THE FUNCTION OF D_1- AND D_2-DOPAMINE RECEPTORS IN BOVINE PINEAL GLAND

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THE FUNCTION OF D_1- AND D_2-DOPAMINE RECEPTORS IN BOVINE PINEAL GLAND

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ABSTRACT

Previous studies from this laboratory have identified and characterized D_1- and D_2-dopamine receptors in bovine pineal glands. The data indicated a density of D_1-dopamine receptors (974 fmol/mg protein) far exceeding that of D_2-dopamine receptors (37 fmol/mg protein). The purpose of this research is to identify the mRNA for both D_1- and D_2-dopamine receptors and to elucidate the status of dopamine and its possible involvement in the pineal function, particularly on melatonin synthesis. The expression of these dopamine receptor subtypes was determined by using a reverse transcriptase-polymerase chain reaction technique with specific pairs of primers to amplify D_1- and D_2-dopamine receptor mRNA. Amplification of RNA from a bovine striatum (positive control) and a bovine pineal gland resulted in products of the predicted lengths of 231 base pairs for D_1- and 333 base pairs for D_2-dopamine receptors. The results indicated that mRNA from both D_1- and D_2-dopamine receptors is present in the bovine pineal gland. The role of dopamine receptors was investigated by studying the effects of selective D_1- and D_2-dopamine agonists and antagonists on the *N*-acetyltransferase activity of cultured bovine pinealocytes.

The data showed that SKF-38393 (a selective D_1-agonist) enhances *N*-acetyltransferase activity and increases the melatonin level and its stimulatory effect is blocked by SCH-23390 (a D_1-selective antagonist). On the other hand, quinpirole (a selective D_2-agonist) was found to inhibit *N*-acetyltransferase activity and decrease the melatonin level, and its inhibitory effect was found to be blocked by spiperone, haloperidol, and domperidone (D_2-selective antagonists). The level of cyclic 3', 5'-adenosine monophosphate was enhanced after a two-hour incubation of bovine pinealocytes with SKF-38393 and inhibited after incubation with quinpirole, while the effect of each was blocked by SCH-23390 and spiperone, respectively. Regarding phosphorylation, it was found that either SKF-38393 or forskolin enhances the phosphorylation of cyclic 3', 5'-adenosine monophosphate responsive element-binding protein, while their effects are inhibited by SCH-23390 and quinpirole, respectively.

In conclusion, this study directly demonstrates for the first time that, in the mammalian pineal gland, dopamine enhances the melatonin level by stimulating *N*-acetyltransferase activity by means of the D_1-dopamine receptor. In the bovine pineal gland I also found dopamine-dependent phosphorylation of the transcription factor of cyclic 3', 5'-adenosine monophosphate responsive element-binding protein, such as norepinephrine. However, the presence of dopamine containing fibers and the localization of specific dopamine receptors in the pineal gland need to be further elucidated.

KEY WORDS : DOPAMINE RECEPTOR / *N*-ACETYLTRANSFERASE / CYCLIC 3', 5' ADENOSINE MONOPHOSPHATE / MELATONIN
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หน้าที่ของตัวรับโดปามีนในต่อมไพเนียลของวัว
(THE FUNCTION OF D_1- AND D_2-DOPAMINE RECEPTORS IN BOVINE PINEAL GLAND)

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บทคัดย่อ

การศึกษาที่ผ่านมาพบว่ามีตัวรับโดปามีนทั้งชนิดที่ 1 และชนิดที่ 2 ในต่อมไพเนียลวัวโดยมีปริมาณตัวรับโดปามีนชนิดที่ 1 มากกว่าตัวรับโดปามีนชนิดที่ 2 ประมาณ 26 เท่า วัตถุประสงค์ในการศึกษานี้จึงต้องการศึกษาถึง mRNA ของตัวรับโดปามีนทั้งสองชนิดและการทำหน้าที่ของตัวรับโดปามีนในต่อมไพเนียลโดยเฉพาะการสังเคราะห์เมลาโทนิน การศึกษาการแสดงออกของยีนตัวรับโดปามีนโดยวิธี RT-PCR จากการใช้คู่ primer ที่เฉพาะเจาะจงต่อการจับ mRNA พบว่าความยาวคู่เบสที่เฉพาะเจาะจงต่อตัวรับโดปามีนชนิดที่ 1 และชนิดที่ 2 คือ 231 และ 333 ตามลำดับ ผลการศึกษาพบว่ามี mRNA ของ ตัวรับโดปามีนทั้งสองชนิดในต่อมไพเนียล

