night, regardless of whether they are day active or night active (Maywood *et al.*, 1993; Besseau *et al.*, 2006). This makes melatonin rhythm an endocrine marker of darkness/night and is also called as 'hormone of darkness' (Hardeland, 2008). In animals and humans, melatonin has been identified as a remarkable molecule with diverse physiological actions, signaling not only the time of the day or year, but also promoting various immunomodulatory and cytoprotective properties (Pandi-Perumal *et al.*, 2006).

Mammals

In mammals, melatonin plays an important role in the neuroendocrine regulation of circadian rhythmicity (Cassone, 1990; Zawilska *et al.*, 2009). The most solidly established role of melatonin in mammals is the control of the reproduction in seasonal breeders (Brandstatter, 2003; Iwasaki *et al.*, 2005; Tsiligianni *et al.*, 2009) exerted through a modulation by melatonin of the neuroendocrine reproductive axis through different mechanisms (Diaz *et al.*, 2005; Boczek-Leszczyk and Juszczak, 2007). Melatonin is anti-gonadal in human (Laughlin, 1991). Melatonin has been reported to also play a role in fetal sheep physiology, signifying prevention of major contraction of cerebral vessels, restrains cortisol release and restricts brown adipose tissue lypolysis during fetal life (Torres-Farfan *et al.*, 2008). Melatonin implants in sheep may perhaps improve reproductive performance and yield an earlier start of breeding season. The stimulating effect of melatonin on GSHPx activity seems to play a protective role against oxidative damage during the first stage of gestation (Andres *et al.*, 2009). Melatonin, due to its ability to readily pass through the placenta easily protects the foetus from oxidative damage as well as the maternal tissues and placenta (Reiter *et al.*, 2009). Melatonin has also been reported to reduce cortisol response to ACTH in humans (Campino *et al.*, 2008). Melatonin is well known for its role as an inhibitor of secretions of gonadrotropins and is involved as a main factor in the photoperiodic regulation of hypothalamo-hypophyseal-gonadal axis (Sanchez-Barcelo *et al.*, 2007). Melatonin has also been reported to attenuate the release of adreno-medullary catecholamines in rats (Budhram and Lau-Cam, 2009). In mammals, melatonin acts as modulator of defense responses *via* hypothalamo-pineal-adrenal axis (Couto-Moraes *et al.*, 2009). In seasonally breeding mammals, brightly coloured secondary sexual characteristics that serve as a sexual attractant are reportedly enhanced by melatonin administration (Reiter *et al.*, 2009). The levels of thyroid hormones are reportedly inhibited following melatonin administration and increased after pinealectomy in mammals (Wajs and Lewiski, 1992; Ozturk *et al.*, 2000).

In mammals, melatonin has been shown to protect against oxidative stress in various tissues. Melatonin's functions as an antioxidant include i) direct free radical scavenging, ii) stimulation of oxidative enzymes, iii) increase in the efficiency of mitochondrial oxidative phosphorylation and reducing electron leakage, and iv) augmentation of the efficiency of other oxidant (Reiter *et al.*, 2003; Liang *et al.*, 2004; Srinivasan *et al.*, 2005; Thomas-Zapico and Coto-Montes, 2007; Ogeturk *et al.*, 2008). Unlike other antioxidants, it has been reported that melatonin can easily cross all morpho-physiological barriers, e.g., the blood brain barrier, and enters cells and sub-cellular compartments in human (Gupta *et al.*, 2003). Melatonin reportedly protects rat brain cells from oxidative damage induced by ionizing radiation (Undeger *et al.*, 2004) and also have potential utility in therapeutic treatment of oxidative stress-induced neuronal dysfunctions (Reiter *et al.*, 2005). Similarly, it also has protective effects against oxidative stress in Fmr1 knockout mice (Romero-Zerbo *et al.*, 2009) and in rats with renal mass reduction. Further, melatonin has also been reported to ameliorate oxidative stress, inflammation, proteinuria, and progression of renal damage (Quiroz *et al.*, 2008). In the liver of rats, melatonin treatment substantially prevents carbon tetrachloride-induced apoptosis and oxidative damage (Ogeturk *et al.*, 2008). Melatonin has also been found to be highly protective against damage to macromolecules resulting from oxygen and nitrogen-based reactants in mammals (Reiter *et al.*, 2004). Melatonin has also been reported to act as an anti- and pro-inflammatory property in human blood (Johe and Osterud, 2005). It also play a role in the prevention of oxidative damage (Taysi *et al.*, 2005) and as a protective effect against ischemia/reperfusion (I/R) injury in skeletal muscle of human and nervous tissue in rats (Erkanl *et al.*, 2005; Sayan *et al.*, 2004). Melatonin also exhibits pronounced anti-stressor properties in rats (Arushanian *et al.*, 2007).

There are also several reports on the role of melatonin on fasting, thermoregulation and hibernation in mammals (Celinski *et al.*, 2009; Saarela and Reiter, 1994; Revel *et al.*, 2007; Chowdhury *et al.*, 2008). Other actions of the hormone are the inhibition of dopamine release from retina (Dubocovich *et al.*, 1997), vasoregulatory activity (Doolen *et al.*, 1998; Ting *et al.*, 1999), and effects on cell growth (Blask *et al.*, 2002; Ram *et al.*, 2002).

The melatonin profile in humans and the activity and/or structure of the elements of the synthesis pathway of the hormone are often altered in some pathophysiological states (Arendt, 2005). The effects of melatonin in humans have most often been examined in biological rhythms (Dawson and van den Heuvel, 1998; Zawilska *et al.*, 2009), sleep disorders (Brown, 1994), mental illness (Pacchierotti *et al.*, 2001), depression and schizophrenia (Maldonado *et al.*, 2009). In humans, jet lag caused by transmeridian travel is attenuated by melatonin administration (Brown *et al.*, 2009; Sack, 2009), presumably by resetting the biological clock to match the new environmental time. Melatonin is highly efficient anti-aging factor and as melatonin levels decrease with age, melatonin treatment may reduce age-related skin changes (Erefolu *et al.*, 2005). Similarly, it has been suggested that melatonin reduces oxidant events in the brain and help in slowing down the effects of ageing process in rats (Petrosillo *et al.*, 2008; Baeza *et al.*, 2009). Melatonin has also been reported to exhibit cytotoxic activity in cancer cells and is, thus, used as a chemotherapeutic drug (Mills *et al.*, 2005). Furthermore, melatonin also plays a role on arterial distensibility and blood pressure in humans (Yildiz and Akdemir, 2009). Melatonin has been reported to have a protective effect against oxidative hepatic injury in human (Bekyarova *et al.*, 2009). Melatonin also has a potential role in breast cancer prevention and treatment (Grant *et al.*, 2009). Melatonin also acts as a strong neuroprotective drugs against cerebral ischemia (Wang *et al.*, 2009). Experiments conducted on rats, cats, and monkeys revealed that melatonin also has the ability to reduce sleep onset time and increase sleep duration. In patients with chronic insomnia melatonin has been reported to promote sleep by regulating the sleep/wake rhythm through their actions on melatonin receptors in the SCN (Srinivasan *et al.*, 2009).

Birds

In birds, in addition to its timekeeping functions (Underwood *et al.*, 2001; Natesan *et al.*, 2002), melatonin has been implicated to have a potential role in the regulation of the retinal clock (Peters and Cassone, 2005), and reproductive functions (Lewis *et al.*, 2006; Singh~~a~~ and Haldar, 2007). It also acts as a direct free radical scavenger (Pablos *et al.*, 1995). In several avian species (e.g., house and Java sparrows, European starling, domestic pigeon etc.), melatonin has been reported to regulate circadian rhythms of locomotor activity, feeding, and body temperature (Chabot and Menaker, 1992; Murakami *et al.*, 2001; Silverin *et al.*, 2009). In chicks, melatonin has been reported to stimulate the anti-oxidative enzyme glutathione peroxidase activity in several tissues (Pablos *et al.*, 1995). Thyroid hormones levels in birds are also reportedly inhibited following melatonin administration and increased after pinealectomy (John *et al.*, 1990; Prakash *et al.*, 1998). While the presence of certain level of melatonin act as an important parameter in the direct activation of the thyroid gland by the immune system of males *Gallus domesticus* (Dzerzhynsky *et al.*, 2006). In the Indian finch *Lal munia*, melatonin influences gonadal activity, body weight growth, and thyroid activity by acting directly at the hypothalamo-hypophyseal complex, it counteracts the effect of LH at the feather papillae level. Further, synthesis and/or release of FSH seem to be more sensitive to melatonin than that of LH (Gupta *et al.*, 1987). It has also been reported that administration of 5-HTP and L-DOPA in Japanese quail, *Coturnix coturnix japonica* at specific time interval and variation in pineal functions that modulate reproductive responses also alter the circadian pattern (acrophase and amplitude) of hypothalamic serotonin and dopamine, maintaining a specific phase relation between these cycles and breeding status. Specific circadian phase relation of serotonergic and dopaminergic oscillations regulates reproduction in Japanese quail, *Coturnix coturnix japonica* (Kumar and Charturvedi, 2009).

Melatonin modulates intercellular communication among cultured chick astrocytes (Peters *et al.*, 2005). The diurnal variation in the cellular and humoral immune responses of Japanese quail is dependent on melatonin (Siopes and Underwood, 2008). In quail neurosteroid, 7alpha-hydroxypregnenolone has been reported to mediate melatonin action on diurnal locomotor rhythms (Tsutsui *et al.*, 2009). Similarly, locomotor rhythms in house sparrows (*Passer domesticus*), which are made arrhythmic by either pinealectomy or maintenance in constant light, have been shown to be synchronized by daily administration of melatonin of different durations to simulate melatonin profiles (Cassone *et al.*, 2008). Lack of melatonin following pinealectomy in chicks has been reported to have a significant effect on trace metal levels (Turgut *et al.*, 2006).

Reptiles

In reptiles melatonin is believed to play an important role in the regulation of various physiological activities such as its effects on circadian rhythmicity (both circadian and circannual), thermoregulation and photoperiodic regulation of reproduction (Larson-Prior *et al.*, 1996; Wiechmann and Wirsig-Wiechmann, 1994). Daily melatonin injections are capable of entraining circadian rhythmicity in *P. sicula* (Bertolucci and Foa, 1998). Melatonin has been reported to inhibit thyroid activity in adult female turtles, *Lissemys punctata punctata* (Sarkar *et al.*, 1997). In a nocturnal snake, *Lamprophis fuliginosus* melatonin levels control the complex interactions between behavior and physiology both daily and seasonal basis (Lutterschmidt *et al.*, 2002). In addition, melatonin also plays a role in the seasonal re-organization of the circadian system in these species (Foa and Bertolucci, 2003). Melatonin has been reported to decrease mean preferred body temperature in nocturnal snake, *L. fuliginosus* (Lutterschmidt *et al.*, 2002). Similarly, in ruin lizard melatonin is centrally involved in determining circadian organization in summer, while it is only marginally involved in autumn-winter (Foa *et al.*, 2006).

It has been reported that in iguana, a negative feedback loop involving both melatonin and dopamine regulates the circadian rhythm of the electroretinogram (ERG) b-wave amplitude that is at least in part generated in the brain (Miranda-Anaya *et al.*, 2002). Nocturnal activity in green turtle, *Chelonia mydas* has been reported to be associated with melatonin profiles which resemble those measured during the day (Jessop *et al.*, 2002). In addition, melatonin has also been reported to significantly reduced courtship behavior in male garter .snake, *Thamnophis sirtalis parietalis* with additive inhibitory effects of melatonin and corticosterone in modulating reproductive behaviour in this species (Lutterschmidt *et al.*, 2004).

Amphibians

Like in other vertebrates, in amphibians also melatonin performs diverse functions. Though the functions of melatonin are vast, the major underlying role of melatonin in amphibians is perhaps acting as a potent lightening agent in amphibian skins reversing in a dose-dependent manner the darkening caused by alpha-melanocyte-stimulating hormone (Filadelfi and Castrucci, 1994). It is also involved in the control of skin coloration (Sugden *et al.*, 2004) and induces desensitization in amphibian melanophores (Rollag and Lynch, 1992). Melatonin also induces melanosomes aggregation in Xenopus laevis (Karlsson *et al.*, 2000).

Melatonin plays a role in metamorphosis in anuran amphibians as a thyroid antagonist, whose level falls at the metamorphic climax when the thyroid hormones reach a peak (Wright and Alves, 2001; Wright, 2002). In the tadpole of Indian skipper frog, *Rana cyanophlyctis*, melatonin treatment reportedly counteract the red-light induce acceleration of metamorphosis (Joshi and Mohinuddin, 2003). Consequently, melatonin has been reported to transduce environ-mental information to regulate endocrine periodicity and larval circadian organization, and influences metamorphic rate (Wright *et al.*, 2003). In amphibians, melatonin has been reported to stimulate the release of growth hormone and prolactin inducting the expression of growth hormone-releasing peptide and its related peptide-2 (Chowdhury *et al.*, 2008), to inhibit dopamine release (Isorna *et al.*, 2005). Melatonin acting *via* MT2 receptors also modulates GABA(C) receptor activity in the optic tectum and that this effect is influenced by the light-dark cycle (Prada *et al.*, 2005). In Xenopus frogs, melatonin treatment *in vitro* has been reported to provoke leydig cell morphological changes, blocks GnRH-antagonist-induced testosterone secretion and decreases relaxin expression (Udin, 2005). Melatonin *via* estrogen receptors seems to interfere with the differentiation and/or proliferation of mast cells induced by estradiol treatment either *in vivo* or *in vitro* in the testis of the frog, *Rana esculenta* (Izzo *et al.*, 2004).

Fishes

In fish, melatonin rhythms are believed to entrain the temporal co-ordination of many physiological processes (Ekstrom and Vanecek, 1992). Melatonin reportedly produces prominent effects on growth and metamorphosis (Omura, 2007; Ziv and Gothilf, 2006), pigmentation (Hayashi *et al.*, 1993; Aspengren *et al.*, 2003), pituitary (Bayarri *et al.*, 2004; Amano *et al.*, 2004; Falcon *et al.*, 2007), thyroid (Gupta and Premabati, 2002; Kulczykowska *et al.*, 2004), adrenal (Agha and Joy, 1989; Cahill, 1997), inter-renal function (Vollrath, 1981), smoltification and reproduction (Amano *et al.*, 2004; Bhattacharya *et al.*, 2007; Iigo *et al.*, 2005; Renuka and Joshi, 2009). In addition, the pineal gland and melatonin in fishes have been involved in the control of daily variations of locomotor activity, sleep-like state and feeding habits (Zachmann *et al.*, 1992a; Boeuf and Falcon, 2001). In the goldfish, pinealectomy had photoperiod-dependent effects on growth (Ekstrom and Meissl, 1997). In the Atlantic salmon parr, pinealectomy reduced specific growth rates during the increasing photoperiod, up until the summer solstice, but it had the opposite effects during the decreasing photoperiod (Mayer *et al.*, 1997). As compared to other vertebtrates where melatonin has been involved in metamorphosis through antagonizing thyroid hormone function; there are fewer reports on the relationship between melatonin system and metamorphosis in fish. A recent report showed an inverse pattern of AA-NAT activity (the rate limiting enzyme of melatonin synthesis) and thyroid hormones levels during metamorphosis in the flatfish, *Solea senegalensis* (Isorna *et al.*, 2009). Melatonin has been reported to exert potent melanosome aggregation in several fish species (Fujii and Oshima, 1986). Addition of melatonin to the bathing medium has been reported to cause blanching of the abdominal skin of the neon tetra *Paracheirodon innesi* and the cardinal tetra *P. axelrodi* (Hayashi *et al.*, 1993). *In vitro* administration of melatonin and noradrenaline in female two-spotted gobies, *Gobiusculus flavescens* has been shown to increased skin transparency, but seem to have negative effect coloration. But treatment of melatonin and MSH, or melatonin and prolactin, increased both coloration and transparency (Skold *et al.*, 2008).

In fishes, melatonin seems to modulate neuroendocrine functions by targeting the pituitary gland itself (Falcon *et al.*, 2007). Melatonin has been reported to modulate growth hormone and prolactin secretion in cultured trout pituitaries (Falcon *et al.*, 2003a). It has been reported that melatonin administration in the masu salmon, *Oncorhynchus masou*, reduced pituitary GnRH and luteinizing hormone content but stimulated pituitary follicle stimulating hormone content (Amano *et al.*, 2004). In the rainbow trout, *Oncorhynchus mykiss*, melatonin administration has been reported to cause reduction in 3, 4-dihydroxyphenylacetic acid (DOPAC) in both the hypothalamus and the pituitary as well as in the pituitary DOPAC/Dopamine ratio (Hernandez-Rauda *et al.*, 2000). More direct evidence was provided by studies in the Atlantic croaker (*Micropogonias undulates*) in which intraventricular administration of melatonin in the preoptic area (POA) decreased luteinizing hormone release by the pituitary gland in animals with fully developed gonads (Khan and Thomas, 1996).

The pineal organ has been reported to play an important role in reproduction in fishes (Joy and Agha, 1991; Popek *et al.*, 1992; Renuka and Joshi, 2009). As in mammals, melatonin appears to exert its effects *via* the hypothalamo-hypophyseal-gonadal axis (Vollrath, 1981; Popek *et al.*, 1992; Ghosh and Nath, 2005). Melatonin treatment has been reported to have a negative effect on eel reproductive function (Sebert *et al.*, 2008). Pinealectomy in *Channa punctatus* accelerated growth of the ovary during preparatory phase, but had no significant effect in prespawning or postspawning phases in (Joy and Khan, 1991). An increase in the number of gonadotrophs in *Notemigonus* after pinealectomy points to an inhibitory influence of the pineal on the pituitary gland. Intra-peritoneal injection of melatonin in the Atlantic Croaker, *Micropogonias undulatus* during late-light phase of the day-night cycle elicited a significant elevation in plasma gonadotropin II secretion (Khan and Thomas, 1996). In female catfish, *Clarias batrachus* melatonin showed variable effects on ovary and plasma gonadotropin and vitellogenin levels in both intact and pinealectomized animals. The effect and/or control of melatonin on reproduction appear to act by blocking the GtH II release (Ghosh and Nath, 2005). Melatonin has also been reported to have an inhibitory effect on ovarian vitellogenesis and inducing atresia in the catfish, *Heteropneustes fossilis* (Joy and Agha, 1991), but has no or little effects on estrogen receptor in the liver of rainbow trout, *Oncorhynchus mykiss* both *in vitro* and *in vivo* (Mazurais *et al.*, 2000). Melatonin treatment in male masu salmon shows inhibitory effects on the gonadosomatic index and plasma testosterone levels (Amano *et al.*, 2004). Melatonin has been show to have potential inhibitory effect on the reproduction of three-spined stickleback, *Gasterosteus aculeatus* (Sokolowska *et al.*, 2004). In a sub-tropical surface dwelling carp *Catla catla*, melatonin play a significant role in the regulation of the annual testicular events which seems to vary in relation to the reproductive status of the fish (Bhattacharya *et al.*, 2007).

In fishes, melatonin also contributes to the regulation of several behaviours and physiological functions such as locomotor activity rhythm (including vertical migration), sleep-like states, and thermal preference etc. (Ekstrom and Meissl, 1997; Zhdanova *et al.*, 2001). Melatonin diminishes locomotor activity and food intake in many fish species (Lopez-Olmeda *et al.*, 2006; Rubio *et al.*, 2008). Melatonin administration induces a sleep-like state in *Danio rerio* and zebrafish (Danilova, 2001; Zhdanova *et al.*, 2001). Melatonin administration in goldfish provoked slight effects on lipid peroxidation and mortality resulting from oxidative stress, with reduction of locomotor activity in relation to the vehicle (Lopez-Olmeda *et al.*, 2006). Melatonin-induced effects on lipid metabolism have been observed in teleost (De Pedro *et al.*, 2008). In *Clarias batrachus*, *in vitro* melatonin administration stimulated tissue respiration (Lynsiang, 1998).

Synthesis of Melatonin

The pathway of melatonin synthesis in the pineal organ of the fish is similar to that in the mammalian pineal gland (Gupta and Premabati, 2002). Melatonin is synthesized from the amino acid tryptophan. During the light phase, tryptophan is converted to 5-hydroxytryptophan by the enzyme tryptophan-5-hydroxylase. Then 5-hydroxytryptophan is decarboxyled by aromatic L-aminoacid decarboxylase enzyme to 5-hydroxytryptamine (serotonin). During the dark phase, serotonin is converted to N-acetylserotonin by the rate-limiting enzyme arylalkylamine-N-acetyltransferase (AA-NAT) and N-acetylserotonin is rapidly converted to N-acetyl-5-methoxytryptamine (melatonin) by the enzyme hydroxyindole-O-methyltransferase (HIOMT). Synthesis of melatonin shows marked daily rhythm with low values during the day and large increase at night (Vollrath, 1981; Zhdanova, and Wurtman, 2005). Although day-night pattern of melatonin synthesis and secretion is highly conserved among vertebrates, the signal transduction cascades and the molecular mechanisms that regulate melatonin production differ strikingly among different groups of vertebrates. These differences are prominent when the pineal of mammals, birds, and fish are compared (Klein *et al.* 1997; Korf *et al.* 1998; Gupta *et al.*, 2005).

The rate of melatonin synthesis is minimum during the daytime (light phase) and maximum during the night time (dark phase) in all vertebrates (Vollrath, 1981; Korf, 1999; Falcon *et al.*, 2009). This is mainly due to photoperiod-dependent cyclicity in the activity of the rate-limiting enzyme AA-NAT present in the pineal of all vertebrates (Privat *et al.*, 1999; Gupta *et al.*, 2005; Gupta and Spessert, 2007). As in other vertebrates, melatonin production in the fish pineal is regulated by the light-dark cycle, and it is also influenced by the environmental temperature (Max and Menaker, 1992; Zachmann *et al.*, 1992b; Samejima *et al.*, 2000).

Arylakylamine-N-acetyltransferase (AA-NAT)

Arylakylamine-N-acetyltransferase (AA-NAT; EC 2.1.3.87 Arendt, 1995) was first cloned independently from sheep and rat pineal glands, respectively (Coon *et al.*, 1995; Borjigin *et al.*, 1995). Subsequently chick, human, fish (Klein *et al.*, 1997) and amphibians (Isorna *et al.*, 2006) clones have been isolated by homology and characterised in detailed. AA-NAT expressed in the vertebrate pineal gland catalyzes the N-acetylation of the serotonin into N-acetylserotonin and is considered to be the rate limiting enzyme of the pineal melatonin synthesis. Circulating melatonin is elevated at night in all vertebrates, because AANAT activity increases in the pineal gland during the dark phase in response to signals from the circadian clock. There are remarkable species differences in the mechanisms for regulation of AANAT activity, including prominent transcriptional and post-transcriptional mechanisms (Stehle *et al.*, 2001, 2002; Ganguly *et al.*, 2002). In most vertebrates, activation of AA-NAT drives a 10-fold increase in melatonin synthesis and release approximately 5-6 h after the beginning of the night/dark phase. The half-life of AA-NAT protein/activity is approximately 3 min (Illnerova *et al.*, 1979; Ho and Chik, 1995; Gastel *et al.*, 1998). Tsuboi *et al.*, 2002, reported that an intramolecular disulfide bond appears to be involved in the regulation of AA-NAT activity and there also seems to be a possibility that intracellular redox conditions participate in the regulation of AA-NAT activity *in vivo* through opening and closing of the catalytic funnel. The nocturnal increase in circulating melatonin in vertebrates is regulated by 10- to 100-fold increases in pineal serotonin *N*-acetyltransferase (AA-NAT) activity.

Adrenergic regulation of AA-NAT in Homeotherms

Regulation of AA-NAT activity in the pineal gland of homeotherms varies considerably among different species, and involves transcriptional, posttranscriptional and posttranslational control mechanisms (Gupta *et al.*, 2005).

Adrenergic regulation of AA-NAT in mammalian pineal

The mammalian pineal is innervated mainly by post ganglionic sympathetic nerve fibres. Light stimulates melopsin-containing cells of retina and generates nerve impulses, which are transmitted by the optic nerves to the pineal gland via hypothalamus and superior cervical ganglia. The nerve endings gets hyper polarized due to the impulse generated by the presence of light. Due to hyper polarization, the release of norepinephrine (NE) in the pineal gland is inhibited during the light phase. With the onset of dark phase, the generation of impulse in the retina is inhibited which leads to depolarization in the sympathetic nerve fibres terminating the pineal gland. The depolarization in the nerve terminus leads to increased release of norepinephrine. The norepinephrine released from the adrenergic nerve terminal acts on the pinealocytes and stimulates AA-NAT activity followed by the increased rate of melatonin synthesis.

As mentioned earlier, melatonin is produced by the pineal in a rhythmic fashion with high levels of the hormone during night-time and low/basal levels during day-time (Vollrath, 1981; Korf, 1999; Falcon *et al.*, 2009), and the rhythmic production of melatonin is controlled directly by the diurnal rhythm of activity of arylalkylamine-N-acetyltransferase (AA-NAT; EC.2.3.1.87) – the rate-limiting enzyme of melatonin biosynthetic pathway in all groups of vertebrates (Coon *et al.*, 1995; Klein *et al.*, 1997; Dyda *et al.*, 2000). The circadian rhythm of melatonin synthesis involves three components, i.e., a photodetector, a circadian clock and the melatonin synthesizing machinery. The anatomical organization of these three components has evolved during the course of evolution. In mammals, retina acts as a photodetector and synchronizes a circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, while the melatonin synthesizing machinery is located in pinealocytes. The stimulatory and/or inhibitory circadian signals from the suprachiasmatic nucleus together with the photic information from the retina are conveyed by a multisynaptic pathway from the SCN through brain stem, spinal cord, superior cervical ganglia and postganglionic sympathetic nerve fibres to the pineal gland to drive the circadian rhythms of AA-NAT activity and melatonin (Drijfhout *et al.*, 1996; Pevet *et al.*, 1997; van Esseveldt *et al.*, 2000). In mammalian pineal, AA-NAT activity is photoperiodically controlled via Retina-SCN-SCG-NE-cAMP-PKA-CREB-CRE pathway (Klein, 1999).

In mammalian pineal, the rate of melatonin synthesis is regulated mainly by the primary neurotransmitter 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. As mentioned earlier, a central oscillator located in the hypothalamic SCN, which acts as the brain's clock, integrates photoperiodic information and precisely drives elevated nocturnal release of NE (van Esseveldt *et al.*, 2000). NE binds to the grooves formed by the transmembrane helices of the adrenergic receptors and activates them. The NE-activated β-adrenergic receptors (β-ARs) stimulate both adenylyl cyclase (AC) and cytoplasmic guanylyl cyclase (GC). First, NE-bound β-ARs activate stimulatory G-proteins (Gs) and facilitate dissociation of α-subunit (Gsα) from the complex of βγ-subunits. Then the Gsα binds to, and activates the enzyme AC leading to a 6-10 fold increase in cAMP accumulation (Vanecek *et al.*, 1985; Sugden *et al.*, 1996). The formation of cAMP activates a cascade of enzymatic reactions responsible for increased AA-NAT activity and melatonin synthesis. Simultaneously, β-ARs-induced activation of Gs (Ho *et al.*, 1989; Sugden and Klein, 1987) is also followed by increased nitric oxide synthase (NOS) activity and increased production of nitric oxide (NO) by a population of pinealocytes and simultaneous diffusion of NO into the cytoplasm of adjacent pinealocytes (Spessert *et al.*, 1998). Then NO binds to the heme group of cytosolic sGC resulting in its activation and a 2-4-fold increase in cGMP accumulation in pinealocytes (Spessert *et al.*, 1995). In contrast, the binding of NE to α_1-adrenergic receptors (α_1.ARs) does not have any measurable effects on cAMP and cGMP accumulation in pinealocytes (Schomerus *et al.*, 2002). However, simultaneous stimulation of α_1- and β-ARs results in more than 50-fold increase in cAMP and over 100-fold increase in cGMP accumulation (Vanecek *et al.*, 1985, 1986; Chik and Ho, 1989; Zawilska *et al.*, 1999). NE-induced increase in cGMP levels transduce signal via cGMP-Protein kinase G (PKG)-mitogen-activated protein kinase (MAPK) pathway. Elevation of intracellular Ca^{2+} and the enzyme protein kinase C (PKC) play critical roles in α_1-adrenergic potentiation of β-adrenergically stimulated cAMP accumulation (Ho *et al.*, 1987; Schomerus *et al.*, 2002). Several fold increases in 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, and on the other, stimulates formation of inhibitory transcription factor inducible cAMP early repressor (ICER) which inhibits *aa-nat* gene induction.

Photic stimulation of the retina leads to activation of melanopsin present in retinal ganglion cells and generates neural impulses. These light-induced impulses are transmitted via the non-visual retino-hypothalamic tract (RHT) to the SCN - the master clock that drives the circadian rhythm of melatonin synthesis in the pineal gland. Simultaneously, SCN also receives photic inputs from the intergeniculate leaflet (IGL) via geniculate-hypothalamic tract (GHT) and the raphe nuclei (Reuss, 2003). It is important to mention that SCN generates its own circadian rhythm, and the photic inputs from the retina only entrain the endogenous circadian rhythm of SCN (Gillette and Mitchell, 2002; Stehle *et al.*, 2003). The SCN generates impulses, which regulate the switching 'on' and 'off' of melatonin synthesis by regulating the rhythmic secretion of

NE in the pineal gland. Based on studies involving measurements of melatonin content and *aa-nat* gene expression following bilateral SCN lesions, PVN lesions and SCG removal, it has been proposed that the circadian rhythm of melatonin synthesis is regulated by a combination of a constant but weak stimulatory and a strong rhythmic inhibitory SCN output to PVN (Perreau-Lenz *et al.*, 2003). It has been found that in rats gamma-aminobutyric acid (GABA) release from SCN is involved in the light-induced inhibition of melatonin synthesis during daytime (Kalsbeek *et al.*, 1999), and blocking of GABA-ergic transmission from SCN to PVN increases melatonin synthesis during daytime (Kalsbeek *et al.*, 2000). In other words, during daytime, neural signals originating from the SCN influence the sympathetic nerve fibres *via* PVN-SCG pathway and inhibit the release of NE from the sympathetic nerve terminals present in the pineal gland. However, with the onset of darkness, SCN withdraws its inhibitory signals resulting in increased secretion of NE in the pineal gland.

The release of NE marks the switch 'ON' of melatonin synthesis. As mentioned earlier, NE acts on pinealocytes *via* α- and β-adrenergic receptors and activates adenylyl cyclase *via* Gs and accelerates formation of cAMP. The free catalytic subunits of activated PKA are translocated to the nucleus where they stimulate phosphorylation of cAMP response element binding protein (CREB). Besides PKA activation in the cytoplasm, cAMP may also enter into the nucleus and activate nuclear PKA, which can stimulate CREB phosphorylation. The phosphorylated CREB (pCREB) becomes capable of binding to the cAMP response element (CRE). The binding of pCREB to CRE leads to the activation of the *aa-nat* gene and formation of the AA-NAT mRNA. Additionally, pCREB also stimulates transcription of CREM gene and formation of ICER mRNA. The increase in the levels of AA-NAT mRNA is followed by a proportionate increase in the activity of the AA-NAT enzyme. The increased activity of the rate-limiting AA-NAT enzyme accelerates acetylation of serotonin to N-acetylserotonin, which is followed by several fold increase in the rate of melatonin synthesis and release. The stimulatory effects of NE on cAMP and AA-NAT activity are reportedly potentiated by several other neurotransmitters [e.g., vasoactive intestinal polypeptide (VIP), δ-sleep inducing peptide (DSIP), adenosine, serotonin etc.] that increase cAMP accumulation directly or indirectly (Gupta *et al.*, 1992; Simonneaux and Ribelayga, 2003; Faluhelyi *et al.*, 2006; Huang *et al.*, 2008).

Besides cAMP-PKA-CREB-CRE-*aa-nat* gene-induced increase in AA-NAT mRNA and AA-NAT activity, adrenergic stimulation has also been reported to switch 'ON' melatonin synthesis by at least four additional mechanisms, which do not involve formation of AA-NAT mRNA in the pineal of some mammals like ungulates, primates and humans (Klein *et al.*, 1997; Ganguly *et al.*, 2001; Coon *et al.*, 2002). These mechanisms are (i) direct cAMP-induced activation of AA-NAT, (ii) inhibition of proteasomal proteolysis of AA-NAT, (iii) activation and protection of AA-NAT due to complex formation with 14-3-3, and (iv) direct and indirect actions of Ca^{2+} on AA-NAT.

Unlike rodent pineals where melatonin synthesis increases slowly, there is very rapid increase in circulating melatonin levels at the beginning of the dark phase in human and some other species (Coon *et al.*, 2002), in which AA-NAT mRNA levels exhibit marginal diurnal fluctuations (Klein *et al.*, 1997). Further, while AA-NAT activity increased, AA-NAT mRNA levels of bovine pinealocytes were not altered following NE treatment (Schomerus *et al.*, 2000). These findings suggest that in some mammals, there is an alternative mechanism through which adrenergic stimulation of cAMP production stimulates melatonin synthesis without increasing *de novo* synthesis or activity of the AA-NAT protein as estimated in broken-cell preparations (Namboodiri *et al.*, 1985a, b). In order to find out whether cAMP can switch 'ON' melatonin synthesis without increasing AA-NAT synthesis or AA-NAT activity measured under V_{max}-conditions, a cell line (1E7) expressing human NAT (hNAT) has been developed. Studies on cAMP regulation in 1E7 cells indicate that treatment with forskolin, dibutyryl cAMP, isobutylmethylxanthine or isoproterenol activate cellular hNAT within intact cells by 8-fold without markedly increasing the abundance of AA-NAT protein or AA-NAT activity in broken cell preparations, while forskolin, isobutylmethyl-xanthine and isoproterenol stimulate cAMP accumulation (Coon *et al.*, 2001). These findings indicate that melatonin synthesis can be switched 'ON' by cAMP without increasing AA-NAT protein.

In mammals whose AA-NAT mRNA levels exhibit marginal or no diurnal fluctuation, cAMP stimulates accumulation of the AA-NAT protein during the night by affecting post-transcriptional processes. In these species, cAMP-dependent inhibition of proteasomal proteolysis of AA-NAT protein following adrenergic stimulation seems to play a dominant role in nocturnal switching 'ON' of melatonin synthesis (Gastel *et al.*, 1998; Fleming *et al.*, 1999). In the bovine pineal, inhibition of proteasomal proteolysis of

AA-NAT alone has been found to increase the enzyme activity by increasing AA-NAT protein levels by five- to ten-fold that may be responsible for switching 'ON' melatonin synthesis (Schomerus *et al.*, 2000). As in ungulates and primates, inhibition of proteasomal proteolysis has also been reported to increase accumulation of AA-NAT protein and activity in fish pineal (Falcon *et al.*, 2001). It seems that under unstimulated conditions AA-NAT protein is continuously synthesized and immediately degraded by the process of proteasomal proteolysis. The adrenergic stimulation of cAMP formation seems to protect AA-NAT protein from proteasomal proteolysis resulting in increased accumulation of the AA-NAT protein and activity followed by increased melatonin synthesis (Gastel *et al.*, 1998; Klein *et al.*, 1997). It has been suggested that the adrenergic signal may protect AA-NAT proteolysis *via* cAMP-dependent phosphorylation of two highly conserved AA-NAT PKA sites - the motifs proposed to be destined to be degraded by proteasomal proteolysis. Alternatively, the adrenergic signal can protect AA-NAT from degradation *via* cAMP-dependent phosphorylation of other proteins involved in targeting AA-NAT for proteasomal proteolysis. As mentioned earlier, adrenergically-induced post-translational modification of the existing AA-NAT protein seems to play a very critical role in increasing/maintaining AA-NAT activity (Klein *et al.*, 2003). Adrenergically regulated cAMP promotes the formation of AA-NAT/14-3-3 complex, which increases the AA-NAT activity and accelerates melatonin production by shielding PKA-phosphorylated AA-NAT from dephosphorylation and/or proteolysis as well as by decreasing the K_m of the enzyme for serotonin (Klein *et al.*, 2003).

Adrenergic stimulation of pinealocytes also leads to influx of Ca^{2+} from the extracellular fluid into the cytoplasm and increased release of the bivalent ion from intracellular storage sites (Marin *et al.*, 1996). The adrenergically increased Ca^{2+} levels are essential for full activation of AA-NAT. The cation binds directly to the AA-NAT protein and increases its affinity for serotonin and thereby enhances catalytic activity, accelerating melatonin synthesis (Gupta *et al.*, 2005). There are also indications that at least a part of the action of Ca^{2+} indirectly affects steps in the induction of AA-NAT activity/melatonin synthesis beyond the accumulation of cAMP (Santana *et al.*, 2001). Similar to cAMP-regulated PKA-induced phosphorylation of CREB in pinealocytes, Ca^{2+} influx has also been found to stimulate CREB phosphorylation *via* PKA-Rap1(Ras-related small G-protein)-ERK/MAPK pathway (Grewal *et al.*, 2000). Thus, in addition to cAMP as an adrenergic second messenger, Ca^{2+} also seems to play a critical supportive role in adrenergic mechanisms responsible for AA-NAT induction/activation and switching 'ON' melatonin synthesis.

The process of melatonin synthesis in mammalian pineal is turned 'off' primarily by SCN *via* a complex mechanism. Under natural conditions, the AA-NAT activity and melatonin synthesis decline rapidly in the second half of the dark phase (dawn). This decrease in AA-NAT activity and melatonin synthesis takes place mainly due to a decrease in SCN-regulated NE release associated with increased PDE activity and faster turnover of cAMP, decreased PKA activity, increased protein phosphatase activity, increased dephosphorylation of pCREB, decreased transcription of the *aa-nat* gene, increased proteasomal proteolysis of AA-NAT, and inhibition of the *aa-nat* gene due to high levels of ICER protein. Acetylcholine (ACh) secreted from the parasympathetic nerve fibres of the central pinealopetal projection may also counteract the adrenergic stimulation and inhibits melatonin synthesis by stimulating glutamate exocytosis (Yamada *et al.*, 1998a). Glutamate acts on pinealocytes *via* metabotropic glutamate receptors (mGluRs), stimulates inhibitory G-protein (Gi) and inhibits cAMP formation by inhibiting adrenergically-stimulated adenylyl cyclase (Yamada *et al.*, 1998b) and down-regulate melatonin secretion (Kim *et al.*, 2008) in the rat pineal. Glutamate seems to act as an auto/paracrine transmitter and neurotransmitter from neurons originating from the central nervous system and this excitatory transmitter may play an important role in modulating melatonin synthesis (Govitrapong *et al.*, 2007). All these inhibitory pathways, acting separately and/or in combination, terminate the adrenergic stimulatory signal, decrease AA-NAT activity and inhibit the process of melatonin synthesis in the pineal gland. Though the rhythm of nocturnal release of NE is closely regulated by the SCN, the rapid decline in AA-NAT activity and melatonin synthesis during the latter half of the dark phase cannot be entirely due to rhythmic nocturnal decrease in the levels of NE and cAMP. Several reports suggest that ICER plays a crucial role in restricting the amplitude of melatonin rhythm by its inhibitory influence on *aa-nat* gene transcription and thus may facilitate switching 'off' the melatonin synthesis.

The adrenergic signal transduction simultaneously activates both *aa-nat* gene and CREM gene via the ARs - cAMP - PKA - CREB - pCREB pathway. However, unlike AA-NAT mRNA levels that attain a peak during the first half of the night (dark phase), the observed increase in ICER mRNA levels precedes the decrease in AA-NAT mRNA, AA-NAT activity and melatonin synthesis. ICER seems to act as a very sensitive natural reporter for stimulated adrenergic pathways in rat pinealocytes, and binds directly to CRE element in the AA-NAT promoter and represses transcription of the *aa-nat* gene resulting in decreased activity of AA-NAT and inhibition of the rate as well as the amplitude of melatonin synthesis (Foulkes *et al.*, 2000; Stehle *et al.*, 2001). It seems that, during the first half of night, the ratio between pCREB (stimulatory TF) and ICER (inhibitory TF) in the rat pineal gland is in favour of the stimulatory TF due to drastic increase in the concentration of pCREB, which overrides ICER protein levels. However, as the duration of the dark phase increases, the intra-pineal pool of pCREB declines despite persistent NE release. The decrease in pCREB is caused probably due to increased amounts and/or activities of protein phosphatases (Maronde *et al.*, 1999b). It has been reported that NE-stimulation induces accumulation of protein serine/threonine phosphatase 1 (PSP1)-catalytic subunit in pineal nuclei, but does not affect the distribution of PSP2A-catalytic subunit (Koch *et al.*, 2003). Dephosphorylation of pCREB by PSPs seems to be an essential mechanism for the down-regulation of AA-NAT induction and melatonin biosynthesis. The decline in pCREB levels is followed by steady increase in ICER protein levels. As a result, the inhibitory effect of ICER overrides the stimulatory influence of pCREB on the *aa-nat* gene transcription, and thereby switches 'off' melatonin synthesis (Stehle *et al.*, 2001). Both AA-NAT mRNA levels and melatonin synthesis are increased drastically after the selective silencing of ICER in rat pinealocytes, probably due to uninhibited transcription of the *aa-nat* gene (Maronde *et al.*, 1999b). The adrenergic induction of the CREM gene and ICER formation is transient because steadily increasing ICER levels attenuate the transcription of cAMP-inducible genes including CREM gene (Foulkes *et al.*, 1996). Thus, ICER seems to repress its own production through a negative autoregulatory mechanism constituting the CREM feedback loop (Coon *et al.*, 2001). A critical analysis of the available information suggests that the balance between the ratio of pCREB (stimulatory TF) and ICER (inhibitory TF) levels determines the transcriptional activity of AA-NAT promoter (Gupta *et al.*, 2005). Due to diurnal shifts in the ratio of pCREB and ICER, the promoter cycles between activated and repressed states as a function of the day-night cycle. ICER levels overwhelm/dominate pCREB levels during the second half of night and switch 'off' the adrenergically stimulated melatonin synthesis.

In addition to ICER, Fos-related antigen 2 (Fra-2) was also supposed to be involved in turning 'off' the melatonin synthesis. While stimulating the transcription of the *aa-nat* gene, the adrenergic signals also simultaneously activate *fra-2* gene *via* pCREB and stimulate accumulation of Fra-2 mRNA that drives the 100-fold rhythm in Fra-2 protein. It seems that Fra-2 containing AP-1 complexes bind to AP-1 site at position -32 in the AA-NAT promoter (Baler *et al.*, 1997). The AP-1 binding site is located in close proximity of the major transcriptional start point, therefore, the binding of Fra-2 containing AP-1 complexes to this site was thought to disrupt the assembly of the basic transcription machinery and to have an inhibitory influence on expression of the *aa-nat* gene and melatonin synthesis. However, a recent report has indicated that Fra-2 expression does not have any inhibitory influence on *aa-nat* gene expression (Smith *et al.*, 2001). The switching 'off' mechanism for melatonin synthesis in the pineal of ungulates and primates may not involve transcriptional inhibition by adrenergically controlled inhibitory TFs like ICER. The inhibition of proteasomal proteolysis of AA-NAT is lifted due to declining levels of cAMP and/or pCREB in the later part of night, leading to increased breakdown of AA-NAT protein and switching 'off' of melatonin synthesis.