การศึกษาผลของตัวกระตุ้นและตัวปิดกั้นตัวรับโดปามีนทั้งสองชนิดที่มีต่อการทำปฏิกิริยาของเอนไซม์ *N*-acetyltransferase ในไพเนียลโลไซท์ของวัวพบว่า SKF-38393 (ตัวกระตุ้นตัวรับโดปามีนชนิดที่ 1) มีผลกระตุ้นปฏิกิริยาของเอนไซม์ *N*-acetyltransferase รวมทั้งสามารถเพิ่มระดับของเมลาโทนินได้โดยที่ผลของการกระตุ้นนี้ถูกยับยั้งโดย SCH-23390 (ตัวปิดกั้นตัวรับโดปามีนชนิดที่ 1) ในขณะที่ quinpirole (ตัวกระตุ้นตัวรับโดปามีนชนิดที่ 2) มีผลยับยั้งปฏิกิริยาของเอนไซม์ *N*-acetyltransferase และการลดระดับของเมลาโทนินโดยที่ผลของมันถูกยับยั้งโดย spiperone, haloperidol, และ domperidone (ตัวปิดกั้นตัวรับโดปามีนชนิดที่ 2) ส่วนการศึกษาระดับของ cAMP (cyclic3', 5'-adenosine monophosphate) ในไพเนียลโลไซท์ของวัวหลังจากใส่ยาลงในน้ำเพาะเลี้ยง 2 ชั่วโมงพบว่า cAMP ถูกกระตุ้นโดย SKF-38393 โดยที่ผลของการกระตุ้นนี้ถูกยับยั้งโดย SCH-23390 ในขณะที่ quinpirole มีผลยับยั้งปฏิกิริยาของ cAMP โดยที่ผลของมันถูกยับยั้งโดย spiperone นอกจากนี้พบว่า SKF-38393 กระตุ้นการเกิด phosphorylation ของ CREB (cAMP response element binding protein)โดยที่ผลของการกระตุ้นนี้ถูกยับยั้งโดย SCH-23390 ในขณะที่ quinpirole สามารถยับยั้งการกระตุ้นของ forskolin ต่อ การเกิด phosphorylation ของ CREB

การศึกษาครั้งนี้สามารถสรุปได้เป็นครั้งแรกว่าโดปามีนในต่อมไพเนียลของสัตว์เลี้ยงลูกด้วยนมมีผลโดยตรงต่อการกระตุ้นเอนไซม์ *N*-acetyltransferase และการเพิ่มระดับของเมลาโทนินจากการกระตุ้นของตัวรับโดปามีนชนิดที่ 1 รวมทั้งมีผลเช่นเดียวกันกับนอร์อิพิเนฟรินในการกระตุ้น phosphorylation ของ CREB และการสร้างเมลาโทนินในต่อมไพเนียลผ่านทางกลไกการถอดรหัสในนิวเคลียส อย่างไรก็ตามเส้นใยประสาทของโดปามีนและตำแหน่งเฉพาะที่ของโดปามีนในต่อมไพเนียลนั้นจะต้องทำการศึกษาวิเคราะห์ต่อไป

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LIST OF ABBREVIATIONS

% control	percentage of control
α_1-AR	alpha-adrenergic receptor
β_1-AR	beta-adrenergic receptor
5-HT	5-hydroxytryptamine
AA-NAT	arylalkylamine-N-acetyltransferase
AC	adenylyl cyclase
ACh	acetylcholine
ANOVA	analysis of variance
ATP	adenosine triphosphate
B_{max}	receptor density
cAMP	adenosine 3', 5'- cyclic monophosphate
cGMP	cyclic guanosine monophosphate
CREB	cAMP response element binding protein
CRE	cAMP responsive element
CREM	CRE modulator
CSF	cerebrospinal fluid
DA	dopamine
DBH	dopamine beta-hydroxylase
DOPAC	3, 4-dihydroxyphenylacetic acid
D_1-R	D_1-dopamine receptor
D_2-R	D_2-dopamine receptor
GABA	gamma aminobutyric acid
Glu	glutamate
HIOMT	hydroxyindole-*0*- methyltransferase
hr	hour
HRP	horseradish peroxidase
IC_{50}	inhibit 50% of the binding

LIST OF ABBREVIATIONS (CONT.)

ICER	inducible cAMP Early Repressor
IBMX	inhibitor 3-isobutyl-1-methylxanthine
K_d	dissociation equilibrium constant
kDa	kilodalton
L-dopa	L-dihydroxyphenylalanine
M	molar or mode per liter
mg	milligram
min	minute(s)
ml	millilter
MT	melatonin
MW	molecular weight
NAS	N- acetylserotonin
NAT	N-acetyltransferase
ND	non-detectable
NE	norepinephrine
ng	nanogram
nm	nanometer
O.D.	optical density
^oC	degree Celcius
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
pCREB	phosphorylation of cyclic adenosine monophosphate responsive element-binding protein
PBS	phosphate buffer saline
PKA	protein kinase A
PI	phosphatidylinositol
PLC	phospholipase C
pmol	picromole

LIST OF ABBREVIATIONS (CONT.)