Adrenergically-induced increase in cAMP and cGMP levels is associated with increased activity of cAMP- and cGMP-dependent phosphodiesterase enzyme (PDE). The increase in the activity of the phosphodiesterase increases hydrolysis of cAMP, and thereby weakens the stimulatory effect of the adrenergic signals on AA-NAT induction and melatonin synthesis. PDE-induced decline in cAMP level decreases PKA activity, which in turn, leads to reduced phosphorylation of CREB and AA-NAT. This cascade leads to reduction in *aa-nat* gene expression and acceleration of dephosphorylation and proteasomal proteolysis of AA-NAT. As mentioned earlier, adrenergically-stimulated cGMP activates the MAPKK-MAPK pathway. Nocturnal and NE-induced expression of MAP kinase phosphatase-1 (MKP-1) and a possible interaction of MKP-1 with MAP kinases in rat pineal also support a functional role of cGMP-PKG-MAPKK-MAPK pathway in regulation of melatonin synthesis (Price *et al.*, 2004; Chansard *et al.*, 2005; Price *et al.*, 2007).

During the early hours of adrenergic stimulation, Ca^{2+} facilitates adrenergic stimulation of melatonin synthesis as discussed earlier. However, prolonged adrenergic signaling may result in accumulation of Ca^{2+} levels to critically higher levels, which might be suppressing AA-NAT activity and leading to commensurate inhibition of melatonin synthesis (Morton, 1989). Whether Ca^{2+}, which acts as a key regulator of gene expressions *via* CREB phosphorylation in other tissues, also influences AA-NAT induction and melatonin synthesis by activating CREM gene in pinealocytes remains to be established.

Adrenergic Regulation of AA-NAT in birds

The general mechanism of biosynthesis of melatonin in birds and mammals appears to be similar. As in mammals, the rhythmic changes in melatonin content of the avian pineal gland has been reported to be regulated primarily by changes in activity of the enzyme AA-NAT (Thomas *et al.*, 1998; Herichova *et al.*, 2001; Iuvone *et al.*, 2002). In the pineal of birds, AA-NAT and melatonin have been reported to exhibit a circadian rhythm with high levels during the night and low levels during the day (Rudeen *et al.*, 1990; Kato *et al.*, 1999; Natesan *et al.*, 2002). However, the mechanism controlling the night-time increase and the daytime decrease in AA-NAT activity has been reported to be regulated differently in mammals and birds (Binkley *et al.*, 1981). In mammalian pineal, the rhythm in melatonin synthesis is entirely driven by the SCN, which acts as a circadian oscillator. However, the bird pineal has been reported to retain a photoreceptive capability and possess a photic input pathway for regulation of melatonin synthesis (Underwood *et al.*, 2001; Natesan *et al.*, 2002). The avian pineal contains both circadian oscillator as well as pacemaker to drive circadian rhythm in the biosynthesis of melatonin and photoreceptors to synchronize the rhythm to environmental lighting (Oishi *et al.*, 2001; Okano and Fukada, 2003; Zawilska *et al.*, 2006). It has been reported that the circadian pacemaker of avian pineal gland oscillates both *in vivo* and *in vitro*, where light exposure suppresses the night-time increase in AA-NAT activity (Hamm *et al.*, 1983; Bailey *et al.*, 2003). When placed in organ and/or cell culture, the avian pineal express at least four circadian cycles of melatonin biosynthesis in the dark or dim red light (Zatz *et al.*, 1988; Lorenc-Duda *et al.*, 2008). Further, the chick pineal *in vitro* responds to environmental light in three ways: phase-shift of the circadian cycle, acute suppression of the melatonin biosynthetic release and increase in amplitude (Zatz *et al.*, 1988).

In birds, both direct photoreception and neurotransmitters have been reported to affect AA-NAT activity and melatonin synthesis (Voisin *et al.*, 1990; Zatz, 1991). In contrast to mammalian pineal, where NE stimulates melatonin biosynthesis *via* elevated cyclic AMP levels (White and Klein, 1995), the avian pineal shows an inhibition of the melatonin synthetic pathway following norepinephrine treatment (Voisin *et al.*, 1990). The different responses to NE of the mammalian and chicken pineal can be readily attributed to different types of adrenergic receptors and G-proteins involved in NE-induced signal transduction. In mammalian pineal, NE acts *via* α_1- and β_1-adrenergic receptors coupled to stimulatory G-proteins (Gs) and stimulates adenylate cyclase resulting in increased AA-NAT activity and melatonin synthesis (Gupta *et al.*, 2005). In the avian pineal, however, NE acts *via* α_2-adrenergic receptors coupled to inhibitory G-proteins (Gi) and inhibits adenylate cyclase activity resulting in inhibition of AA-NAT activity and melatonin synthesis (Zatz and Mullen, 1988; Zawilska and Iuvone, 1990; Nowak *et al.*, 1997). The avian pineal clock generates a rhythm in the abundance of *aa-nat* mRNA that can account for the free running rhythm in AA-NAT activity (Bernard *et al.*, 1997). In avian pineal, the transcriptional regulation of the *aa-nat* gene is mediated by the clock genes. Changes in *aa-nat* expression are mediated by the E-Boxes that have been found in the promoter region of the *aa-nat* gene (Natesan *et al.*, 2002). It has been reported that the clock-dependent nocturnal increase in *aa-nat* mRNA requires gene expression for the synthesis of melatonin (Bernard *et al.*, 1997; Natesan *et al.*, 2002). In mammals, both transcriptional activation and protection of AA-NAT protein against proteasomal proteolysis are provided by NE-induced cAMP formation. However, the transcriptional regulation of the *aa-nat* gene in chick pineal is mediated primarily by the clock genes, whereas protection of AA-NAT activity from proteosomal degradation is mediated by cAMP (Gastel *et al.*, 1998; Ganguly *et al.*, 2002; Natesan *et al.*, 2002).

Regulation of melatonin synthesis in reptiles and amphibians

As in mammals and birds, daily and seasonal variations in AA-NAT activity and melatonin content have been reported in pineal organ of both reptiles and amphibians, with the maximum value occurring during the night (Tosini and Menaker, 1996; Lutterschmidt *et al.*, 2002; Chiba *et al.*, 2005). However, unlike in mammals and birds, there is paucity of information on the mechanism of regulation of melatonin synthesis in reptiles and amphibians. Photoperiod and temperature seem to play an important role in the regulation of AA-NAT activity and melatonin production in pineals of reptiles and amphibians (Tilden and Hutchinson, 1993; d'Istria *et al.*, 1994; Moyer *et al.*, 1997). Pinealectomy lead to abolition of the circadian rhythm of plasma melatonin in the ruin lizard, *Podarcis sicula* (Foa *et al.*, 1992). It has been reported that both light and temperature are important modulators of pineal function in gecko (Moyer *et al.*, 1997). The pineal organ of reptiles can act as a photo- and thermo- transducer, which translates information on light and temperature into an internal cue in the form of pineal melatonin rhythm (Firth *et al.*, 1991; Hyde and Underwood, 2000). Melatonin rhythm in the sleepy lizard, *Tiliqua rugosa* has been reported to persist for at least 6 days at temperatures of 25 and 33 °C in constant dark (Firth *et al.*, 2006). Pinealectomy was reported to affect plasma melatonin level in the neotenic tiger salamander (Gern and Norris, 1979). In *Rana perezi*, increase in environmental photoperiod and temperature induced a day-night rhythm of plasma melatonin levels, while a decrease in environmental temperature abolished the melatonin rhythm (Isorna *et al.*, 2005). In the frog, *Rana tigrina*, the retino-pineal gland pathway appears to produce light-induced changes in pineal glands of frogs 1-month-old or older, but this pathway does not appear to function in 6-month-old frogs (Lee *et al.*, 1997). In cultured pineal of the green frog, *Rana perezi* AA-NAT activity and melatonin rhythms were similar to that observed *in vivo* under natural environmental conditions, where forskolin increased AA-NAT activity up to 2-fold and melatonin production upto 4-fold irrespective of lighting conditions. Similarly addition of cycloheximide to the pineal culture significantly reduced both nocturnal AA-NAT activity and melatonin release (Alonso-Gomez *et al.*, 2000).

Regulation of melatonin synthesis in fishes

The melatonin biosynthesis pathway and its molecular components in fishes are similar to other vertebrates (Vollrath, 1981; Kroeber *et al.*, 2000). In mammalian pinealocytes, the diurnal rhythm in AA-NAT expression is controlled by neural signals originating from the suprachaismatic nucleus of the hypothalamus which acts as 'Zeitgeber" (Foulkes *et al.*, 1997b). However, unlike mammalian pineal, the fish pineal contains a complete melatonin rhythm generating system, incorporating a photodetector, a circadian clock and melatonin synthesis machinery (Bolliet *et al.*, 1997; Falcon *et al.*, 2001). The fish pineal reportedly contains precursors of melatonin like 5-hydroxyl-tryptophan and 5-hydroxytryptamine (Kroeber *et al.*, 1998). In several species of fishes, the pineal has been reported to secrete melatonin as well as intermediate compounds (like 5-methoxytryptophol, 5-methoxyindoleacetic acid and 5-methoxy-tryptamine) of melatonin biosynthetic pathway (Falcon *et al.*, 1989; Max and Menaker, 1992). In fishes also AA-NAT is the rate-limiting enzyme in the melatonin biosynthesis pathway (Coon *et al.*, 1999; Benyassi *et al.*, 2000; Falcon *et al.*, 2001). The rhythm in AA-NAT activity in fishes, as in most vertebrates, is driven by circadian clocks, and the photoperiod resets and entrains the clocks (Coon *et al.*, 1999; Besseau *et al.*, 2007). The presence of AA-NAT activity has been reported in a number of piscine species (Morton and Forbes, 1988; Benyassi *et al.*, 2001). Although a single AA-NAT gene has been found in mammals, two *aa-nat* genes are expressed in fishes, which are designated as *aa-nat-1* and *aa-nat-2* (Coon *et al.*, 1999; Mizusawa *et al.*, 2000; Klein, 2006). Recently in teleost fish a third *aa-nat* gene has been reported (Coon and Klein, 2006). In pike, it has been reported that AA-NAT-1 is exclusively expressed in the retina and AA-NAT-2 in the pineal gland (Coon *et al.*, 1999; Gothilf *et al.*, 1999). Further, AA-NAT gene activity, AA-NAT activity and melatonin synthesis increase during darkness, and light inhibit AA-NAT activity and melatonin synthesis (Begay *et al.*, 1998; Coon *et al.*, 1999; Falcon *et al.*, 2001). In the rat pineal, light acts independently via downstream mechanism to turn off AA-NAT activity by initiating proteasomal proteolysis of AA-NAT protein (Gastel *et al.*, 1998). Similar proteasome-based mechanisms may function widely as selective molecular switches in vertebrate neural systems. In mammalian pineal, NE stimulates β-adrenergic receptors while α-adrenergic receptor potentiates the β-adrenergic receptor for the formation of cAMP (Chick and Ho, 1989). However, in fish pineal, the activation of β-adrenergic receptor has been reported to increase cAMP formation and NAT induction, while activation of α-adrenergic receptors inhibit AA-NAT activity (Falcon *et al.*, 1991; Thibault *et al.*, 1993; Gupta and Kharshiing, unpublished).

In the fish pineal, the nocturnal rise in melatonin synthesis is associated with an increase in cAMP production and Ca^{2+} entry through voltage gated channels, and light inhibits melatonin synthesis by decreasing cAMP content and closure of Ca^{2+} channels (Falcon and Gaildrat, 1997). It is noteworthy that the cAMP content of pineal is increased during darkness and decreased in the presence of light, and the diurnal rhythm of cAMP in the fish pineal is influenced by the light-dark cycle (Falcon and Gaildrat, 1997). In static organ culture, cAMP levels have been reported to be increased following treatment with forskolin (an activator of stimulatory G-protein) as well as by isobutylmethylxanthine and theophylline (inhibitors of phosphodiesterases) (Thibault *et al.*, 1993). In the rat pineal, the increased concentration of cAMP leads to phosphorylation of cAMP response element binding protein (CREB) and formation of phospho-CREB (pCREB) (Foulkes *et al.*, 1997b; Maronde *et al.*, 1999a). Then, pCREB activates the NAT gene followed by the formation of aa-*nat* mRNA and AA-NAT protein. Further, elevation of cAMP by 8-Bromo-cAMP- forskolin and 3-isobutyl-1 methylxanthine has been reported to increase the level of pCREB and melatonin in light or dark-adapted pineal of both rat and trout (Kroeber *et al.*, 2000). It has been reported that in pike pineal, light induced decrease in AA-NAT 2 protein in photoreceptor cells and light-induced decrease was blocked by inhibitors of the proteasomal proteolysis (Falcon *et al.*, 2001). Further, if the pike pineal glands were maintained under light at night, the treatment with these inhibitors increased AA-NAT 2 protein and activity (Falcon *et al.*, 2001). These findings suggest that proteasomal proteolysis is a conserved element in the regulation of AA-NAT activity in the vertebrate pineal.

In addition to cAMP, calcium has also been reported to play a crucial role in regulation of NAT activity and melatonin synthesis in the fish pineal (Begay *et al.*, 1994a,b; Meissl *et al.*, 1996; Gasser and Gern, 1997). The voltage-gated L-type calcium channels seem to play an important role in the regulation of calcium in both oscillating and non-oscillating fish pineal photoreceptor cells (Korf *et al.*, 1997). Dark-induced melatonin synthesis is reportedly inhibited following calcium depletion by antagonists of L-type and N-type voltage sensitive calcium channels (Gasser and Gern, 1997). Further, calcium channel antagonist-induced inhibition of dark-induced melatonin synthesis was abolished following treatment with dibutyryl cAMP. This suggests that Ca^{2+} acts upstream of cAMP in regulating melatonin synthesis the fish pineal. Ca^{2+} also seems to regulate fish pineal cAMP content *via* calciproteins. It has been reported that Ca^{2+} and/or Ca^{2+}-calciprotein complexes might be acting in fishes at two different sites, one involving regulation of cAMP metabolism and the other being independent of cAMP (Falcon, 1999; Begay *et al.*, 1994a, b).

Cholinergic Regulation in AA-NAT activity and melatonin synthesis in vertebrates

The role of cholinergic signal transduction cascades in pineal function, both homeotherms and poikilotherms especially in the regulation of melatonin production, is not fully understood. Even though, the muscarinic cholinergic receptors (mAChR) have been characterized in the pineal gland of sheep (Taylor *et al.*, 1980), rat (Finocchiaro *et al.*, 1989) which was also verified by autoradiography study revealed very low density of muscarinic receptors in the rat pineal gland (Laitinen *et al*, 1989), and cow (Govitrapong *et al.*, 1989). The presence of a parasympathetic innervation of the pineal gland has long been debated (Pujito *et al.*, 1999). However, localization of pinealopetal fibers originating in the pterygopalatine and the optic ganglia (Shiotani *et al.*, 1986; Møller and Liu, 1999) together with the demonstration of pineal cholinergic fibers in the rat (Hernandez *et al.*, 2004), chicken (Kasahara *et al.*, 2002), ferret (David *et al.*, 1973), rabbit (Romijn, 1973), monkey (David and Kumar, 1978), cow (Pujito *et al.*, 1991) and fish (Ekstrom and Korf, 1986; Samejima *et al.*, 1994). Moreover, the expression of the muscarinic receptor subtype, M1, was demonstrated in the rat pineal by in situ hybridization (Pujito *et al.*, 1994). Recently, nAChR has been cloned and expressed in the zebrafish brain (Ackerman *et al.*, 2009).

Reports on the presence of high affinity muscarinic cholinergic binding sites of a specific choline acetyltransferase, along with an inhibitory action of cholinomimetic agents on the activity of serotonin N-acetyltransferase, suggested that muscarinic cholinergic fibers may modulate the synthesis and actions of pineal melatonin (Pujito *et al*, 1991). In mammalian pineal gland, acetylcholine has been demonstrated to exert various effects including an inhibitory action on the NE-induced stimulation of melatonin production. In the pineals of Long Evans rat acetylcholine acting *via* muscarinic receptors possess the capacity to stimulate melatonin release during early ontogeny (Wagner *et al.*, 2000). Biochemical investigations shows binding sites for cholinergic ligands (Govitrapong *et al.*, 1989) in the bovine pineal gland. In contrast to rat pinealocytes, however, cholinergic and glutamatergic stimuli do not inhibit NE-induced increases in AANAT activity or in melatonin production in bovine pinealocytes. Thus, rat and bovine pinealocytes also differ with regard to cholinergic signalling. In neonatal pinealocytes, acetylcholine elevates Ca^{2+} *via* muscarinic rather than nicotinic acetylcholine receptors. In the second postnatal week, pinealocytes gain responsiveness to nicotine and gradually lose responsiveness to muscarinic cholinergic stimuli (Schomerus *et al.*, 1999). It appears that cholinergic innervation play an important role in pineal gland physiology by inhibiting melatonin synthesis via the activation of nAChRs (Stankov *et al.*, 1993; Yamada *et al.*, 1998a). In bovine pinealocytes, activation of both mAChR and nAChR increases intracellular Ca^{2+} (Schomerus *et al.*, 2002). Serotonin nerve endings in rat brain were also found to be sensitive to cholinergic stimulation (Rausch, *et al.*, 1985). More recent studies have demonstrated a cholinergic innervation of the pineal gland in the rat is from the parasympathetic nervous system (Korf *et al.*, 1996; Larsen *et al.*, 1998; Schafer *et al.*, 1998) and possibly from the medial habenula within the brain (Schafer *et al.*, 1998). The M1 receptor agonist carbachol was found to significantly increase both PKG activity and cGMP production in the SCN *via* a cGMP/PKG pathway to induce phase shifts (Liu *et al.*, 1997).

Unlike in mammals, there is paucity of information on the muscarinic cholinergic regulation of the pineal and AA-NAT activity and/or melatonin synthesis in other vertebrates. Though both muscarinic and nicotinic receptors have been reported to be distributed uniformly throughout the pineal gland of trout with the highest value for the high-affinity choline transporter occurred in the proximal portion in rainbow trout pineal organ (Samejima *et al.*, 1994). Putative cholinergic neurons have also been reported in the photosensory pineal organ of a cyprinid teleost, the European minnow (Ekstrom and Korf, 1986). Immuno-histochemically studies in the brain of two species of lampreys, *petromyzon marinus* and *lampetra fluviatilis* (Pombal *et al.*, 2001) also clearly shows that both the receptors are widely distributed in compared to studies carried in other vertebrates, suggesting that major cholinergic pathways are present in lampreys. On the whole these reports revealed that the organization of many cholinergic systems in the lamprey comprises features of the anamniote brain that remain common to all living amniotes studied so far (Pombal *et al.*, 2001). Recent studies have demonstrated that zebrafish have at least two muscarinic receptor genes (Hsieh and Liao, 2002).

Although activation of AA-NAT and melatonin biosynthetic pathway in vertebrates is mainly centered on norepinephrine stimulating adrenergic receptors, however, various other transmitters are also involved, directly and/or indirectly, in the modulation of AA-NAT activity and melatonin synthesis in the pineal gland of most vertebrates (Simonneaux and Ribelayga, 2003; Alonso-Gomez *et al.*, 2000; Cahill, 1997) which are also involved in day-time inhibition and night-time stimulation of pineal metabolism (Gariduo *et al.*, 2001). Neurotransmitters, particularly dopamine (Zawilska *et al.*, 2004; Iuvone *et al.*, 2005; Zilberman-Peled *et al.*, 2006; Kumar and Chaturvedi, 2009), acetylcholine (Yamada *et al.*, 1998a,b), serotonin (Hayashi *et al.*, 1999; Huang *et al.*, 2008) and Gamma-aminobutyric acid (Rosenstein *et al.*, 1990; Brandstatter, and Hermann, 1996). Neuropeptides such as vasopressin (Simonneaux *et al.*, 1990), vasoactive intestinal peptide (Nowicki *et al.*, 2002; Faluhelyi *et al.*, 2006), pituitary adenylate cyclase-activating peptide (Simonneaux *et al.*, 1990; Csernus *et al.*, 2004), δ-sleep inducing peptide, adenosine, neuropeptide Y etc are able to modulate the noradrenergic stimulation of melatonin synthesis in the pineal of vertebrates (Olcese, 1991; Gupta *et al.*, 1992; Simonneaux *et al.*, 1994; Simonneaux and Ribelayga, 2003). Similarly, substance P has been reported to inhibit the NE-induced increase in AANAT activity and melatonin secretion in mammals (Mukda *et al.*, 2009).

A critical review of the preceding information indicates that, unlike in homotherms, there is scarcity of information on the adrenergic signal transduction responsible for AA-NAT induction in the pineal organ of any poikilothermic vertebrates, particularly in the fish pineal. There is no information on the role of subtypes of α- and β-adrenergic receptors in mediation of NE action on AA-NAT induction. The role of cholinergic input in the regulation of AA-NAT activity in fish pineal remains to be investigated. Similarly, there is no information on the nature of G-proteins involved in adrenergic and cholinergic signal transduction in the fish pineal. Since melatonin influences a wide spectrum of fish physiology (e. g., breeding, migration, intermediary and oxidative metabolism, pigmentation, electrolyte balance, growth and development etc.) (Gupta and Premabati, 2002), a sound knowledge of the role of adrenergic and cholinergic signal transduction pathway involved in the regulation of AA-NAT activity and melatonin synthesis is essential. Similarly, there is also practically no information on the role of calcium ions, PKC, phosphodiestarases and protein phosphatases in the regulation of AA-NAT activity as well as in the fish pineal melatonin synthesis. Therefore, keeping in view the scarcity of information and critical phylogenetic position of the fish, it was thought worthwhile to undertake a comprehensive investigation to explore the adrenergic and cholinergic pathways in regulation of AA-NAT activity in the pineal organ of the air-breathing cat fish, *Clarias gariepinus*.

CHAPTER - I

Materials and methods

Introduction

Fishes are a group of vertebrates that has fully adapted to the aquatic mode of life and differs in many aspects of habits and habitats from the terrestrial vertebrates. During the course of evolution, they developed a special mechanism by which they could extract dissolved oxygen from water through their gills. The freshwater air breathing fishes supplement their aquatic respiration primarily by utilizing atmospheric oxygen (O_2) and release carbon dioxide (CO_2) aquatically (Hazel *et àl.*, 1993). The bodily functions of fish are directly or indirectly subjected to fluctuations in the environment.

All the experiments of this Ph. D. dissertation were conducted on the air-breathing fish, *Clarias gariepinus* due to its easy availability, excellent survival under laboratory conditions and reasonable cost throughout the year at Shillong. A brief introduction of the fish, techniques used for the measurement of AA-NAT activity, and the biostatistical methods used for the analysis of the data is presented in the following sections.

Clarias gariepinus

Clarias gariepinus (Burchell, 1822) is a teleost, which is widely distributed all over India and other tropical countries. This species usually live in a variety of freshwater environments, including quiet waters like lakes, ponds, and pools. They are also found in flowing rivers, rapids, and around dams (Teugels 1986). They are very adaptive to extreme environmental conditions, and can live in pH range of 6.5-8.0 (Ivoke *et al.*, 2007). They are able to live in very turbid waters and can tolerate temperatures of 8-35 °C. Their optimal temperature for growth is 28-30 °C (Teugels 1986).

C. gariepinus are bottom dwellers and do most of their feeding there. They are also obligate air breathers, which mean they do spend some time on the surface. This species can live in very poorly oxygenated waters and is one of the last species to live in such an uninhabitable place (Pienaar 1968). They are also able to secrete mucus to prevent drying and are able to burrow in the muddy substrate of a drying body of water (Skelton, 2001). This species can attain sizes of up to 1.7 meters including the tail and can weigh up to 59 kg when fully grown (Skelton, 2001). *Clarias gariepinus* breeds during monsoon (Suchiang, 2008). 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 propitious time of the year ensuring maximum survival and faster growth of the young ones. At Shillong, gonadal activity remains minimum (quiescent phase) during the months of January and February, which increases gradually during the months of March to May (progressive phase). Breeding occurs from June to August (breeding phase). Then gonads undergo regression during the months of November and December (regressive phase). This cyclic change in the gonadal activity is possibly cued by the external factors (e.g. water temperature, photoperiod etc) (Suchiang, 2008).

For this dissertation, adult male *Clarias gariepinus* (body weight: 90-100g; body length: 23-27 cm) were purchased from the local fish suppliers. Fishes were maintained in clear plastic tubs and acclimatized before starting any experiment at least for 15 days in the laboratory under natural climatic conditions at Shillong (Latitude 25°.30′ N, Longitude 91°.52′ E; Altitude 1450 ASL; Minimum water temperature: 4° C; maximum water temperature: 24.5 °C). During acclimatization, the fishes were fed daily with minced earthworms and commercial fish food ad libitum. Water was changed everyday to avoid infections.

Chemicals

All fine chemicals including hormones used in the experiments were purchased from Sigma-Aldrich, USA. ^{14}C-acetyl coenzyme A was purchased from Amersham Pharmacia Biotech (U. K).

Collection of pineals

It has been found in our laboratory that the AA-NAT activity in the photoreceptive pineal of *Clarias gariepinus* does not 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 immediately after the sunset. In order to collect pineal, the fish was decapitated under dim red light and the pineal window was quickly exposed with the help of a sterilized surgical blade. The pineal was rapidly removed, washed in culture medium and placed in the culture medium in a well of multi-well culture plate (Corning cell wells-25820, New York, USA).

Pineal organ culture

Dulbecco's Modified Eagles Medium (DMEM) supplemented with Bovine Serum Albumin (BSA), Calcium carbonate (CaCo3), Ascorbic acid and Penicillin-Streptomycin was used for the pineal culture using multi-well culture plate. After pre-incubation for one hour, the medium was removed and replaced with medium containing desired concentration of agonists/antagonists/inhibitors. The pineals were incubated for 6 hours at 25^0 C in an atmosphere of 85% O_2, 5% CO_2 and 95% relative humidity with the help of O_2-CO_2 gas incubator (Heraeus: Cytoperm). Pineals incubated in DMEM without any drugs were treated as control. After incubation, the pineals were removed and placed in numbered Eppendorf tubes, which were immediately frozen in liquid nitrogen for the measurement of AA-NAT activity.

Measurement of pineal arylalkylamine-N-acetyltransferase (AA-NAT) activity

Arylalkylamine-N-acetyltransferase (AA-NAT) activity was measured with the help of radio-enzymatic assay as described by Deguchi and Axelrod (1972) with slight modifications. For the measurement of AA-NAT activity, the pineals were sonicated in 75 μl of homogenization buffer (phosphate buffer: pH 6.5 with 6 nM acetyl Co-enzyme A). The 15 μl samples (in duplicate) of the sonicated pineals were incubated for 20 min at 25°C with 5 µl of the reaction mixture (tryptamine hydrochloride solution, phosphate buffer, 14C-acetyl Co-A). 100 µl of borate buffer was added to stop the reaction. In order to extract acetylated tryptamine, a mixture of isoamyl alcohol and toluene (3:97) was added to the tubes, and the tubes were rotated in a rotary mixer (Stuart Scientific, U. K) for 15 minutes, and centrifuged at 3000 rpm for 5 minutes. Two ml of the upper phase of the mixture was transferred to a scintillation vial containing 5 ml of scintillation fluid. The radioactivity in each sample was counted in terms of DPM with the help of a liquid scintillation counter (Wallace). Vials (in duplicates) treated as 'Blanks' contained homogenization buffer, reaction mixture, extraction solution, and scintillation fluid only. After calculations, AA-NAT activity was expressed as nmol/pineal/hour.

Analysis of data:

The data were analyzed statistically with the help of One-way ANOVA and regression analysis (Snedecor, 1961). A $p < 0.05$ was considered as significant.

These standard methods were followed in all experiments. The detail of the experimental protocol is described as follows:

Major steps in the measurement of AA-NAT activity

Remove pineal from the fish and transfer it to Eppendorf tube

↓

Store the tubes in liquid nitrogen

↓

Sonicate pineal in homogenization buffer

↓

Add reaction mixture to homogenate and incubate for 20 minutes

↓

Add borate buffer to stop the reaction

↓

Add extraction solution and mix for 15 minutes using rotary mixer

↓

Centrifuge for 5 minutes at 3000 rpm

↓

Take the upper phase

↓

Add scintillation fluid

↓

Count radioactivity (DPM) using liquid scintillation counter

↓

Calculate results

CHAPTER - II

In vitro studies on differential role of α and β adrenergic receptors and cholinergic receptors in adrenergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

Introduction

The effects of norepinephrine (NE) at the cellular level are mediated by G-protein coupled receptors (GPCRs) which are called adrenergic receptors or adrenoceptors. Both α- and β-adrenergic receptors are present on the membrane of mammalian and avian pinealocytes (Little *et al.*, 1996; Suh *et al.*, 1999; Gupta *et al.*, 2005; Zawilska, 1994; Nowak *et al.*, 1997). In addition, α- and β-adrenergic receptors are also present in the photoreceptor cells of the mammalian retina, which possess AA-NAT and produce melatonin (Wikberg-Matsson *et al.*, 1996; Lashbrook and Steinle, 2005).

In mammals, NE binds to both α_1- and β-adrenergic receptors present on the pinealocyte membrane and initiates adrenergic signal transduction *via* cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) generating pathways (Gupta *et al.*, 2005). In all mammalian species, including man, activation of β_1-adrenergic receptors leads to increased production of cAMP (Howell and Morgan,

1991) and AA-NAT induction (Vanecek *et al.*, 1985), while activation of α_1-adrenergic receptors does not influence AA-NAT induction in the pineal (Chik and Ho, 1989). However, simultaneous activation of α_1- and β_1-adrenergic receptors leads to several fold increase in cAMP and AA-NAT activity in mammalian pineal (Roseboom *et al.*, 1996; Klein *et al.*, 1997; Klein, 1999). On the other hand, α_1-adrenergic receptors have been reported to potentiate the action of β-adrenergic receptors on AA-NAT induction in the mammalian pineal (Klein *et al.*, 1983; Alphs and Lovenberg, 1984). In rat pineal, the nocturnal melatonin synthesis was reportedly suppressed by α_2.adrenoceptor agonists and/or possibly through a non-adrenergic mechanism of central origin (Mustanoja *et al.*, 1997, 1999, 2000). On the other hand, avian pineal AA-NAT activity and melatonin synthesis seems to be inhibited *via* the postsynaptic α_2-adrenergic receptors (Pratt and Takahashi, 1987; Voisin *et al.*, 1990; Zawilska, 1994; Holthues and Vollrath, 2004). It has been found that α_1-adrenergic receptors mediate inhibition of AA-NAT activity, while α_2-adrenergic receptors stimulate melatonin synthesis in the pineal of pigeon (Vakkuri *et al.*, 1992). The inhibition of AA-NAT activity in chick pineal cells by α_2-adrenergic agonist has been found to be strongly correlated with depolarizing concentrations of KCl (Voisin *et al.*, 1987) and an inhibition of cAMP accumulation (Voisin *et al.*, 1990; Nowak *et al.*, 1997).

The role of cholinergic signal transduction cascades in mammalian and avian pineal melatonin synthesis is not fully understood. However, localization of cholinergic fibres has been reported in the pineal gland of both mammals and birds (Laitinen *et al*, 1989, 1995; Han *et al.*, 2000; Sato and Wake, 1984; Kasahara *et al.*, 2002). In the rat pineal gland acetylcholine (Ach) level has been reported to exhibit day-night rhythm (Wessler *et al.*, 1997), and seems to play a modulatory role in melatonin production and adrenergic receptor function (Wagner *et al.*, 2000; Schenda and Vollrath, 1998). Further, it has also been reported that activation of nicotinic and muscarinic acetylcholine receptors did not seem to alter cAMP levels neither on AA-NAT activity nor melatonin production in the bovine pinealocytes (Schomerus *et al.*, 2002).

As in avian and mammalian pineal, both α- and β-adrenergic receptors are found in the fish pineal (Falcon *et al.*, 1991; Korf *et al.*, 1997). Further, as in the mammalian pineal, activation of β-adrenergic receptor in the fish pineal by NE has been reported to increase cAMP accumualtion and AA-NAT induction (Thibault *et al.*, 1993). However, unlike in mammals, activation of α-adrenergic receptors has been reported to inhibit AA-NAT activity (Falcon *et al.*, 1991). However, unlike in mammals and birds, there is practically no information on the differential role of α- and β-adrenergic receptors in mediation of adrenergically-induced AA-NAT induction in the fish pineal. Similarly, there is also paucity of information on the effects of simultaneous activation of both α- and β-adrenergic receptors on the adrenergic stimulation/induction of AA-NAT activity in the fish pineal AA-NAT activity.

Muscarinic cholinergic fibers are reportedly present in the pineal gland and brain of fishes (Samejima *et al.*, 1994; Zikopoulos and Dermon, 2005; Pombal *et al.*, 2001; Anadon *et al.*, 2000; Ackerman *et al.*, 2009), and seem to modulate the synthesis of pineal melatonin (Phansuwan-Pujito *et al*, 1991). Acetylcholine (Ach) has been shown to constitute a postsynaptic modulation of photoreceptor signals (Brandstatter *et al.*, 1995) and an increase ganglion cell activity in the trout pineal organ (Brandstatter and Hermann, 1996). However, there is practically no information on the cholinergic regulation of AA-NAT and melatonin in any fish species. There is also no information on whether cholinergic stimulation plays any role in adrenergic stimulation on AA-NAT activity in the fish pineal.

Therefore, keeping in view the scarcity of information, it was thought worthwhile to investigate the role of α- and β-adrenergic receptors and cholinergic receptors in the regulation of AA-NAT activity in the pineal of the air-breathing catfish, *Clarias gariepinus.*

Materials and Methods

For this study, *in vitro* experiments were conducted on the pineal of adult male air-breathing fish, *Clarias gariepinus.* Fishes were purchased from local fish suppliers, maintained in plastic tubs and acclimatized at least for 15 days in the laboratory under natural climatic conditions before starting any experiment. During acclimatization, the fishes were fed daily with minced earthworms and commercial fish food *ad libitum.* Water was changed every day to avoid infections.

In order to collect pineal, the fish was decapitated under dim red light and the pineal window was quickly exposed with the help of a sterilized surgical blade. The pineal was rapidly removed, washed in culture medium (DMEM) and placed in the culture medium in a well of multi-well culture plate (Corning Cell Wells, New York, USA) for organ culture and treatment with different agonists/antagonists/inhibitors.

The following experimental protocol was undertaken for the proposed study:

Experimental Protocol

Experiments	Agonists/Antagonists	Doses/Concentrations of Agonists/Antagonists
i) Dose-dependent *in vitro* effect of NE on AA-NAT activity	Norepinephrine (NE)	10^{-6} M, 10^{-4} M and 10^{-3} M
ii) *In vitro* effect of α_1- and β-adrenergic agonists and antagonists on AA-NAT activity during summer & winter	Isoproterenol (Iso)	10^{-3} M
	Phenylephrine (Phe)	10^{-3} M
	Propranolol (Prop)	10^{-3} M
	Prazosin	10^{-3} M
	Iso+ Phe	10^{-3} M Each
	Prop +Iso+Phe	10^{-3} M Each
	Prazosin + Iso+ Phe	10^{-3} M Each
iii) *In vitro* effects of α_2-adre-nergic agonist and anta-gonist on AA-NAT activity	NE	10^{-4} M
	Clonidine	10^{-4} M
	Yohimbin (Yohim)	10^{-4} M
	Yohim + NE	10^{-4} M Each
	Yohim + Clonidine	10^{-4} M Each
iv) Dose-dependent *in vitro* effect of carbachol on AA-NAT activity	Carbachol	10^{-6} M, 10^{-4} M and 10^{-3} M
v) *In vitro* effect of different doses of norepinephrine and carbachol on AA-NAT activity	NE +Carbachol (Carb)	NE (10^{-4} M) + Carb (10^{-3} M) NE (10^{-4} M) + Carb (10^{-6} M) NE (10^{-6} M) + Carb (10^{-3} M)

Pineal organ culture

The fish pineal were cultured following the process described in Chapter 1. The pineals were pre-incubated for one hour, after which the medium was removed and replaced with medium containing desired concentration of the agonists/antagonists. The pineals were then incubated for 6 hours at 25^0 C in an atmosphere of 85% O_2, 5% CO_2 and 95% relative humidity with the help of O_2-CO_2 gas incubator (Heraeus:Cytoperm, Germany). Pineals incubated in DMEM without any drugs were treated as control. After incubation, the pineals were removed and placed in numbered Eppendorf tubes, which were immediately frozen in liquid nitrogen for the measurement of AA-NAT activity.

Measurement of AA-NAT activity

AA-NAT activity was measured following the method described in detail in Chapter 1.

The data were analyzed statistically with the help of One-way ANOVA and regression analysis (Snedecor, 1961). A $p < 0.05$ was considered as significant.

Results

Dose-dependent *in vitro* effect of NE on AA-NAT activity:

The data are presented in Table 2:1; Fig. 2:1. Norepinephrine significantly increased AA-NAT activity in a dose-dependent manner. Regression analysis of the data indicates a positive correlation between the increasing doses of NE (10^{-6} M, 10^{-4} M and 10^{-3} M) and pineal AA-NAT activity.

In vitro effect of α_1- and β-adrenergic agonists and antagonists on AA-NAT activity:

The data are presented in Table 2:2; Fig. 2:2. *In vitro* treatment of the fish pineal with isoproterenol (a specific β_1 adrenergic receptor agonist) significantly stimulated fish pineal AA-NAT activity during both winter and summer months. However, when the pineals were treated with a combination of isoproterenol (10^{-3} M) and phenylephrine (a specific α_1-adrenergic receptor agonist), phenylephrine (Phe) did not potentiate the stimulatory effect of isoproterenol (Iso) on pineal AA-NAT activity irrespective of the seasons. When the pineals were first treated with propranolol (a specific β_1 adrenergic receptor antagonist) for 15 minutes followed by treatment with a combination of Iso (10^{-3} M) and Phe for 6 h, propranolol (Prop) completely blocked the isoproterenol-induced increase in AA-NAT activity during both the seasons. This treatment also dramatically reduced the Iso + Phe-induced increases in AA-NAT activity to levels below those found in unstimulated pineals during both the seasons. When pineals were pre-treated with 10^{-3} M solution prazosin (an α-adrenergic receptors antagonist) for 15 minutes prior to treatment with a combination of Iso + Phe (10^{-3} M) for 6 hr, prazosin treatment drastically reduced Iso + Phe-induced increase in AA-NAT activity during both the summer and winter seasons.

In vitro effects of α_2-adrenergic agonist and antagonist on AA-NAT activity:

The data are presented in Table 2:3; Fig. 2:3. Clonidine, (10^{-4} M) (specific α_2-receptor agonist) significantly increased AA-NAT activity, while yohimbin (10^{-4} M) (specific α_2-receptor antagonist) treatment drastically reduced basal AA-NAT activity. When the pineal were pre-treated with yohimbin for 15 minutes followed by 6 hr treatment with NE in one group and with clonidine in another group, in both the groups yohimbin counteracted the stimulatory effect of both NE and clonidine on pineal AA-NAT activity.

Dose-dependent *in vitro* effect of carbachol on AA-NAT activity:

The data are presented in Table 2:4; Fig. 2:4. *In vitro* treatment with three concentrations (10^{-6} M, 10^{-4} M and 10^{-3} M) of carbachol a specific agonist of the muscarinic M1 receptor significantly increased pineal AA-NAT activity in a dose-dependent manner. Regression analysis of the data revealed a positive correlation between the doses of carbachol and AA-NAT activity.

In vitro effect of combinations of different doses of NE and carbachol on AA-NAT activity:

When the fish pineals were treated with different combinations of high and low doses of NE and carbachol [NE (10^{-4}M) + Carbachol (10^{-3}M)], [NE (10^{-4} M) + Carbachol (10^{-6}M)], [NE (10^{-6}M) + Carbachol (10^{-3}M)], AA-NAT activity was not increased by any concentration of NE and carbachol in combination (Table 2:5; Fig. 2:5).

Discussion

To the best of our knowledge, this might be the first study of its kind in which attempts have been made to investigate the nature and role of subtypes on adrenergic and cholinergic receptors involved in regulation of AA-NAT activity in the pineal organ of any fish species. The results of the present study seem to suggest that both adrenergic and cholinergic mechanisms are directly involved in regulation of AA-NAT activity (hence melatonin synthesis) in the fish pineal (Tables 2.2 & 2.4; Figs. 2.2 & 2.4). A positive correlation between the increasing doses of NE or carbachol and AA-NAT activity (Tables 2.1 & 2.4; Figs. 2.1 & 2.4) seems to indicate a direct relation between the extent of adrenergic and cholinergic activations and AA-NAT activity, and hence melatonin synthesis.

Though both the adrenergic and cholinergic agonists, when administered *in vitro* separately, invariable stimulated the AA-NAT activity significantly (Tables 2:1 & 2:4; Figs. 2:1 & 2:4), however, none of the combinations of the adrenergic and cholinergic agonists could alter AA-NAT activity significantly (Table 2:5; Fig. 2:5). These observations seem to suggest that the adrenergic and the cholinergic mechanisms are separately capable of stimulating the enzyme activity, but these mechanisms counteract the stimulatory effect of each other when activated simultaneously.

In the present investigation, AA-NAT activity in the fish pineal was stimulated by both β_1-adrenergic and α_2-adrenergic agonists and α_1-adrenergic agonist failed in potentiating the enzyme activity (Table 2:2 & 2:3; Fig. 2:2 & 2:3). Further, propranolol completely blocked the Iso-induced increase in AA-NAT activity during both the seasons, and partially blocked the stimulatory effect of Iso + Phe during summer but not during winter (Table 2:2; Fig. 2:2). Similarly, prazosin treatment drastically reduced

Iso + Phe-induced increase in AA-NAT activity during both summer and winter seasons (Table 2:2; Fig. 2:2). It is important to note that clonidine significantly increase AA-NAT activity, while yohimbin treatment drastically reduced basal AA-NAT activity as well as also blocked the stimulatory effect of clonidine on AA-NAT activity (Table 2:3; Fig.2:3). On the basis of these findings it can be suggested that NE stimulates AA-NAT activity in the photoreceptive fish pineal organ mainly *via* β-adrenergic receptors, while α_1-adrenergic receptors seem to be essential for the stimulation of AA-NAT activity *via* β-adrenergic receptors. Further, α_2-adrenergic receptor also seems to be involved in adrenergic stimulation of AA-NAT activity. Though the β_1-adrenergic receptors are involved in the neural switching on of the AA-NAT activation, functional α_1- adrenergic receptors seem to be necessary for the stimulatory effects of NE on AA-NAT activity in the fish pineal. This might also be the first report indicating involvement of muscarinic M1 receptors in regulation of AA-NAT activity in the photoreceptive pineal in a fish species. In the mammalian pineal, only β_1-adrenergic agonists (but not the α_1-adrenergic agonists) stimulate AA-NAT activity, and α_1-adrenergic agonists reportedly potentiate the stimulatory effects of β_1-adrenergic agonists (Sugden *et al.*, 1984; Vanecek *et al.*, 1985; Chik and Ho, 1989; Klein *et al.*, 1983; Alphs and Lovenberg, 1984).