RIA	radioimmunoassay
SCG	superior cervical ganglia
SCGx	superior cervical ganglionectomy
SCN	suprachiasmatic nucleus
sec	second
S.E.M	standard error of mean
TH	tyrosine hydroxylase
UV	ultraviolet
μCi	microcuries
μg	microgram
μl	microliter
μM	micromolar

CHAPTER 1
INTRODUCTION

The mammalian pineal gland has several unique features. These include being considered part of the central nervous system although outside the blood–brain barrier, receiving both central and peripheral innervations, and synthesis of melatonin. Melatonin is a liposoluble secretory product, which is released on a continuous basis and crosses the blood–brain barrier to interact with other neurotransmitter systems such as those of dopamine and serotonin (Ebadi and Govitrapong, 1986). Melatonin synthesis is known to be regulated by a light dependent release of norepinephrine which increases the formation of cyclic AMP and the activity of serotonin N-acetyltransferase (NAT), which is the rate-limiting enzyme in the synthesis of melatonin. The activity of NAT and the concentration of melatonin exhibit a marked 24-hr rhythm, being low during daytime, and increasing at night (Ebadi and Govitrapong, 1986). However, recent findings suggest that hydroxyindole-o-methyltransferase activity may be more related to determining the amount of melatonin formed than NAT (Borjigin et al., 2002). Physiologic and pharmacologic criteria have divided dopamine receptors into D_1- and D_2-subtypes (Kebabian and Calne, 1979). At least five genes encoding subtypes of dopamine receptors have been isolated and categorized as D_1- and D_2-like according to their nucleotide sequences and the pharmacological profiles of the expressed proteins. The D_1-like receptors include the D_1- and D_5-receptors, whereas the D_2-like receptors include the two isoforms of the D_2-receptors, differing in the length of their predicted third cytoplasmic loop, dubbed D_2-short (D_2S) and D_2-long (D_2L), and the D_3- and D_4-receptors (Missale et al., 1998).

The presence of a dopaminergic function in the pineal gland was first suspected by Axelrod et al. (1969), who demonstrated that in addition to L-norepineprine, dopamine also stimulated the production of [^{14}C]melatonin in the cultured pineal gland. Furthermore, the subcutaneous administration of 150 mg/kg L-dopa increased the activity of NAT whereas this activation was not seen in rats

pretreated with MK-486, an inhibitor of aromatic L-amino acid decarboxylase, suggesting that the L-dopa-mediated stimulation of NAT was because of the formation of a catecholamine. In addition, following superior cervical ganglionectomy, which denervated the peripheral innervation of pineal gland, the L-dopa-mediated stimulation of NAT became potentiated (Deguchi and Axelrod, 1972b). Additionally, studies by Lynch et al. (1973) showed that the administration of L-dopa increased the concentration of melatonin, and this effect was potentiated by pretreatment with 6-hydroxydopamine, which is known to destroy catecholaminergic nerve terminals (Tranzer and Thoenen, 1967, 1968). Furthermore, the studies have shown that the bovine pineal gland possesses dopamine D_2-like receptors with a B_{max} value of 37 fmol/mg protein (Govitrapong et al., 1984). The previous study reported that the bovine pineal gland also possessed D_1-dopamine receptors, which were characterized by using [^{3}H]SCH 23390, the selective D_1-dopamine receptor antagonist. The receptor density (B_{max}) was 974 fmol/mg protein receptors (Simonneaux et al., 1990). In addition, in a previous autoradiographic localization of dopaminergic and noradrenergic receptors in the bovine pineal gland, it has been shown that the density order of the receptor was dopamine D_1 > α_1 adrenergic > dopamine D_2 > β_1-adrenergic receptor (Simonneaux et al., 1991). Dopamine may have a synergistic effect with that of norepinephrine in stimulating the activity of serotonin-*N*-acetyltransferase (Axelrod et al., 1969; Deguchi and Axelrod, 1972a; Lynch et al., 1973).

The regional distribution of these receptor sites in the pineal of various species may vary vastly from one another. For example, in rats, norepinephrine stimulated NAT activity 30- to 50-fold and the maximum stimulation was seen 4 hr after the addition of norepinephrine. In bovine pineal glands, norepinephrine stimulates NAT activity moderately, between twofold and fivefold, and the maximum stimulation was seen 2 hr after the addition of norepinephrine (Chan and Ebadi, 1980). The moderate density of β_1 adrenergic receptor sites in the bovine pineal gland (Simonneaux et al., 1991) is also consistent with the moderate stimulation of NAT by norepinephrine in this species (Chan and Ebadi, 1980).