Unlike in the mammalian and the avian pineals where AA-NAT activity is regulated only by adrenergic mechanisms that involve mainly β_1.adrenergic receptors and α_2-adrenergic receptors, respectively (Vanecek *et al.*, 1985; Voisin *et al.*, 1990; Vakkuri *et al.*, 1992), in the fish pineal organ AA-NAT activity is regulated by both adrenergic and cholinergic mechanisms (findings of the present study). Further, the adrenergic stimulation of AA-NAT activity seems to involve β_1-, α_1- and α_2-adrenergic receptors. It, thus, seems that AA-NAT activity and melatonin in the fish pineal is regulated by a primitive non-specific regulatory mechanism involving both adrenergic and cholinergic inputs, and three subtypes of the adrenergic receptors. The regulatory mechanism probably became advanced during the course of evolution and involved only one subtype of adrenergic receptors and eliminated the role of cholinergic mechanism.

Table 2:1- Dose dependent *in vitro* effect of norepinephrine on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

Treatment	**AA-NAT activity (nmol/pineal/h)**
Control	1.08 ± 0.24 *
Norepinephrine (10^{-6}M)	2.04 ± 0.22 [a]
Norepinephrine (10^{-4}M)	3.12 ± 0.13 [b]
Norepinephrine (10^{-3}M)	4.20 ± 0.15 [b]

Correlation Coefficient (r) = 0.98

*All values are expressed as Mean ± Standard Error (S. E.); N = 4.

[a, b] Differ significantly from the control group: $p < 0.05$ and 0.01, respectively.

Table 2:2- *In vitro* effects of agonists and antagonists of α_1- and β_1-adrenergic receptors on AA-NAT activity in the pineal of catfish, *Clarias gariepinus* during summer and winter

Treatment	**AA-NAT activity (nmol/pineal/h)**	
	Summer	**Winter**
Control	0.83 ± 0.15[*]	0.70 ± 0.01
Isoproterenol (Iso)	1.41± 0.10 [b]	1.71 ± 0.07 [a]
Propranolol + Iso	0.31± 0.07 [f]	0.73 ± 0.08 [f]
Iso + Phenylephrine (Phe)	1.00 ± 0.07 [e]	1.49 ± 0.17 [a]
Propranolol + Iso + Phe	0.44 ± 0.07 [l]	1.22 ± 0.08 [a]
Prazosin + Iso + Phe	0.09 ± 0.07 [c, m]	0.18 ± 0.07 [a, m]

[*] All values are expressed as Mean ± Standard Error (S.E.); N = 4.

[a, b, c] Differ significantly from the control group: $p < 0.05$, 0.02, 0.01 and 0.001, respectively.

[e, f] Differs significantly from isoproterenol treated group: $p < 0.02$ and 0.001, respectively.

[l, m] Differ significantly from isoproterenol + phenylephrine treated group: $p < 0.02$ and 0.001, respectively.

Table 2:3- *In vitro* effects of α_2-adrenergic agonist and antagonist on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

Treatment	**AA-NAT activity (nmol/pineal/h)**
Control	$1.16 \pm 0.21^{*}$
Norepinephrine (NE; 10^{-4} M)	2.54 ± 0.12^{b}
Clonidine (10^{-4} M)	$2.25 \pm 0.14^{b, i}$
Yohimbin (10^{-4} M)	$0.45 \pm 0.12^{a, i}$
Yohimbin (10^{-4} M) + NE (10^{-4} M)	1.09 ± 0.08^{f}
Yohimbin (10^{-4} M) + Clonidine (10^{-4} M)	1.40 ± 0.10^{b}

[*] All values are expressed as Mean ± Standard Error (S. E.); N = 4.

[a, b] Differ significantly from the control group: $p < 0.02$ and 0.001, respectively.

[f] Differs significantly from yohimbin treated group: $p < 0.001$.

[i] Differs significantly from clonidine + yohimbin treated group: $p < 0.001$.

Table 2:4- Dose dependent *in vitro* effect of carbachol on AA- NAT activity in pineal of catfish, *Clarias gariepinus*

Treatment	AA-NAT activity (nmol/pineal/h)
Control	0.86 ± 0.04*
Carbachol (10^{-6} M)	0.70 ± 0.09
Carbachol (10^{-4} M)	1.08 ± 0.05 [b]
Carbachol (10^{-3} M)	1.14 ± 0.09 [a]

Correlation Co-efficient (r) = 0.78

*All values are expressed as Mean ± Standard Error (S. E.); N =4.

[a, b] Differ significantly from the control group: $p < 0.05$ and 0.01, respectively.

Table 2:5- *In vitro* effects of combinations of different doses of norepinephrine (NE) and carbachol on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

Treatment	**AA-NAT activity (nmol/pineal/h)**
Control	0.81 ± 0.05 *
NE (10^{-4} M) + Carbachol (10^{-3} M)	1.06 ± 0.12
NE (10^{-4} M) + Carbachol (10^{-6} M)	1.19 ± 0.18
NE (10^{-6} M) + Carbachol (10^{-3} M)	0.88 ± 0.06

* All values are expressed as Mean ± Standard Error (S. E.); N = 4.

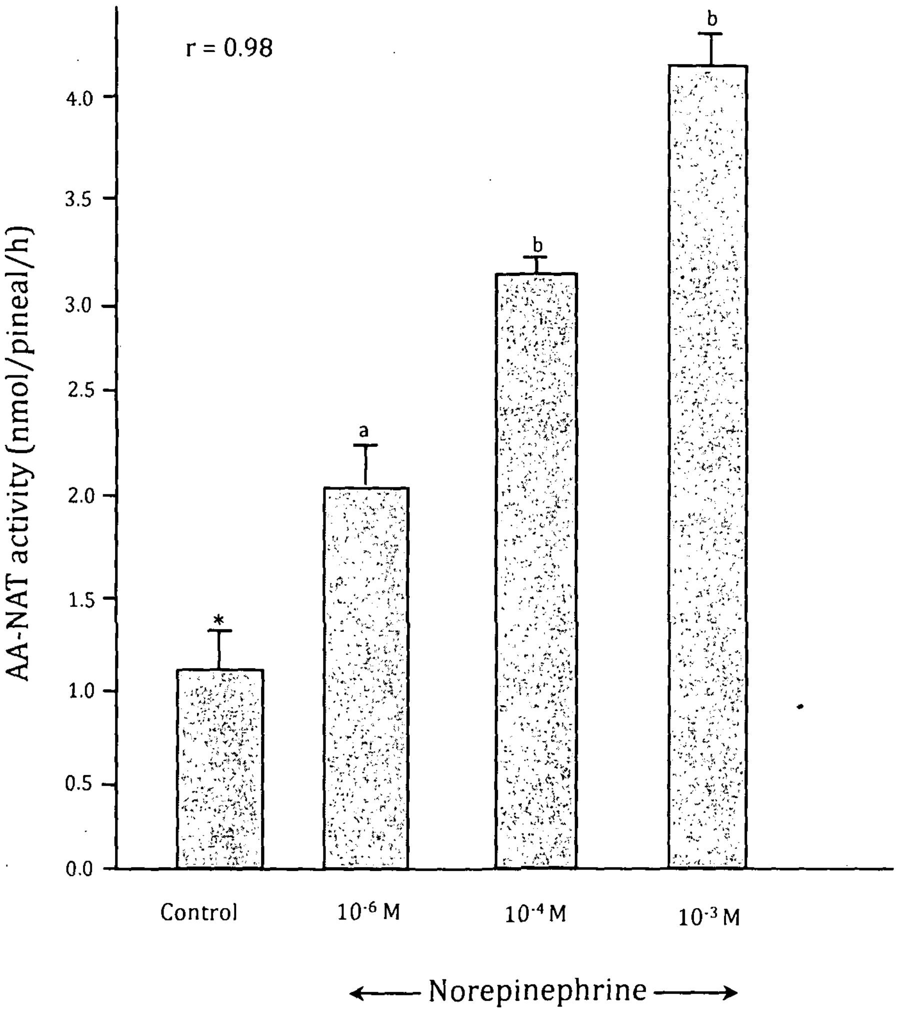

Figure 2:1- Dose-dependent *in vitro* effects of norepinephrine on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

*All values are expressed as Mean ± Standard Error (S. E.); N = 4

[a, b] Differ significantly from the control group: $p < 0.05$ and 0.01, respectively

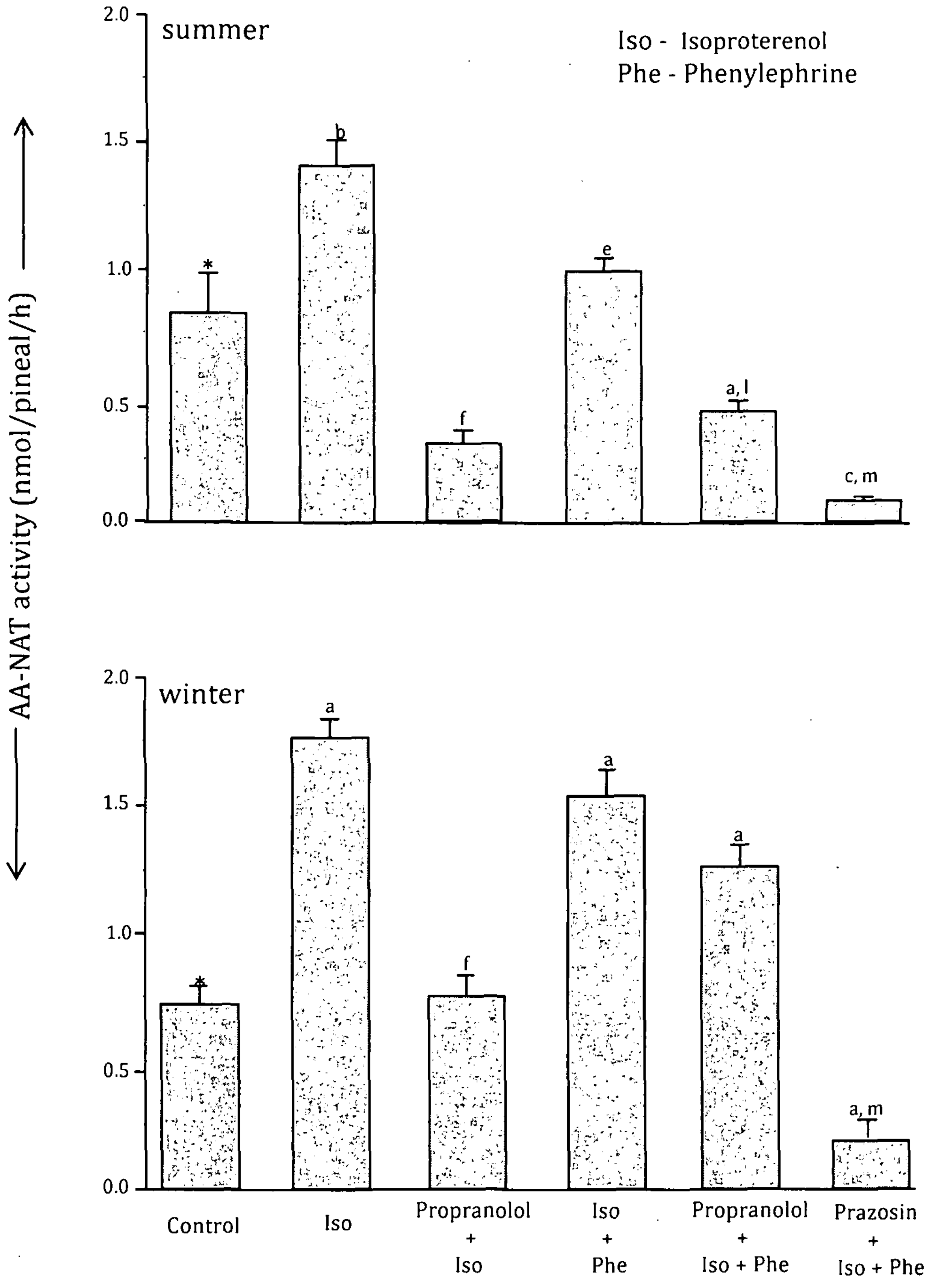

Figure 2:2 - *In vitro* effects of agonists and antagonists of α_1- and β_1-adrenergic receptors on AA-NAT activity in the pineal of catfish, *Clarias gariepinus* during summer and winter

All values are expressed as Mean ± Standard Error (S.E.); N = 4.

$^{a, b, c, d}$ Differ significantly from the control group: $p < 0.05, 0.02, 0.01$ and 0.001, respectively.

$^{e, f}$ Differs significantly from isoproterenol treated group: $p < 0.02$ and 0.001, respectively.

$^{l, m}$ Differ significantly from isoproterenol + phenylephrine treated group: $p < 0.02$ and 0.001, respectively.

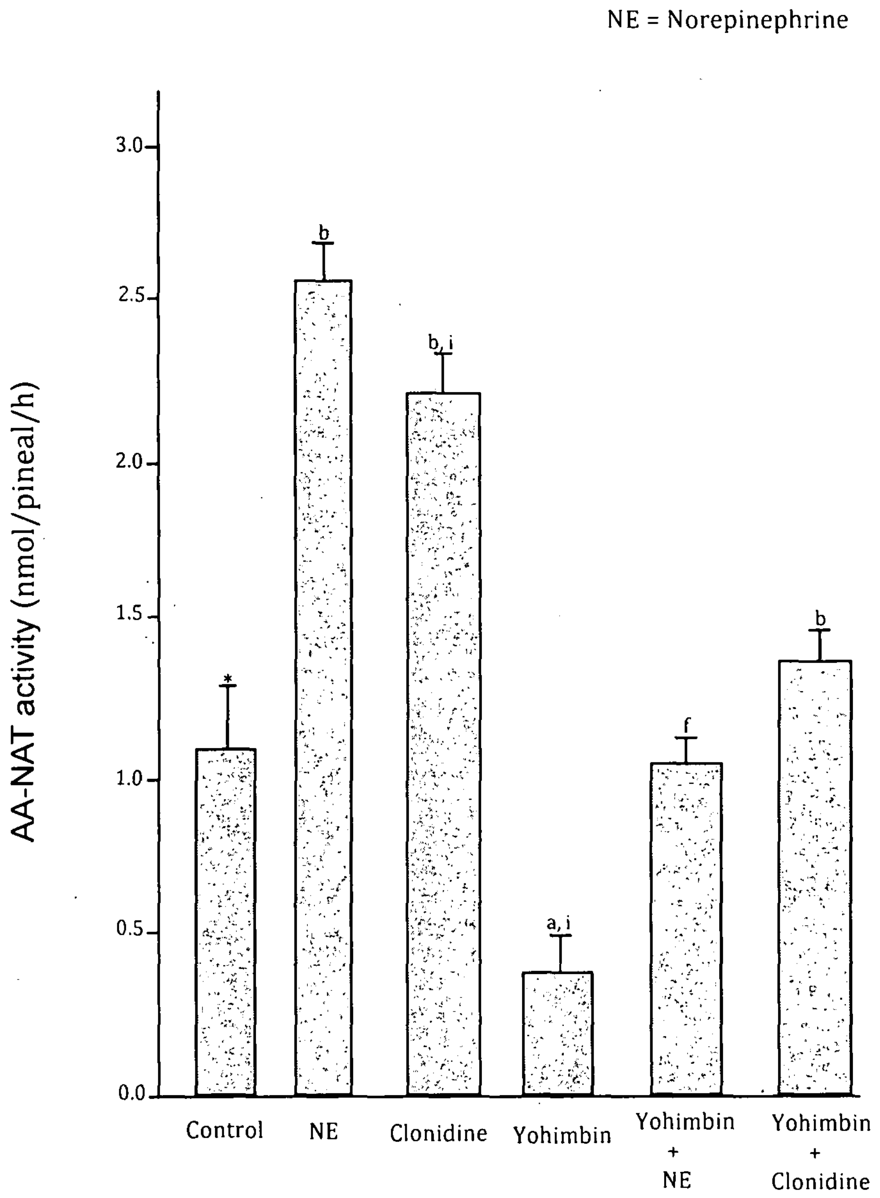

Figure 2:3 - *In vitro* effects of α_2-adrenergic agonist and antagonist on AA-NAT activity in the pineal of *Clarias gariepinus*

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
^{f} Differs significantly from yohimbin treated group: $p < 0.001$.
^{i} Differs significantly from clonidine + yohimbin treated group: $p < 0.001$.

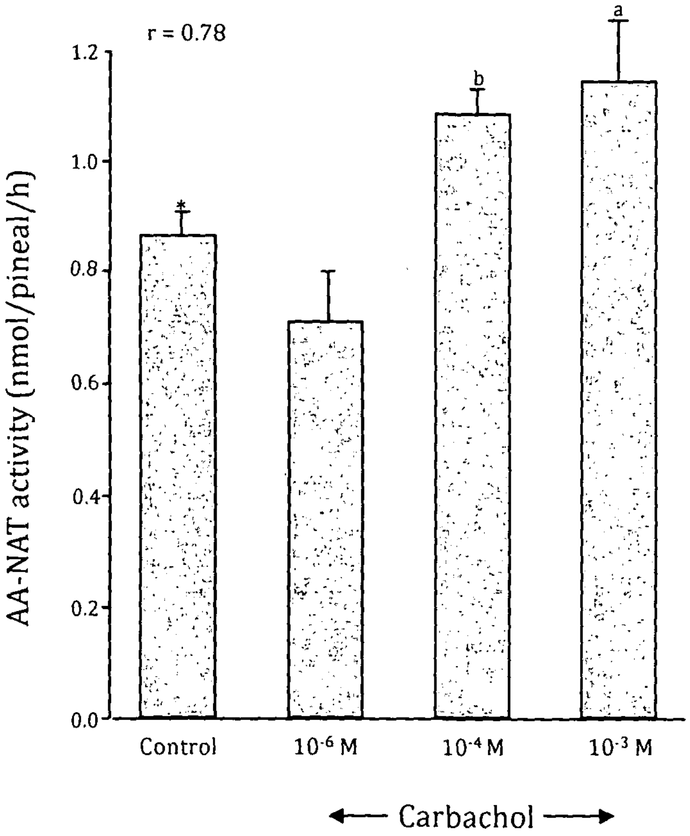

Figure 2:4 – Dose dependent *in vitro* effect of carbachol on AA-NAT activity in pineal of catfish, *Clarias gariepinus.*

*All values are expressed as Mean ± Standard Error (S. E.); N = 4.
^{a,b} Differ significantly from the control group.$p < 0.05$ and 0.01, respectively.

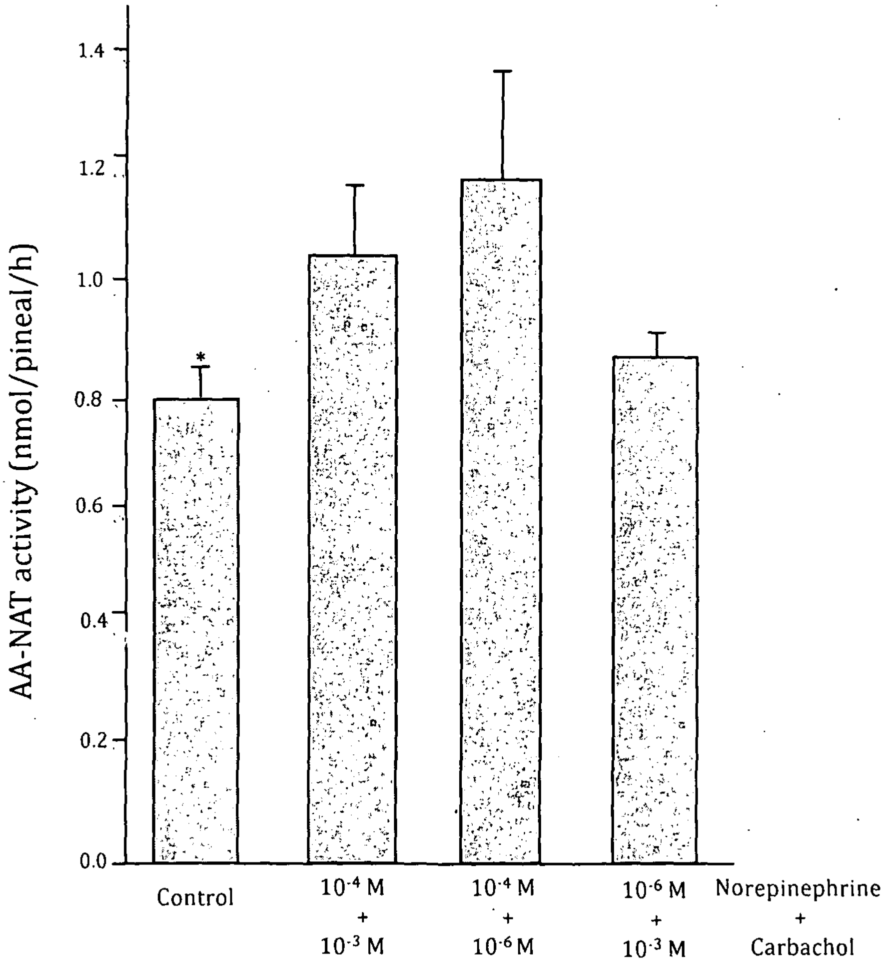

Figure 2:5 - *In vitro* effects of combinations of different doses of norepinephrine and carbachol on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

*All values are expressed as Mean ± Standard Error (S. E.); N = 4.

Chapter III

In vitro studies on the nature of G-protein (s) involved in adrenergic and cholinergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

Introduction

G-proteins play an important role in hormonal signal transduction by acting as regulatory links between cell-membrane-bound receptors and second messengers such as cAMP, cGMP, and certain phospholipids derivatives (Yoon *et al.*, 2008; Levin, 2009). According to their function, G-proteins are classified as (i) stimulatory G-proteins (G_s), which stimulate effectors (enzymes), (ii) inhibitory G-proteins (G_i), which inhibit effectors, and (iii) novel G-proteins (G_o) whose functions are not well established (Jiang and Bajpayee, 2009). Mammalian pinealocytes have been reported to contain G_s, G_i and Go (Sugden, 1990; Babila *et al.*, 1992; Gupta *et al.*, 2001).

In all mammalian species, melatonin production is regulated by the primary neurotransmitter norepinephrine (NE) which is released from sympathetic nerve fibres exclusively at night. NE binds to α_1- and β-adrenergic receptor present on the membrane of pinealocytes and triggers the adrenergic signal transduction *via* cAMP and cGMP in the pineal. The NE-activated β-ARs activate stimulatory G-proteins (Gs) and facilitate dissociation of α-subunit (Gsα) from the complex of βγ-subunits. Then the Gsα binds to and activates the enzyme adenylate cyclase (AC) leading to a 6-10 fold increase in cAMP accumulation (Vanecek *et al.*, 1985; Sugden *et al.*, 1996). The formation of cAMP activates a cascade of enzymatic reactions responsible for increased AA-NAT activity and melatonin synthesis (Roseboom *et al.*, 1996; Klein *et al.*, 1997; Klein, 1999). Simultaneously, β-ARs-induced activation of Gs (Ho *et al.*, 1989; Sugden and Klein, 1987) is also followed by increased nitric oxide synthase (NOS) activity and increased production of nitric oxide (NO) by a population of pinealocytes and simultaneous diffusion of NO into the cytoplasm of adjacent pinealocytes (Spessert *et al.*, 1998; Gupta *et al.*, 2005). Then NO binds to the heme group of cytosolic sGC resulting in its activation and a 2-4-fold increase in cGMP accumulation in pinealocytes (Spessert *et al.*, 1995). It is important to mention that β-adrenergic receptor potentiates the effects of nitroprusside on cGMP accumulation in intact rat pinealocytes through a mechanism involving Gs (White and Klein, 1995). Several fold increase in cAMP and cGMP levels follows with simultaneous stimulation of both α_1- and β-ARs (Vanecek *et al.*, 1985, 1986; Chik and Ho, 1989; Zawilska *et al.*, 1999) leading 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.

Unlike in mammals, NE shows inhibitory effect on melatonin release in the avian pineal (Zatz and Mullen, 1988). In birds, NE binds to α_2-adrenoceptors which is functionally coupled by PTX-sensitive G-protein (G_i) to adenylate cyclase (Pratt and Takahashi, 1988), inhibits cAMP accumulation and, in turn, inhibits AANAT activity and melatonin production during daytime (Kumar, 1996; Zatz *et al.*, 2000; Natesan *et al.*, 2002). G_{11} has been reported to mediate pineal phototransduction for entrainment of the pineal clock (Fukada and Kojima, 2007).

There is scarcity of information on the nature of G-proteins involve in cholinergic signal transduction in the pineal of vertebrates. However, cloning and expression of muscarinic M1 receptors in rat cell line has been reported to mediate multiple responses in the cell by coupling to different effectors, possibly to different G-proteins (Stein *et al.*, 1988). Acetylcholine (Ach) seems to play a modulatory role in the rat pineal (Schenda and Vollrath, 1998) by depressing noradrenaline release from intrapineal sympathetic fibres and hence melatonin synthesis (Wessler *et al.*, 1997). Ach acting through both M1AChR and M3AChR has been reported to mediate control of mouse thalamic activity *via* G(q)/G(11) G-proteins (Broicher *et al.*, 2008). M1 muscarinic acetylcholine receptor (mAChR) has been reported to couple to $G_{q/11}$ in chicken pineal cells in cultured (Kasahara *et al.*, 2002)

Retinal photoreceptors in mammals and birds and pineal photoreceptors of birds seem to share common components of G-protein signal transduction (Lolley *et al.*, 1992; Kasahara *et al.*, 2000). A Gα subunit of cone transducin has been localized on the outer segment of bovine cones (Lee *et al.*, 1992). Similarly, presence of a 70-kDa PKA-dependent phosphorylation of the 33-kDa protein complex with $G_{\beta\gamma}$ has been reported in the rat retina and in cow and sheep pineal glands (Reig *et al.*, 1990). Presence of putative G-proteins in the chicken pineal cells appears to be PTX-insensitive G-protein(s) (Okano and Fukada, 1997). Similarly, subsequent reports also show the endogenous photoreceptive molecule pinopsin in the avian pineal has been reported to functionally coupled with two types of G-proteins alpha (i.e. $G_{t1}\alpha$ and $G_{q/11}\alpha$) (Kasahara *et al.*, 2000) mediates the phase-shifting effect of light *via* a G-protein signaling pathway (Max *et al.*, 1995). Further, cDNA clones encoding $G_{i2}\alpha$, $G_{i3}\ \alpha$, and $G_{o1}\alpha$ and its splicing variant $Go_2\alpha$ in the chicken pineal gland has been isolated having potential site for pertussis toxin-catalyzed ADP-ribosylation (Okano *et al.*, 1997). Expression of G protein gammaT1 subunit gene has been reported in the pineal and retina of zebrafish during development (Chen *et al.*, 2007).

Notwithstanding these reports on the role of the different types of G-proteins in the mammalian and avian pineal, there is paucity of information on the nature of G-proteins involved in the adrenergic and cholinergic regulation/modulation of AA-NAT activity and/or melatonin synthesis in any fish species. Therefore, it was thought worthwhile to investigate the types of G-proteins involved in both adrenergic and cholinergic regulation of AA-NAT activity using specific agonists and antagonists.

Materials and Methods:

For *in vitro* investigations on pineal organ, adult male air-breathing fish, *Clarias gariepinus* (for other details, please see Chapter I). Fishes were purchased from the local fish suppliers and maintained in plastic tubs and acclimatized at least for 15 days before starting any experiment in the laboratory conditions. During acclimatization, the fishes were fed daily with minced earthworms and commercial fish food *ad libitum*. Water was changed every day to avoid infections.

In order to collect pineal, the fish was decapitated under dim red light and the pineal window was quickly exposed with the help of a sterilized surgical blade. The pineal was rapidly removed, washed in culture medium (DMEM) and placed in the culture medium in a wells of multi-well culture plate (Corning Cell Wells, New York, USA) for organ culture and *in vitro* treatment with NE and specific inhibitors of different types of G-proteins.

The following experimental protocol was followed for the proposed investigations:

Experimental Protocol

Experiments	**Agonists/Inhibitors**	**Concentrations of Agonists/inhibitors**
i) *In vitro* effects of norepinephrine, N-ethylmaleimide (NEM; specific inhibitor of sulfhydryl G-proteins), pertussis toxin (PTX; specific inhibitor of inhibitory G-proteins), and Guanosine 5′-[β-thio] diphosphate trilithium salt (G7637; specific stimulatory G-proteins) on adrenergic stimulation of pineal AA-NAT activity	Norepinephrine	10^{-4} M
	NEM	10^{-4} M
	NEM + NE	10^{-4} M + 10^{-4} M
	PTX	1μg/ml
	PTX + NE	1μg/ml + 10^{-4} M
	G7637	10^{-4} M
	G7637 + NE	10^{-4} M + 10^{-4} M
ii) *In vitro* effects of carbachol, N-ethylmaleimide, pertussis toxin, and G7637 on cholinergic stimulation of pineal AA-NAT activity	Carbachol	10^{-4} M
	NEM	10^{-4} M
	NEM +Carbachol	10^{-4} M + 10^{-4} M
	PTX	1μg/ml
	PTX + Carbachol	1μg/ml + 10^{-4} M
	G7637	10^{-4} M
	G7637+ Carbachol	10^{-4} M + 10^{-4} M

Pineal organ culture:

The fish pineal were cultured following the process described in Chapter 1. The pineals were pre-incubated for one hour, after which the medium was removed and replaced with medium containing desired concentration of NE and inhibitors as indicated in the "Experimental Protocol". The pineals were then incubated for 6 hours at 25^0 C in an atmosphere of 85% O_2, 5% CO_2 and 95% relative humidity with the help of O_2-CO_2 gas incubator (Heraeus:Cytoperm, Germany). Pineals incubated in DMEM without any drugs were treated as control. After incubation, the pineals were removed and placed in numbered Eppendorf tubes, which were immediately frozen in liquid nitrogen for the measurement of AA-NAT activity.

Measurement of AA-NAT activity:

AA-NAT activity was measured following the method described in detail in Chapter-1.

Statistical Analysis of Data: The data were analyzed statistically with the help of One-way ANOVA and regression analysis (Snedecor, 1961). A $p < 0.05$ was considered as significant.

Results

In vitro effects of norepinephrine, N-ethylmaleimide, pertussis toxin, and G7637 on AA-NAT activity:

The data are presented in Table 3:1; Fig. 3:1. The pineals were pre-treated *in vitro* with the different inhibitors 15 minutes prior to stimulation by norepinephrine. *In* *vitro* treatment of the fish pineal with norepinephrine (10^{-4} M) significantly increased AA-NAT activity. However, treatment of the fish pineal with N-Ethylmaleimide (NEM; specific inhibitor of sulfhydryl G-proteins), pertussis toxin (PTX; specific inhibitor of inhibitory G-proteins) and Guanosine 5′-[β-thio] diphosphate trilithium salt (G7637; inhibitor of stimulatory G-proteins) significantly inhibited the basal activity of AA-NAT (Table 3:1; Fig. 3:1). While NEM completely blocked the stimulatory effect of NE on AA-NAT activity, NE could stimulate AA-NAT activity partially but significantly in the pineal organs pretreated with the PTX and G7637.

In vitro effects of carbachol, N-ethylmaleimide, pertussis toxin, and G7637 on AA-NAT activity

The data are presented in Table 3:2; Fig. 3:2. The pineals were pre-treated *in vitro* with the different inhibitors 15 minutes prior to stimulation by carbachol. *In vitro* treatment of the fish pineal with 10^{-4} M carbachol significantly increased AA-NAT activity. Treatment of the fish pineal with 10^{-4} M solution of N-Ethylmaleimide (NEM; specific inhibitor of sulfhydryl G-proteins), 1μg/ml solution of pertussis toxin (PTX; specific inhibitor of inhibitory G-proteins) and 10^{-4} M solution of Guanosine 5′-[β-thio] diphosphate trilithium salt (G7637; inhibitor of stimulatory G-proteins) significantly inhibited basal AA-NAT activity. Carbachol stimulated AA-NAT activity significantly but partially as compared to the 10^{-4} M NEM and 1μg/ml PTX-treated groups. 10^{-4} M G7637 completely blocked the carbachol-induced increase in the enzyme activity.

Discussion

On the basis of the present findings, we suggest that all the three types of G-proteins (i.e., sulfhydryl G-proteins, PTX-sensitive G-proteins and Stimulatory G-proteins are involved in adrenergic and cholinergic stimulation of AA-NAT activity in the fish pineal (Tables 3:1 & 3:2; Figs. 3:1 & 3:2). The strong inhibitory effect of N-ethylmaleimide (NEM) on the NE-induced increase in AA-NAT activity (Table 3:1; Fig. 3:1) seems to indicate that sulfhydryl G-proteins play a major stimulatory role in adrenergic regulation of AA-NAT activity in the fish pineal. Conversely, NE could stimulate AA-NAT activity partially but significantly the pineal pretreated with PTX or G7637. These observations seem to suggest that PTX-sensitive G-proteins and stimulatory G-proteins are also involved in adrenergic stimulation of AA-NAT activity in the photoreceptive pineal organ of the fish. In the mammalian pineal, sulphydryl G-proteins and phospholipase A_2-associated G-proteins sub-types exert a clear modulatory effect on adrenergic signal transduction (Gupta *et al.*, 2005; Zhan-Poe and Craft, 1999) and members of G_i/G_o family play a minor role in the adrenergic signal transduction (Akiba *et al.*, 1992; Igarashi *et al.*, 1993) which seems to be in line with the present finding in the fish (Table 3:1; Fig. 3:1). However, in contrast to avian pineal, where PTX-sensitive G-protein (G_i) *via* α_2-adrenergic receptor is involved in the adrenergic regulation of AA-NAT activity and melatonin synthesis (Takahashi and Pratt, 1988). Thus, the earlier reports in mammals and present findings (Table 3:1; Fig. 3:1), when considered together indicated that while sulfhydryl G-proteins play an important role in adrenergic stimulation of AA-NAT activity, while PTX-sensitive G-proteins and stimulatory G-proteins are also involved in adrenergic stimulation of AA-NAT activity in the photoreceptive pineal of fish. This might be the first report of its kind establishing the nature of G-proteins involved in the adrenergic stimulation of AA-NAT activity in the photoreceptive pineal of a fish species.

G7637, specific inhibitor of stimulatory G-proteins significantly inhibited AA-NAT activity as well as completely blocked the carbachol-induced increased in the enzyme activity (Table 3:2 & Fig. 3:2). Further, carbachol could stimulate AA-NAT activity partially but significantly as compared to both NEM and PTX treated groups, respectively (Table 3:2 & Fig. 3:2). On the basis of the present findings, it can be suggested that the role of stimulatory G-protein appear to be more important in the cholinergic stimulation of AA-NAT activity, although PTX-sensitive and sulfhydryl G-proteins also seem to be involved in cholinergic stimulation of AA-NAT activity the photoreceptive pineal organ of the fish. It, thus, seems that the M1 receptors might regulate AA-NAT (please see Chapter II) through activation of stimulatory G-protein which differs from the mammalian system, where, inhibitory G-proteins have been reported to play a primary role in the muscarinic-cholinergic signal transduction in the pineal (Hare *et al.*, 1998).

It, thus, seems that the molecular mechanisms involved in the adrenergic and cholinergic regulation of AA-NAT activity in the photoreceptive fish pineal get diversified at the level of G-proteins. It remains to be established whether the dependence of the adrenergic and cholinergic mechanisms on different types of G-proteins results in counteraction of stimulatory action of these mechanisms on AA-NAT activity as reported in Chapter II.

Table 3:1-*In vitro* effects of norepinephrine, N-ethylmaleimide, pertussis toxin and G7637 on AA-NAT activity in the pineal of *Clarias gariepinus*

Treatment	**AA-NAT activity (nmol/pineal/h)**
Control	4.15 ± 0.23 [*]
Norepinephrine (NE; 10^{-4} M)	6.87 ± 0.69 [a]
N-ethylmaliemide (NEM; 10^{-4} M)	2.43 ± 0.04 [b]
NEM (10^{-4} M) + NE (10^{-4} M)	1.97 ± 0.01[b]
Pertussis Toxin (PTX; 1 μg/ml)	1.58 ± 0.01 [b]
PTX + NE (10^{-4} M)	2.82 ± 0.04 [a, d]
G7637 (10^{-4} M)	2.87 ± 0.07 [b]
G7637 (10^{-4} M) + NE (10^{-4} M)	3.04 ± 0.23 [b, g]

[*] All values are expressed as Mean ± Standard Error (S. E.); N = 4.

[a, b] Differ significantly from the control group: $p < 0.01$ and 0.001, respectively.

[d] Differs significantly from PTX treated group: $p < 0.001$.

[g] Differs significantly from G7637 treated group: $p < 0.01$.

Table 3:2- *In vitro* effects of carbachol, N-ethylmaleimide, pertussis toxin and G7637 on AA-NAT activity in the pineal of *Clarias gariepinus*

Treatment	**AA-NAT activity (nmol/pineal/h)**
Control	2.72 ± 0.02*
Carbachol 10^{-4} M)	2.98 ± 0.02 [b]
N-ethylmaliemide (NEM; 10^{-4} M)	1.27 ± 0.02 [b]
NEM (10^{-4} M) + Carbachol (10^{-4} M)	1.66 ± 0.04 [a, d]
Pertussis Toxin (PTX; 1μg/ml)	0.85 ± 0.02 [b]
PTX (1μg/ml) + Carbachol (10^{-4} M)	1.33 ± 0.07 [b, g]
G7637 (10^{-4} M)	1.39 ± 0.27 [b]
G7637 (10^{-4} M) + Carbachol (10^{-4} M)	0.97± 0.16 [b, j]

[*] All values are expressed as mean ± standard error (S. E.); N = 4.

[a, b] Differ significantly from the control group: $p < 0.02$ and 0.001, respectively.

[d] Differs significantly from NEM treated group: $p < 0.02$.

[g] Differs significantly from PTX treated group: $p < 0.001$.

[j] Differs significantly from G7637 treated group: $p < 0.001$.

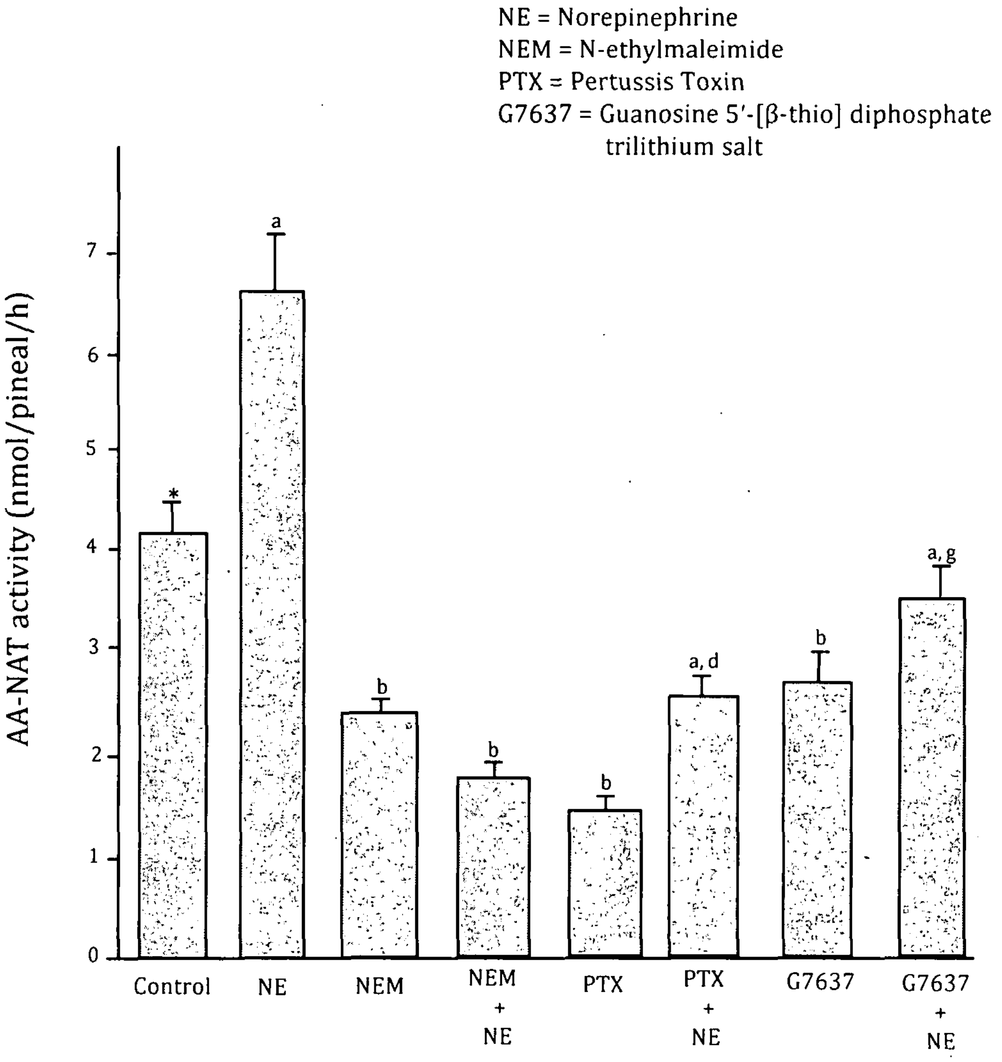

Figure 3:1 - *In vitro* effects of norepinephrine, N-ethylmaleimide, pertussis toxin and G7637 on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

*All values are expressed as Mean ± Standard Error (S. E.); N = 4.

[a, b] Differ significantly from the control group: $p < 0.01$ and 0.001, respectively.

[d] Differs significantly from PTX treated group: $p < 0.001$.

[g] Differs significantly from G7637 treated group: $p < 0.01$.