In the bovine pineal gland, dopamine concentration is equal to or higher than norepinephrine concentration (Govitrapong et al., 1989). In addition, the tyrosine hydroxylase and dopamine beta-hydroxylase activities are also higher during the night

than during the day. Compared to that at midday, the tissue content of endogenous norepinephrine at midnight was enhanced by 50% and that of dopamine by 45%. The existence of an increased dopaminergic turnover during the night supported a role for dopamine in the regulation of melatonin synthesis and in the synchronization of the pineal functions (Miguez et al., 1996). Furthermore, the reduction of NAT activity, which may be linked to impaired pineal catecholaminergic neurotransmission, was suggested (Miguez et al., 1998). Govitrapong et al. (2003) using [^{3}H]GBR 12935 investigated the existence of dopamine transporter in the bovine pineal gland. Drugs that blocked the uptake of dopamine were effectively in displacing [^{3}H]GBR 12935 from bovine gland whereas drugs that blocked the uptake of serotonin, norepinephrine and mixed reuptake inhibitor affecting serotonin and norepinephrine were ineffective. Furthermore, specific binding to bovine pineal membrane was higher than in striatum.

In the rat, and apparently also in other rodents, melatonin synthesis is primarily regulated through transcriptional mechanisms. NE activates pinealocytes through the beta-adrenergic/cyclic adenosine monophosphate (cAMP) pathway (Klein et al., 1997; Korf et al., 1998). Elevated cAMP levels result in activation of protein kinase A which phosphorylates the transcription factor cAMP response element-binding protein (CREB) (Roseboom and Klein, 1995; Tamotsu et al., 1995; Maronde et al., 1999). Phosphorylated CREB (pCREB) binds to cAMP response elements (CREs) in the promoter regions of various genes. In the rat pineal organ, pCREB is of central importance for initiation, maintenance, and termination of the rhythmic melatonin biosynthesis (Maronde et al., 1999). One crucial target of pCREB is the gene for the arylalkylamine N-acetyltransferase (AANAT), the key enzyme of melatonin synthesis. In bovine pinealocytes, Aanat mRNA is constitutively expressed, and AANAT protein is continually synthesized. In unstimulated cells, however, AANAT protein is immediately destroyed by proteasomal proteolysis. NE appears to act via cAMP to protect AANAT from proteolytic destruction, resulting in accumulation of the protein and increased melatonin production (Schomerus et al., 2000). In addition, immunoblots (Schomerus et al., 2003) showed that the NE-induced immunoreaction was due to phosphorylation of the transcription factor CREB and another protein, presumably the activating transcription factor 1 (ATF-1). This result indicated that NE-induced CREB phosphorylation represented a conserved element in pineal

physiology of mammal. However, the genes targeted by pCREB may vary from one mammalian species to the other (Schomerus et al., 2003).

The previous study of dopamine D_1- and D_2-receptors in the bovine pineal gland (Govitrapong et al., 1984; Simonneaux et al., 1991; Simonneaux et al., 1990), the involvement of dopamine in regulating melatonin synthesis in rat pineal glands (Govitrapong et al., 1989), and the existence of dopamine transporter in the bovine pineal gland (Govitrapong et al., 2003) were caused to identify mRNAs of both D_1- and D_2-dopamine receptors in bovine pineal glands. In addition, an attempt has been made in the present study to clarify the mechanism of dopamine in controlling melatonin production in the mammalian pineal gland via cAMP accumulation and the dopamine-dependent phosphorylation of the transcription factors cyclic adenosine monophosphate responsive element-binding protein (CREB) in cultured bovine pinealocytes.

CHAPTER 2
OBJECTIVES

Several lines of evidence have been reported an interaction among pineal gland, melatonin and dopamine. From the previous studies (Govitrapong et al., 1984, 1989; Simmuneaux et al., 1990, 1991) indicated a density of pineal D_1-dopamine receptorss (974 fmol/mg protein) far exceeds that of D_2-dopamine receptors (37 fmol/mg protein) on the rat pineal glands. Furthermore, the dopamine transporter has been recently identified and characterized in the bovine pineal gland by our group (Govitrapong et al., 2003). The present data indicated that DA is not only the precursor of NE, but also a true pineal neurotransmitter and might have an independent function in regulating melatonin synthesis. Therefore, this study has been identified the gene expression and function of dopamine receptor subtypes by using bovine pineal glands and pinealocytes as a model. Thus, the objectives are as follows:

1. To determine the gene expression of corresponding D_1, D_2 dopamine receptors in bovine pineal glands.
2. To study the status of D_1- and D_2-dopamine receptors and its possible involvement in the pineal function, particularly on melatonin synthesis.
3. To clarify how melatonin production was regulated in the vertebrate pineal gland by the study of influence of dopaminergic stimuli on cAMP accumulation and CREB phosphorylation in the bovine pinealocyte.