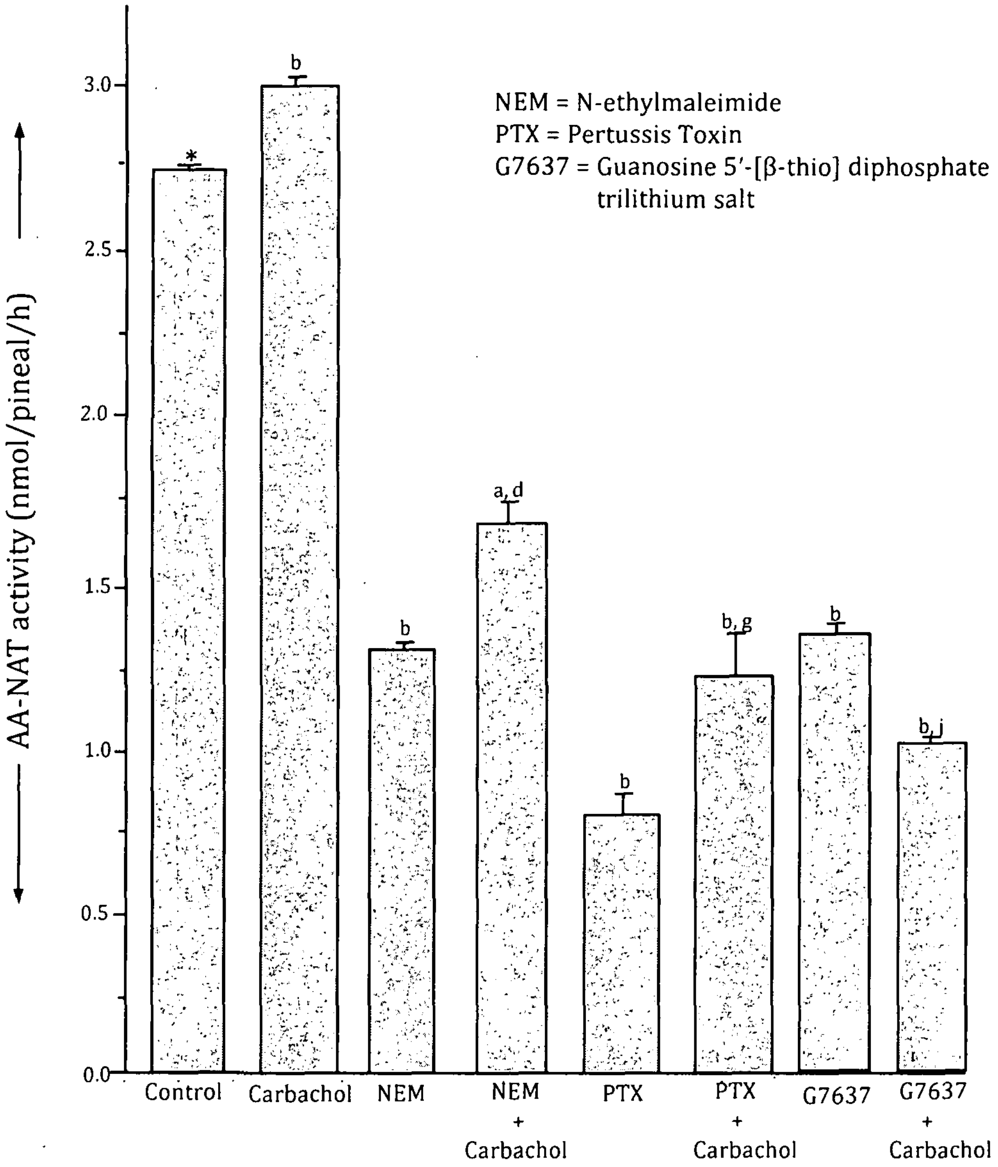

Figure 3:2 - *In vitro* effects of N-ethylmaleimide, pertussis toxin and G7637 on AA-NAT activity in the pineal of *Clarias gariepinus*

* 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.

^{d} Differs significantly from NEM-treated group: $p < 0.02$.

^{g} Differs significantly from PTX-treated group: $p < 0.001$.

^{j} Differs significantly from G7637-treated group: $p < 0.001$.

CHAPTER-IV

In vitro studies on the role of Ca^{2+}, protein kinase C, phosphodiesterase and serine/threonine phosphatase in adrenergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

Introduction

Calcium ions play an important role in the regulation of the sensitivity of α_1-adrenergic potentiation of β_1-adrenergic stimulation of cAMP and cGMP accumulation, AA-NAT induction, and the synthesis and release of melatonin in the mammalian pineal (Negishi *et al.*, 1998; Ho *et al.*, 2001; Chansard *et al.*, 2006; Lee *et al.*, 2006). Adrenergic stimulation of pinealocytes in mammals leads to influx of Ca^{2+} from the extracellular fluid into the cytoplasm as well as increased release of the Ca^{2+} from intracellular storage sites (Marin *et al.*, 1996; Yamada *et al.*, 2002; Barbosa *et al.*, 2008). There are also indications that at least a part of the action of Ca^{2+} indirectly affects steps in the induction of AA-NAT activity beyond the accumulation of cAMP (Santana *et al.*, 2001). In rat pinealocytes, Ca^{2+} influx triggered by NE significantly stimulated the protein level of AA-NAT, and is responsible for maintaining the Ca^{2+} response after repetitive stimulation (Lee *et al.*, 2005). Similarly, presence of voltage-dependent calcium channels, mainly of the L-type, was demonstrated in chick pineal cells (Harrison and Zatz, 1989), which are involved in the regulation of melatonin synthesis and secretion (D'Souza, and. Dryer, 1994). The nocturnal increase of AA-NAT activity in hen retina and pineal gland has been reported to be regulated *in vivo* by Ca^{2+} (Zawilska and Nowak, 1990). The effect of Ca^{2+} influx on the induction and stability of AA-NAT is at least partially mediated by increased levels of cyclic AMP (Zawilska *et al.*, 1992).

Activation of Calcium-dependent protein kinase C (PKC) 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 (Ho and Klein, 1987). Similarly, norepinephrine is well known to activate PKC (Abdel-Latif, 1986). PKC also directly phosphorylated the rat recombinant AA-NAT *in vitro* (Choi *et al.*, 2004).

In mammalian pineal, the enzyme cyclic nucleotide phosphodiesterase (PDE) stimulates breakdown of both cAMP and cGMP and thereby regulates cyclic nucleotide-dependent physiological processes (Kim *et al.*, 2007). In mammalian pinealocytes, intracellular levels of cAMP and cGMP are controlled by NE through both α_1- and β_1-adrenergic receptors in a synergistic manner *via* cAMP generating pathway, which also regulates AA-NAT activity and melatonin synthesis (Vanecek *et al.*, 1985; Klein *et al.*, 1997). Phosphodiesterases in the mouse pineal gland are unlikely to suppress AA-NAT induction because a phosphodiesterase inhibitor itself had no effect on the mRNA levels (Fukuhara *et al.*, 2005). The expression and characterization of PDE 6 in the pineal gland of chicken has also been reported, and the enzyme reportedly plays a role in the inhibition of AA-NAT activity and melatonin synthesis (Morin *et al.*, 2001).

Protein phosphatases (PSPs) have been reported to control AA-NAT protein levels and melatonin biosynthesis in mammalian pinealocytes (Spessert *et al.*, 2000, 2001) with a direct effect on the phosphorylation state of the AA-NAT protein (Hunter, 1995; Spessert *et al.*, 2001). Dephosphorylation of phosphorylated cAMP response element binding protein (pCREB) by PSPs seems to be an essential mechanism for the down-regulation of AA-NAT induction and melatonin biosynthesis in the mammalian pineal (Stehle *et al.*, 2001; Gupta *et al.*, 2005). Similarly, the expression of serine/threonine protein phosphatase 2A has also been reported in the chick pineal, which is highly circadian under both *in vivo* and *in vitro* (Olcese, 2003). Pharmacological inhibition of PSP-2A enzyme in chick pineal tissue leads to elevated phosphoCREB levels and concomitant melatonin secretion, indicating that this enzyme participates at some level in the control of nocturnal pineal melatonin synthesis (Olcese, 2003).

The nocturnal rise in melatonin synthesis in the fish pineal also seems to be associated with Ca^{2+} entry through voltage gated channels (Falcon and Gaildrat, 1997). In trout pinealocytes, Ca^{2+} and low calcium/high magnesium buffer reduced melatonin release through an action on the calcium concentration (Meissl *et al.*, 1996). Activation of the voltage-dependent L-type channel in these cells also modulates melatonin secretion (Begay *et al.*, 1994b) and synthesis (Abbink *et al.*, 2008). It has been reported that calcium ions act upstream of cAMP in regulating melatonin synthesis in the trout pineal photoreceptor cells (Gasser and Gern, 1997). Notwithstanding these reports on the physiological action of calcium in fish pineal, there is paucity of information on the role of calcium in the adrenergic regulation of AA-NAT activity and/or melatonin synthesis in any fish species. Elevation of cAMP levels following treatment with 8-

Bromo-cAMP, forskolin and 3-isobutyl-1 methylxanthine increased the levels of pCREB and melatonin in light or dark-adapted fish pineal organs (Kroeber *et al.*, 2000).

As compared to mammalian and avian pineal, there is practically no information on the role of PKC, PDE and serine/threonine phosphatases on adrenergic induction of AA-NAT and melatonin synthesis in the pineal organ of poikilothermic vertebrates in general and of fishes in particular.

A critical review of the preceding information indicates that, unlike in mammalian and avian pineal, there is lack of information on the role of Ca^{2+}, PKC, PDE and serine/threonine phosphatases in the regulation of AA-NAT activity and/or melatonin synthesis in the pineal organ of any fish species. Therefore, it was decided to investigate the role of Ca^{2+}, PKC, PDEs and serine/threonine phosphatases in adrenergic regulation of AA-NAT activity and melatonin synthesis in the photoreceptive pineal of catfish, *Clarias gariepinus*.

Materials and Methods

For this study, *in vitro* experiments were conducted on the pineal of adult male air-breathing fish, *Clarias gariepinus*. Fishes were purchased from local fish suppliers, maintained in plastic tubs and acclimatized at least for 15 days in the laboratory under natural climatic conditions before starting any experiment. During acclimatization, the fishes were fed daily with minced earthworms and commercial fish food *ad libitum*. Water was changed every day to avoid infections.

In order to collect pineal, the fish was decapitated under dim red light and the pineal window was quickly exposed with the help of a sterilized surgical blade. The pineal was rapidly removed, washed in culture medium (DMEM) and placed in the culture medium in a well of multi-well culture plate (Corning Cell Wells, New York, USA) for organ culture and treatment with different agonists/antagonists/inhibitors.

The following experimental protocol was followed for the proposed study:

Experimental Protocol

Experiments	**Agonists/Inhibitors**	**Concentration of Agonists/Inhibitors**
i) *In vitro* effects of nitrendipine (L-type voltage sensitive calcium channel blocker) on norepinephrine-induced AA-NAT activity	Norepinephrine (NE) Nitrendipine (NT) NT + NE	10^{-4} M Each
ii) *In vitro* effect of chelerythrine (specific inhibitor of PKC) on norepinephrine-induced AA-NAT activity	Norepinephrine (NE) Chelerythrine (Chel) Chel + NE	10^{-4} M Each
iii) Incubation period-dependent *in vitro* effect of NE and theophylline (specific inhibitor of PDE) on AA-NAT activity	Norepinephrine (NE) Theophylline (Theo) (Incuabation time: 30 min, 1 h, 4h & 6h)	10^{-4} M Each

iv) *In vitro* effects of okadaic acid (specific inhibitor of serine/threonine phosphatise 1), calyculin A (specific inhibitor of serine/threonine phosphatase 2A) and cypermethrin (specific inhibitor of serine/threonine phosphatase 2B) on norepinephrine-induced AA-NAT activity	Norepinephrine (NE) Okadaic Acid (OA) OA + NE Calyculin A (CA) CA + NE Cypermethrin (Cyp) Cyp + NE	10^{-4} M Each

Pineal organ culture

The fish pineal were cultured following the process described in Chapter I. The pineals were pre-incubated for one hour, after which the medium was removed and replaced with medium containing desired concentration of NE and specific inhibitors. The pineals were then further incubated for 6 hours at 25^0 C in an atmosphere of 85% O_2, 5% CO_2 and 95% relative humidity with the help of O_2-CO_2 gas incubator (Heraeus:Cytoperm, Germany). Pineals incubated in DMEM without any drugs were treated as control. After incubation, the pineals were removed and placed in numbered Eppendorf tubes, which were immediately frozen in liquid nitrogen for the measurement of AA-NAT activity.

Measurement of AA-NAT activity

AA-NAT activity was measured following the method described in detail in Chapter I.

Statistical Analysis

The data were analyzed statistically with the help of One-way ANOVA and regression analysis (Snedecor, 1961). A $p < 0.05$ was considered as significant.

Results

In vitro effects of nitrendipine on NE-induced AA-NAT activity:

The data are presented in Table 4:1 and Figure 4:1. The fish pineals were pre-treated *in vitro* with 10^{-4} M solution of nitrendipine (L-type voltage sensitive calcium channel blocker) for 15 minutes prior to stimulation by norepinephrine. Nitrendipine treatment significantly reduced the basal activity of the enzyme. Further, nitrendipine also completely blocked the NE-induced increase in the pineal AA-NAT activity.

In vitro effect of chelerythrine on NE-induced AA-NAT activity:

The data are presented in Table 4:2 and Fig. 4:2. 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 significantly decreased AA-NAT activity as compared to that of the control group. However, the chelerythrine chloride did not block the NE-induced increase in the enzyme activity.

Incubation period-dependent *in vitro* effects of norepinephrine and theophylline on AA-NAT activity:

The data are presented in Table 4:3 and Figure 4:3. *In vitro* incubation of the fish pineals with NE and theophylline (specific inhibitor of PDE) significantly increased

AA-NAT activity, and 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 AA-NAT activity:

The data are presented in Table 4:4 and Figure 4:4. 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; specific inhibitor of serine/threonine phosphatase 1) and calyculin A (CA; specific inhibitor of serine/threonine phosphatise 2A) 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, had no significant effect either on basal AA-NAT activity or on NE-induced increase in the enzyme activity.

Discussion

To the best of our knowledge, the present study seems to be the first of its kind in which the role of calcium ions, Ca^{2+}-dependent Protein kinase (PKC), phosphodiesterase and serine/threonine phosphatase (type-1, 2A and 2B) has been investigated in regulation of AA-NAT activity in photoreceptive pineal organ of a fish species. The results of the present study indicate that both Ca^{2+} and Ca^{2+}-dependent Protein kinase (PKC) act as an important component in the adrenergic regulation of

AA-NAT activity, and hence melatonin synthesis in the fish pineal organ (Table 4.1 & 4.2; Fig. 4.1 & 4.2). Blocking of the Ca^{2+} channel by nitrendipine significantly inhibited NE-induced AA-NAT activity and the enzyme activity was seen to reduce by nearly half following the treatment (Table 4:1 & Fig. 4.1). Likewise, AA-NAT activity in the fish pineal was significantly decreased following blocking of the PKC activity by chelerythrine (Table 4.2 & Fig. 4.2). The results suggest that the influx of calcium *via* the L-type voltage sensitive calcium channels seems to be essential for maintaining basal as well as for NE-induced AA-NAT activity. Similarly, PKC also seems to be involved in maintaining the basal activity of AA-NAT, but it does not seem to play any role in the adrenergic stimulation of AA-NAT activity since the PKC inhibitor, chelerythrine was unable to block the NE-induced increase in the enzyme activity (Table 4:2 & Fig. 4:2).

As in the case of mammalian and avian pineal, where L-type voltage sensitive calcium channels has been reported to be involved in the regulation of AA-NAT activity and melatonin synthesis (Lee *et al.*, 2005; Afeche *et al.*, 2006; D'Souza and Dryer, 1994; Zawilska and Nowak, 1990), melatonin synthesis in the pineal of some fishes has been associated with an increase in cAMP production and Ca^{2+} entry through L-type voltage gated channels (Falcon and Gaildrat, 1997; Korf *et al.*, 1997). Further, the present findings indicate that Ca^{2+}-dependent PKC is also involved in the regulation of AA-NAT activity in the fish pineal organ as reported in the mammalian pineal, where PKC has been reported to be involved in adrenergic stimulation of pineal cAMP accumulation (Ho *et al.*, 1987; Schomerus *et al.*, 2002), cGMP (Ho *et al.*, 1987) and AA-NAT activity (Choi *et al.*, 2004).

In the present investigation, AA-NAT activity in the fish pineal organ was significantly stimulated following treatment with norepinephrine and theophylline (PDE inhibitor). The stimulatory effect of the PDE inhibitor on AA-NAT activity was found 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 4:3 & Fig. 4:3). It is noteworthy that in the mammalian pineal, administration of theophylline in the dark phase cause a rise in pineal cAMP (Olivieri and Daya, 1992) and both AA-NAT activity and melatonin increased following treatment with PDE inhibitors (IBMX or aminophylline) irrespective of the time of the day (Zurawska and Nowak, 1992). It, thus, seems that as in mammals, the cyclic nucleotide-dependent phosphodiesterase plays a crucial role in regulation of AA-NAT activity (hence melatonin synthesis) in the fish pineal even in the absence of adrenergic stimulation.

Both Ser/Thr phosphatase 1 and 2A (PSP 1 and PSP 2A) significantly inhibited basal AA-NAT activity in the fish pineals organ (Table 4:4; Fig. 4:4). However, calyculin A was more effective in suppressing AA-NAT activity as compared to okadaic acid (Table 4.4; Fig. 4.4). 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 (Table 4.4 & Fig. 4.4). On the other hand, serine/threonine phosphatase 2B had no significant effect either on basal AA-NAT activity or on NE-induced increases in the enzyme activity (Table 4.4 & Fig. 4.4). It is important to mention that protein phosphatase in the mammalian pinealocytes control

AA-NAT protein levels and melatonin biosynthesis (Spessert *et al.*, 2000, 2001; Stehle *et al.*, 2001; Gupta *et al.*, 2005). Further, PSP-2A has also been reported to control the nocturnal pineal melatonin synthesis in the chicken pineal (Olcese, 2003). Thus, on the basis of the present findings, it can be suggested that Ser/Thr phosphatase 1 and 2A are essential for the basal as well as for adrenergic stimulation of AA-NAT activity in the fish pineal organ, and the presence of the PSP-1 and PSP-2A might serve as a key regulatory component in regulation of AA-NAT protein in the fish pineal. There is also a possibility 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 regulation of AA-NAT activity in the fish pineal organ.

On the basis of the present findings, we suggest that Ca^{2+}, calcium-dependent PKC, PDE and serine/threonine phosphatase 1 and 2A are involved in the regulation of AA-NAT activity and, hence melatonin synthesis in the photoreceptive fish pineal.

Table 4:1- *In vitro* effects of norepinephrine and nitrendipine on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

Treatment	**AA-NAT activity (nmol/pineal/h)**
Control	1.64 ± 0.04 [*]
Norepinephrine (NE; 10^{-4} M)	2.23 ± 0.11 [a, e]
Nitrendipine (NT; 10^{-4} M)	0.94 ± 0.01 [a]
NT (10^{-4} M) + NE (10^{-4} M)	0.67 ± 0.05 [a, d]

[*] All values are expressed as Mean ± Standard Error (S.E); N = 4

[a] Differs significantly from the control group: $p < 0.01$.

[d, e] Differ significantly from Nitrendipine treated group: $p < 0.02$ and 0.001, respectively.

Table 4:2- *In vitro* effect of norepinephrine and chelerythrine on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

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 4: 3- Incubation period-dependent *in vitro* effects of norepinephrine and theophylline on AA-NAT activity in the photoreceptive pineal of catfish, Clarias gariepinus

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^{c}	2.61 ± 0.21^{c}	3.33 ± 0.04^{b}	4.35 ± 0.02^{b}
Theophylline (10^{-4} M)	1.22 ± 0.08^{c}	1.53 ± 0.05^{c}	3.49 ± 0.01^{b}	4.58 ± 0.06^{c}

*All values are expressed as Mean ± Standard Error (S. E.); N = 4.

$^{a,\, b,\, c}$ Differ significantly from the control group: $p < 0.02$, 0.01 and 0.001, respectively.

Table 4:4- *In vitro* effects of norepinephrine, okadaic acid, calyculin A and cypermethrin on AA-NAT activity in the pineal of catfish, *Clarias gariepinus*

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

*All values are expressed as Mean ± Standard Error (S. E); N = 3.

[a, b, c] Differ significantly from the control group: $p < 0.05$, 0.01 and 0.001, respectively.

[d] Differs significantly from okcadaic acid treated group: $p < 0.001$.

[g] Differs significantly from calyculin A treated group: $p < 0.001$.

[j] Differs significantly from cypermethrin treated group: $p < 0.001$.

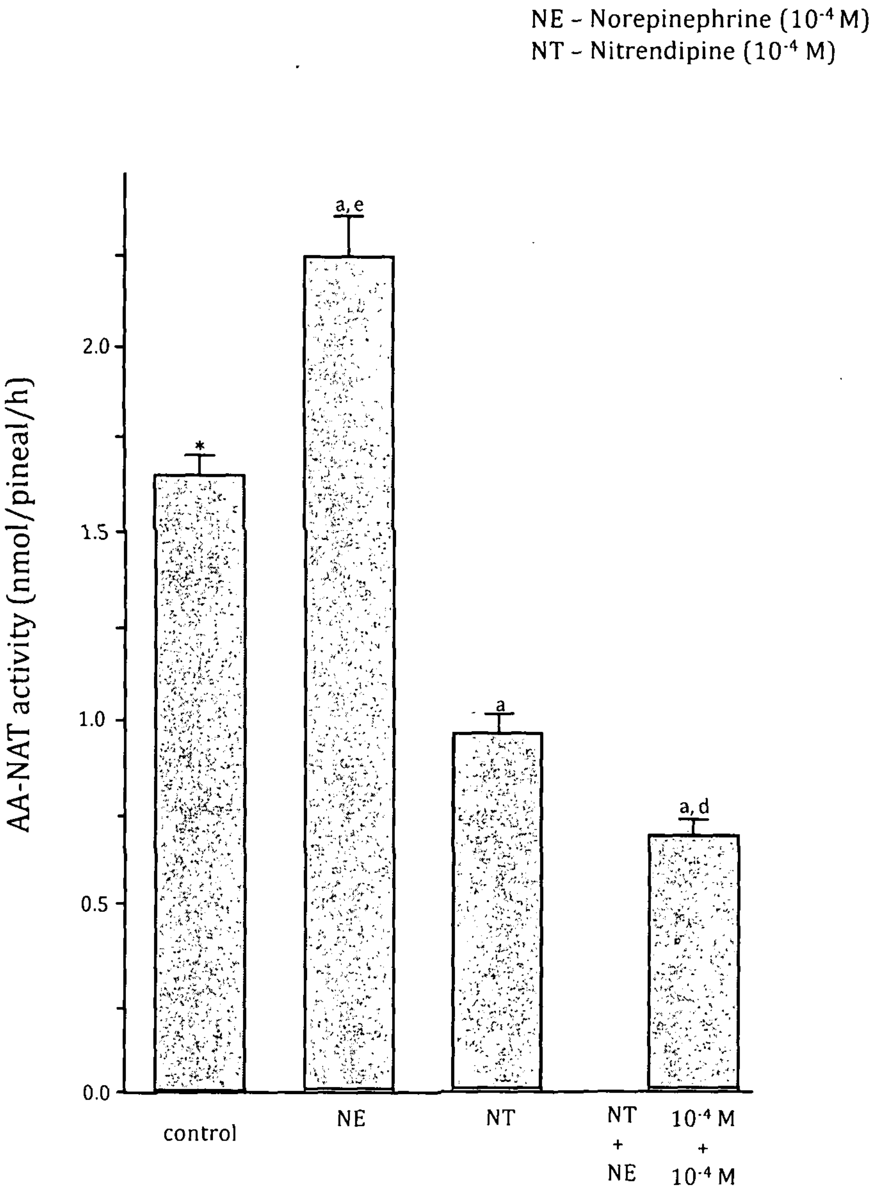

Figure 4:1 - *In vitro* effects of norepinephrine and nitrendipine on AA-NAT activity in the pineal of *Clarias gariepinus*

*All values are expressed as Mean Standard ± Error (S.E.); N= 4.

[a] Differs signficantly from the control group: $p < 0.01$.

[d] Differ significantly from the nitredipine treated group: $p < 0.02$ and 0.001, respectively.

NE = Norepnephrine
Chel = Chelerythrine

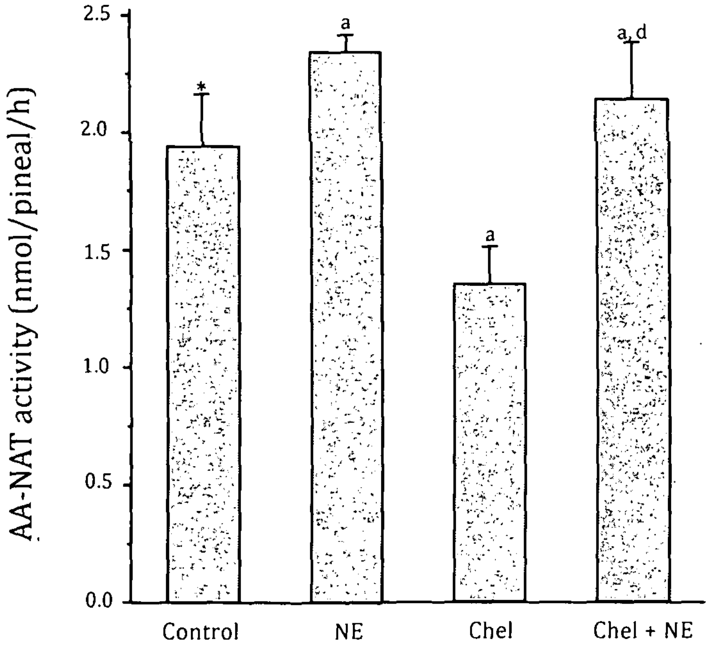

Figure 4:2 - *In vitro* effect of norepinephrine and chelerythrine on AA-NAT activity in the pineal of catfish, *Clarias gariepinus.*

* 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: $p < 0.05$.

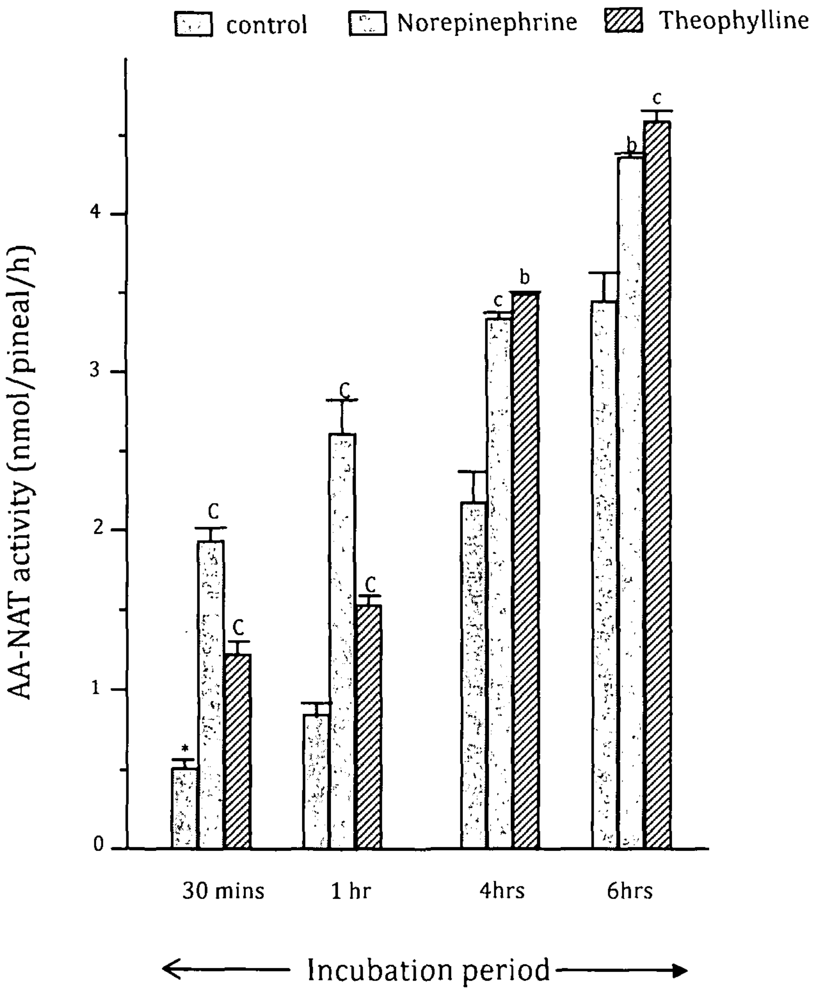

Figure 4:3 - Incubation period-dependent *in vitro* effect of norepinephrine and theophylline on AA-NAT activity in the pineal of *Clarias gariepinus*

*All values are expressed as Mean ± Standard Error (S.E); N = 4.
[a,b,c] Differ significantly from the control group: $p < 0.02$, 0.01, and 0.001, respectively.

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

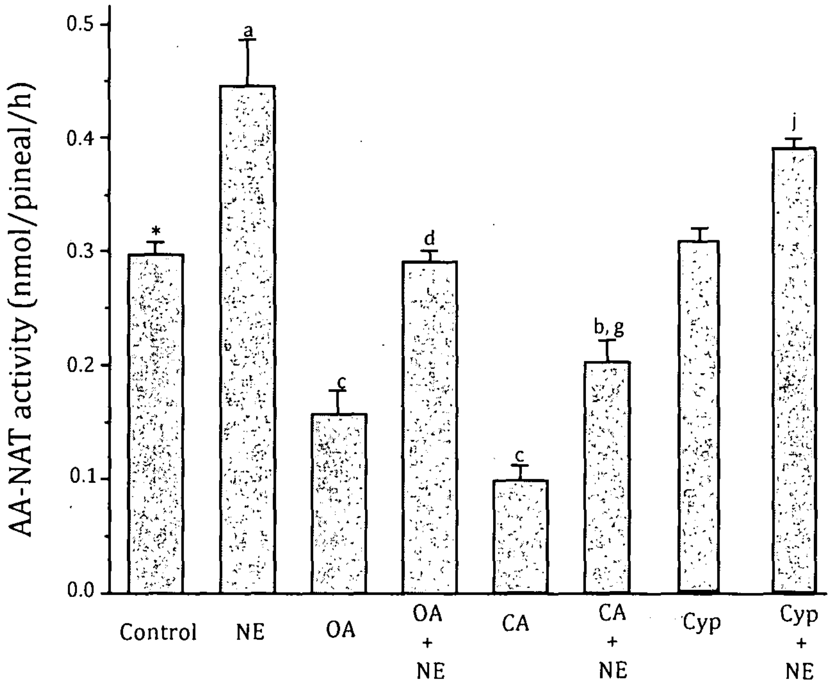

Figure 4:4 - *In vitro* effects of norepinephrine, okadaic acid, calyculin A and cypermethrin on AA-NAT activity in the pineal of *Clarias gariepinus.*

* All values are expressed as mean ± standard error (S. E.); N = 4.

[a, b, c] Differ significantly from the control groups: $p < 0.05$, 0.01 and 0.001, respectively.

[d] Differs significantly from the okadaic acid treated group: $p < 0.001$.

[g] Differs significantly from the calyculin A treated group: $p < 0.001$.

[j] Differs significantly from the cypermethrin treated group: $p < 0.001$.

CHAPTER - V

Summary and Conclusions

Arylalkylamine N-acetyltransferase (AA-NAT) is the key enzyme in the biosynthesis of melatonin in the pineal gland and retinal photoreceptors. AA-NAT is the rate-limiting enzyme of melatonin biosynthesis pathway which catalyzes the penultimate step in the melatonin synthesis accelerating conversion of serotonin to N-acetylserotonin (Klein *et al.*, 1997). AA-NAT controls daily changes in melatonin production by the pineal gland, and thereby plays a unique role in biological time-keeping in vertebrates. In mammalian pineal, primary neurotransmitter norepinephrine NE, by activating both α- and β-adrenergic receptors, causes 100-fold increases in cAMP and cGMP accumulation (Klein *et al.*, 1985) leading to increased AA-NAT activity and melatonin synthesis (Klein *et al.*, 1997; Gupta et al., 2005). However, there is paucity of information on the nature of neural regulation of AA-NAT activity in the photoreceptive fish pineal. There is also no information on adrenergic and cholinergic signal transduction pathways responsible for regulation of AA-NAT activity in the pineal of any poikilothermic vertebrates in general and in the fish pineal in particular. Though fish pineal also receives parasympathetic nerve fibers (Gupta and Premabati, 2002), but there is no information on effects of cholinergic stimulation separately or in combination with adrenergic stimulation on AA-NAT activity ~~on~~ the fish pineal. Further there is also no information on the nature of G-proteins involved in adrenergic signal transduction and stimulation of

AA-NAT activity in the fish pineal. Similarly, there is also practically no information on the role calcium, proteinkinase C (PKC), phosphodiesterases (PDEs) and serine/threonine phosphatases in the adrenergic stimulation of AA-NAT activity in the fish pineal organ. Therefore, keeping in view the paucity of information on the role of adrenergic and cholinergic mechanism, G-proteins, calcium ions, PKC, PDEs and serine/threonine phosphatases in the adrenergic and cholinergic regulation of AA-NAT activity in the fish pineal, a comprehensive investigation was undertaken to study *in vitro* the role of adrenergic and cholinergic mechanisms, subtypes of adrenergic and cholinergic receptors, types and role of G-proteins, PKC, PDEs and phosphatases in regulation of AA-NAT activity in the pineal organ of the fish, *Clarias gariepinus* using specific agonists, antagonists and inhibitors.

The present Ph. D. dissertation has been divided into five chapters. A brief summary of the chapters has been given below.

Chapter - I: Materials and Method

This chapter deals with the details of the experimental fish, maintenance of the experimental fishes under laboratory conditions, organ culture of the fish pineal, mode of *in vitro* treatment, methods used for measurement of AA-NAT activity and the biostatistical methods used for analyzing the data.

Chapter - II: *In vitro* studies on differential role of α and β adrenergic receptors and cholinergic receptors in adrenergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

This chapter deals with the study of *in vitro* effects of different concentrations of norepinephrine (potent adrenergic sitmulator), carbachol (a specific agonist of muscarinic M1 receptors) and different agonists and antagonists of both α1 and β1 adrenergic receptors, i.e, phenylephrine (a specific agonist of α1 adrenergic receptors), isoproterenol (a specific agonist of β1-adrenergic receptors), clonidine (a specific agonist of α2 adrenergic receptors), prazosin (a specific antagonist of α1 adrenergic receptors), propranolol (a specific antagonist of β1-adrenergic receptors) and yohimbin (a specific antagonist of α_2 adrenergic receptors) on AA-NAT activity in the pineal organ of fish, *Clarias gariepinus* maintained under natural climatic conditions. The major findings and conclusions of the experiments included in this chapter are mentioned below:

1. Isoproterenol (specific β1-adrenergic receptors agonist) significantly increased AA-NAT activity in the photoreceptive fish pineal during both winter and summer seasons.
2. Phenylephrine (specific α1-adrenergic receptors agonist) did not potentiate the stimulatory effect of isoproterenol irrespective of the seasons.
3. Propranolol (β1-adrenergic receptors antagonist) completely blocked the isoproterenol-induced increase in AA-NAT activity during both the seasons.

Propranolol also blocked the stimulatory effect of Isoproterenol + Phenylephrine during summer but not during winter.

4. Prazosin (specific α1-adrenergic receptors antagonist) drastically reduced isoproterenol-induced increase in AA-NAT activity during both the summer and winter seasons.
5. Clonidine significantly increased AA-NAT activity, while yohimbin drastically reduced basal AA-NAT activity as well blocked the stimulatory effect of clonidine on AA-NAT activity.
6. Norepinephrine and Carbachol significantly increased AA-NAT activity in a dose-dependent manner. However, different combinations of high and low doses of norepinephrine and carbachol had no effect on pineal AA-NAT activity.
7. Regression analysis indicated a strong positive correlation between different doses of both norepinephrine and carbachol and pineal AA-NAT activity.

On the basis of these findings it can be suggested that NE stimulates AA-NAT activity in the photoreceptive fish pineal organ mainly *via* β-adrenergic receptors, while α_1-adrenergic receptors seem to be essential for the stimulation of AA-NAT activity *via* β-adrenergic receptors. Further, α_2-adrenergic receptor also seems to be involved in adrenergic stimulation of AA-NAT activity. Though the β_1-adrenergic receptors are involved in the neural switching on of AA-NAT activation, functional α_1- adrenergic receptors seem to be necessary for the stimulatory effects of NE on AA-NAT activity in the fish pineal. This might also be the first report indicating involvement of muscarinic

M1 receptors in regulation of AA-NAT activity in the photoreceptive pineal in a fish species.

It, thus, seems that AA-NAT activity and melatonin in the fish pineal is regulated by a primitive non-specific regulatory mechanism involving both adrenergic and cholinergic inputs, and three subtypes of the adrenergic receptors. The regulatory mechanism probably became advanced during the course of evolution and involved only one subtype of adrenergic receptors in mammalian and avian pineal and eliminated the role of cholinergic mechanism.

Chapter - III: *In vitro* studies on the nature of G-proteins involved in adrenergic and cholinergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

This chapter deals with the study of *in vitro* effects of G7637 (Guanosine 5′-[β-thio] diphosphate trilithium salt; a specific inhibitor of stimulatory G-proteins), N-ethylmaleimide (a specific inhibitor of sulfhydryl G-proteins) and pertussis toxin (a specific inhibitor of inhibitory G-protiens) on norepinephrine (NE-) and carbachol-induced increase in pineal AA-NAT activity in the fish *Clarias gariepinus*. The major findings and conclusions based on the experiments included in this chapter are mentioned below:

1. N-Ethylmaleimide (NEM), pertussis toxin (PTX) and G7637 (Guanosine 5′-[β-thio] diphosphate trilithium salt) significantly inhibited the basal activity of AA-NAT.
2. While NEM completely blocked NE-induced increase in AA-NAT activity, NE could stimulate AA-NAT activity partially but significantly as compared to the PTX- and G7637-treated group.
3. Carbachol significantly but partially stimulated AA-NAT activity in the pineal pretreated with NEM or PTX.
4. G7637 significantly inhibited the basal AA-NAT activity as well as completely blocked the carbachol-induced increased in the enzyme activity.

These findings seem to suggest that all the three G-proteins (i.e., sulfhydryl G-proteins, PTX-sensitive G-proteins and stimulatory G-proteins) are essential for maintaining basal AA-NAT activity, and are involved in adrenergic and cholinergic stimulation of AA-NAT activity in the fish pineal organ. While sulfhydryl G-proteins seem to play a major role in adrenergic stimulation of AA-NAT activity, PTX-sensitive G-proteins and stimulatory G-proteins are also involved in adrenergic stimulation of AA-NAT activity in the photoreceptive pineal of fish. However, in the case of cholinergic stimulation of AA-NAT activity, stimulatory G-proteins seem to play a major role although PTX-sensitive and sulfhydryl G-proteins also seem to be involved. It, thus, seems that the molecular mechanisms involved in the adrenergic and cholinergic regulation of AA-NAT activity in the photoreceptive fish pineal get diversified at the level of G-proteins. This might be the first report of its kind establishing the nature of

G-proteins involved in the adrenergic stimulation of AA-NAT activity in the photoreceptive pineal of a fish species.

Chapter - IV: *In vitro* studies on the role of Ca^{2+}, protein kinase C, phosphodiesterase and serine/threonine phosphatases in adrenergic stimulation of arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of catfish, *Clarias gariepinus*

This chapter deals with the studies on *in vitro* effects of nitrendipine (specific blocker of L-type voltage calcium channels), chelerethrine chloride (specific inhibitor of protein kinase C), theophylline (specific inhibitor of phosphodiesterase), okadaic acid (specific inhibitor of ser/thr phosphatase 1), calyculin A (specific inhibitor of ser/thr phosphatase 2A) and cypermethrin (specific inhibitor of ser/thr phosphatase 2B) on norepinephrine (NE)-induced increase in AA-NAT activity in the fish pineal organ. The major findings and conclusions of the experiments included in this chapter are mentioned below:

1. Nitrendipine significantly reduced basal activity of the enzyme as well as completely blocked the NE-induced increase in AA-NAT activity in the fish pineal organ.
2. Chelerythrine significantly decreased pineal AA-NAT activity. The PKC inhibitor, however, did not block the NE-induced increase in the enzyme activity.

3. Norepinephrine and Theophylline significantly increased AA-NAT activity, and the stimulatory effect of theophylline on AA-NAT activity was found to increase with the increase in the incubation time.
4. Okadaic acid (OA) and calyculin A (CA) significantly inhibited basal AA-NAT activity.
5. NE reversed the inhibitory effects of the OA and CA on the enzyme activity partially but significantly.
6. Cypermethrin had no significant effect either on basal AA-NAT activity or on NE-induced increase in the enzyme activity.

On the basis of these findings, we conclude that calcium ions play an important role in adrenergic stimulation of AA-NAT activity in the fish pineal organ. Ca^{2+}-dependent protein kinase (PKC) seems to be involved in maintaining basal activity of AA-NAT, but it does not seem to play any role in the adrenergic stimulation of AA-NAT activity. Further, the cyclic nucleotide-dependent phosphodiesterase (PDE) also seem to play a crucial role in adrenergic regulation of AA-NAT activity. Inhibition of PDE activity can stimulate fish pineal organ AA-NAT activity in a time-dependent manner even in the absence of adrenergic stimulation.Ser/Thr phosphatase 1 and 2A are also essential for regulation of basal as well as for adrenergic stimulation of AA-NAT activity in the fish pineal organ.

CONCLUSIONS

On the basis of the present findings of the present Ph. D. dissertation, it can be concluded that AA-NAT activity in the photoreceptive pineal of *Clarias gariepinus* can be activated separately by both adrenergic and cholinergic agonists *via* α_2, β-adrenergic and M1 cholinergic receptors, respectively. However, simultaneous activations of both adrenergic and cholinergic receptors do not potentiate but counteract the stimulatory action of each other on the enzyme activity, and hence seem to follow different signal transduction pathways.

Findings of the present study also indicated that sulfhydryl G-proteins, PTX-sensitive G-proteins (G_i/G_o) and stimulatory G-proteins are involved in the adrenergic and cholinergic regulation of AA-NAT activity and, hence melatonin synthesis in the photoreceptive fish pineal organ. While, sulfhydryl G-proteins seem to be a pre-requisite for adrenergic stimulation of AA-NAT activity, stimulatory G-proteins seems to be more important for cholinergic stimulation of fish pineal AA-NAT activity. This might be the first report of its kind establishing the nature of G-proteins involved in the adrenergic and cholinergic stimulation of AA-NAT activity in the photoreceptive pineal of a fish species.

Further, Ca^{2+}, PKC, PDE and Ser/Thr phosphatases also play an important role in the adrenergic regulation of AA-NAT activity in the fish pineal organ *in vitro*. Ca^{2+} seems to play an important role in the adrenergic stimulation of AA-NAT activity in the fish pineal. Influx of calcium *via* the L-type voltage sensitive calcium channels is essentials for basal as well as for NE-induced AA-NAT activity in the fish pineal organ. Ca^{2+}-dependent Protein kinase (PKC) seems to be involved in maintaining basal activity of AA-NAT, but it does not seem to play any role in the adrenergic stimulation of AA-NAT activity. PDE also play a crucial role in regulation of AA-NAT activity in the fish pineal. Though Ser/Thr phosphatase 2B do not seem to play any role in the adrenergic stimulation of AA-NAT activity in the fish pineal organ, Ser/Thr phosphatase 1 and 2A are essential for normal as well as for adrenergic stimulation of AA-NAT activity in the fish pineal organ.

To the best of our knowledge, these findings present for the first time a complete picture of adrenergic and cholinergic regulation of arylalkylamine-N-acetyltransferase (AA-NAT) activity in the photoreceptive pineal organ of a fish species.

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APPENDIX

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