CHAPTER 3
LITERATURE REVIEW

An overview of the pineal gland

The pineal organ was first described by the Alexandrian anatomist Herophilus, in the 3rd century BC, who thought that it was a valve controlling the flow of memories from storage in the fourth ventricle up to the "conscious brain." The pineal was recognized as a gland by the Roman physician Galen, in the 2nd century AD. He observed that the pineal structure appeared different to that of nervous tissue but very similar to that of the other glands. The pineal gland was studied intensively by Descartes during the 17th century. He described the pineal gland as the third eye, not by analogy to its role in the control of the photoperiod, which he had no knowledge of, but because it is, in the Cartesian dualist vision, the place in the body where the soul exerts its control (the seat of imagination and common sense), and not the seat of the soul as it has often been referred to. "The reasonable soul," according to Descartes, "is lodged in the body, but not only as a pilot on its ship, which is necessary that it is united with its body." Descartes was the first to propose a "physiological" explanation for the functioning of the central nervous system, including the pineal gland, for the perception of the environment. At the beginning of the 20th century the physiological role of the pineal gland was studied (Simonneaux and Ribelayga, 2003). The pineal gland has been studied in great detail in animals. In general, the functions of this organ are to regulate daily, developmental, reproductive, and seasonal changes in the neurologic and hormonal activities of the animal in relation to its external environment. It has a complex list of regulatory factors which includes light, neural inputs, and a variety of receptors for catecholamines and steroid and peptide hormones (Erlich and Apuzzo, 1985).

Ascending the evolutionary ladder, there is a gradual change in the anatomy and physiology of the gland. In fish and amphibians, the pineal gland is a photoreceptor organ, converting light into electrical impulses in extensive neural

connections that leave the gland and pass to the brain and body. In birds, the pineal is a photo endocrine transducer, converting light into hormones released into the blood. In mammals, the pineal is a photoneuroendocrine transducer which rhythmically produces and secretes melatonin during the night in response to photoperiodic stimuli and signals from the circadian oscillator in the suprachiasmatic nucleus of the hypothalamus (Korf, 1994; Arendt, 1995). The rhythm in melatonin is essential for seasonal reproduction and maternal-fetal communication; it influences activity and sleep and modulates the function of the endogenous rhythm generator which plays an important role also in human physiology and pathology (sleep-wake cycle, shift work, rapid travel across several time zones, seasonal affective disorders (Arendt, 1995).

Anatomically, the pineal gland is shaped like a pine cone (Figure 3.1). The gland is derived from the neural tube and located at the border between the mesencephalon and the diencephalon of the brain that develops as an evagination of the diencephalic roof. Neuroanatomically, the pineal is described as a part of the epithalamus and thereby as a part of the diencephalon (Moller and Baeres, 2002). It projects posteriorly from the brain stem to which it is attached by a thin stalk lying between the superior colliculi. The third ventricle lies immediately anterior to it and extends into the pineal stalk via a small ependymal lined recess (Reiter, 1981). In most mammals it forms a solid mass between the habenular and posterior commissures, but in rodents, whereas a deep and small part stays close to ventricle III, the major portion of the gland migrates in a dorso-caudal direction to form the superficial pineal, both parts being connected by the pineal stalk.

The pineal gland is divided by connective tissue trabeculae into lobules. The lobules are populated by specialized neurosecretory cells called pinealocytes (Preslock, 1984) that consists of a cell body (7-12 μm in diameter) from which three to five processes emerge. In the electron microscope, dense-core granules are present with the highest density of granules in club-shaped expansions adjacent to capillary blood vessels (Moller and Baeres, 2002). The pinealocytes are the most abundant population in the mammalian pineal gland, accounting for more than 95% of all cells and there is no blood-brain barrier in the gland (Erlich and Apuzzo, 1985; Preslock, 1984). The pinealocytes express several retinal antigens (Schomerus et al., 1994), for example opsin, recoverin, and the retinal S-antigen. By using the S-antigen as a

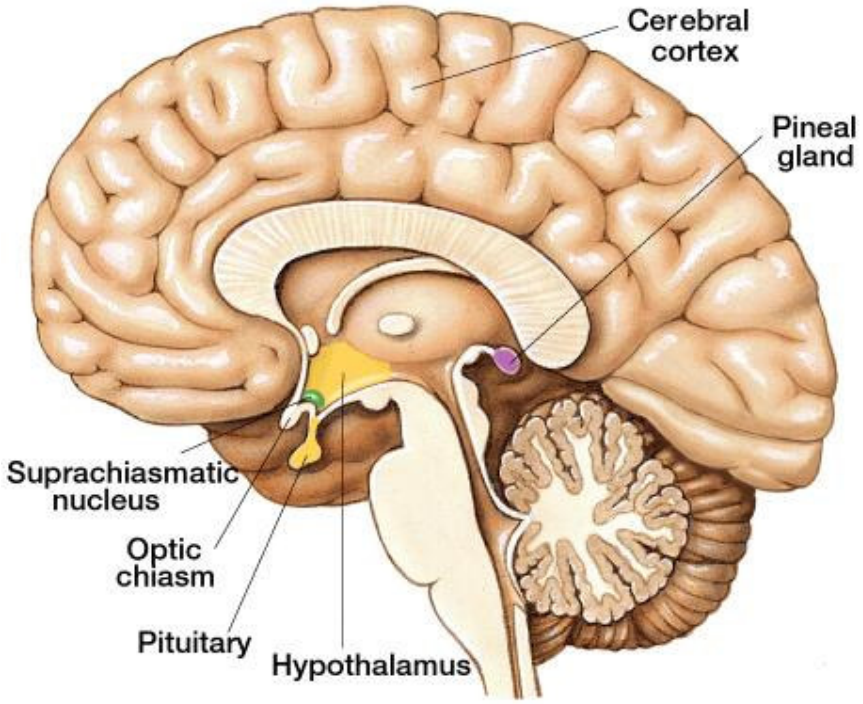


Figure 3.1 Anatomical demonstration of the pineal gland

marker, some of these pinealocytes establish synapse-like contacts with neurons in the brain (Korf et al., 1990) suggesting a direct communication between pinealocytes and neurons in the brain.

Neural inputs to the mammalian pineal gland

The mammalian pineal gland is a neuroendocrine structure targeted by numerous transmitters arriving via neural or endocrine pathways. The pineal gland is innervated with nervous fibers of various origins. Recently, by using the retrograde in vivo trans-synaptic virus-tracing technique, the total pathway was retrogradely mapped from the pineal gland, via the superior cervical ganglion, to the intermediolateral nucleus of the thoracic spinal cord and further to the paraventricular nucleus and finally to the suprachiasmatic nucleus (SCN) (Figure 3.2) (Larsen et al., 1998; Buijs et al., 1999; Moller and Baeres, 2002). The main pathway consists of a complex route named the retino-hypothalamo-pineal pathway, ending with the sympathetic input to the pineal parenchyma. The pineal gland also receives neural inputs of central and parasympathetic origins. These pineal nerve endings contain a large variety of neurotransmitters.

1. Retino-Hypothalamo-Pineal Pathways

The rhythm in melatonin synthesis depends essentially upon three interdependent factors: the endogenous circadian oscillator located in the SCN, the light/day cycle that synchronizes the endogenous oscillator, and the light that acutely inhibits nocturnal melatonin synthesis. It is now well established that there exists a multi-synaptic neural pathway among the retina, SCN, and pineal gland. Various experiments (lesion, neuronal tracing) have allowed researchers to draw the general diagram of the main innervation of the mammalian pineal gland, especially in the rat (Moore and Klein, 1974; Klein and Moore, 1979; Moore, 1996; Larsen, 1999).

a. The Retino-Hypothalamic Tract

Photic information is conveyed from the retina to the ventro-lateral zone of the SCN via the retino-hypothalamic tract (RHT). The light-sensitive cells forwarding the light/ dark information do not appear to be the rod and cone photoreceptors (Lucas et

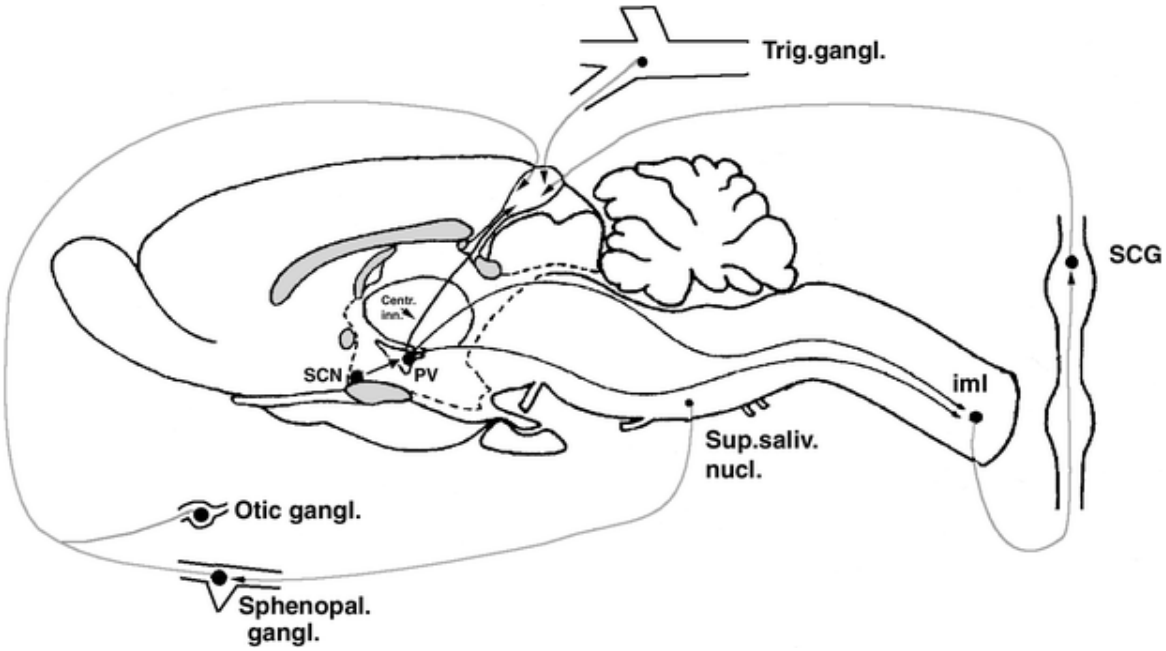


Figure 3.2 Multineuronal innervations of the pineal gland.

(Moller and Baeres, 2002)

al., 1999), but rather are a small subset of retinal ganglion cells containing the photopigment melanopsin (Moore et al., 1995; Berson et al., 2002; Hannibal et al., 2002). The RHT neurotransmitters are mainly glutamate (Glu) and pituitary adenylate cyclase activating peptide (PACAP) (Hannibal et al., 1997), but not substance P (sP), mediating light signaling to the clock. Other inputs originating from the thalamic IGL, containing neuropeptide Y (NPY), enkephalin (Enk), and GABA (Card and Moore, 1982; Moore and Speh, 1993; Morin and Blanchard, 2001) and from the raphe nucleus, containing 5-HT (Moore et al., 1978) also carry photic and nonphotic information to the SCN (Mrosovsky, 1996).

b. The Hypothalamic Endogenous Circadian Oscillator

In mammals, several experiments have demonstrated the presence of an endogenous circadian oscillator in the SCN (Ralph et al., 1990; Takahashi, 1995; Turek et al., 1995) probably located in every SCN neuron showing an endogenous oscillation in firing rate (Welsh et al., 1995). This endogenous activity is higher during the subjective day and synchronized to exactly 24 hr by the photic inputs. Several proteins (PER1–3, TIM, CLOCK, BMAL/MOP3, TAU/type I) work as transcription factors and enzymatic regulators in an autoregulatory transcriptional feedback loop constituting the core of the circadian pacemaker (Simonneaux and Ribelayga, 2003). SCN neurons are mainly peptidergic cells containing vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), and somatostatin (SOM), but also GABA (Buijs et al., 1994, 1995; van Esseveldt et al., 2000). The integration of the photoperiod by the SCN has been proposed to involve two components (one recognizing variations of the dawn, the other of the dusk) with the increase (in the evening) and the diminution (in the morning) of melatonin synthesis being regulated separately during photoperiod changes. The phase relationship between these two oscillator components would determine the duration of the nocturnal melatonin peak. However, an alternative view proposes that the photoperiod may be integrated into every SCN cell, into the molecular mechanism of the circadian clock itself. By affecting the daily profile of the light-sensitive *Per* expression (long under LP, short under SP), photoperiod may, in turn, affect the kinetics of the expression of the clock proteins and consequently the expression of all the clock-regulated genes (Hastings, 2001). Although it has been demonstrated that photoperiod clearly regulates the daily

profile of *Per1* (Messager et al., 2000) and PER1 (Nuesslein-Hildesheim et al., 2000) in the SCN, the link between changes in the clock-gene expression profile and SCN outputs remains to be established.

c. Suprachiasmatic Nucleus of the Hypothalamus Outputs to the Pineal Gland

It is generally considered that the ventro-lateral part of the SCN is the clock input area for the synchronizing events while the dorso-median part contains the oscillator and the output of the timing information. Actually, various SCN neurons project mainly to different hypothalamic structures to transmit the timing information to different functional axes, especially the hypothalamo-pituitary-adrenal axis (rhythmic secretion of corticosterone) and the hypothalamopineal axis (rhythmic secretion of melatonin). Recently, the link between the SCN output and the circadian rhythm in locomotor activity was proposed to be the transforming growth factor acting on the hypothalamic subparaventricular zone (Kramer et al., 2001). The increasing use of cDNA microarrays will help to identify new clock-controlled genes in various tissues (Akhtar et al., 2002; Duffield et al., 2002; Humphries et al., 2002). In the rat, the SCN neurotransmitters involved in the clock output would be essentially VP and GABA (Moore and Speh, 1993; Buijs et al., 1994; Kalsbeek et al., 1995; 1996a). VP appears to be a good clock-controlled transmitter since 1) it displays a circadian rhythm of synthesis and release (Murakami et al., 1991; Kalsbeek et al., 1995; Watanabe et al., 2000); 2) its gene promoter, containing an "E-box," is under the direct control of the clock genes (Jin et al., 1999); and 3) it acts on the dorsomedial hypothalamus to control the circadian rhythm of corticosterone synthesis and release (Kalsbeek et al., 1996b). In addition, VIP (Teclemariam- Mesbah et al., 1997), glutamate (Cui et al., 2001), or another unknown diffusible substance (Silver et al., 1996; Allen et al., 2001) may also be non-neural outputs of the molecular clock. As far as the regulation of melatonin synthesis is concerned, the hypothalamic paraventricular nuclei (PVN) are an essential relay between the SCN and the pineal gland. GABA appears to be involved in transmitting signals from the SCN to the PVN since infusion of a GABA antagonist during the subjective day in the PVN area stimulates melatonin synthesis, whereas infusion of GABA during the night inhibits nighttime melatonin secretion (Kalsbeek et al., 1996a, 1999, 2000). These data

indicate that the SCN is a daytime inhibitor (via GABA) of the PVN stimulation of melatonin synthesis, and is probably also a nighttime stimulator (Kalsbeek et al., 2000). ACh is the main neurotransmitter released in the SCG (Kasa et al., 1991), but other neurotransmitters, especially SOM, vasoactive intestinal polypeptide (VIP), histidine isoleucine peptide (PHI), and calcitonin gene-related peptide (CGRP) are potential candidates in the transmission of information to the SCG. The mammalian pineal gland is characterized by a very dense sympathetic innervation (Moller, 1999). In the rat (Zhang et al., 1991) and sheep (Cozzi et al., 1992) pineal gland most of the tyrosine hydroxylase (TH; the rate-limiting enzyme for NE synthesis) immunoreactive fibers disappear after the SCG removal (SCGx). In addition to the catecholaminergic innervation the pineal organ of mammals also receives a peptidergic innervation (Moller et al., 1996). The sympathetic fibres from the superior cervical ganglia also convey neuropeptide Y (Mikkelsen and Moller, 1999). Other fibres, from central origin, convey vasoactive intestinal polypeptide (VIP) and the peptide histidine-isoleucine (PHI), or substance P, calcitonin gene-related peptide, somatostatine, vasopressin and neuropeptide Y (Moller et al., 1996). These fibers enter the distal part of the pineal via the conarian nerves (*nervi conarii*). The putative transmitters involved have not been identified, but this observation suggests that the pineal gland could exert its influence by a neuronal pathway in addition to the melatonin endocrine pathway.

2. Central Pathways

The direct innervation with nerve fibers originating from the brain is called the *central innervation* of the pineal gland. The numerous studies using electrophysiological and neuroanatomical techniques demonstrated that the mammalian pineal gland receives a diversified central innervation although it is less dense than the sympathetic innervation. These observations have led to the hypothesis that various central structures play a physiological role in the regulation of the metabolic activity of the mammalian pineal gland (Moller et al., 1996, Moller, 1999; Moller and Baeres, 2002). The early ultrastructural observations had already suggested the presence of extra-sympathetic fibers since 1) the pineal gland exhibits synaptic buttons containing large (100 nm) granular vesicles (peptidergic type) or small (40–60

nm) clear vesicles (cholinergic type); 2) myelinated fibers observed in the pineal gland are still preserved after SCGx (Lin et al., 1975; Schneider et al., 1981; Moller and Korf, 1983); and 3) lesions of the habenular area induces the degeneration of fibers and nerve endings in the rodent pineal gland (David and Herbert, 1973; Ronnekleiv and Moller, 1979; Moller and Korf, 1983). Use of the horseradish peroxidase (HRP) tracing technique has confirmed the existence of neural connections between the brain and pineal gland in several rodent species. When tracer was injected into the pineal gland, HRP-positive fibers were observed in the proximal part of the gland continuing either via the posterior commissure or via the habenular commissure. HRP-positive neurons were observed in the habenular nuclei, the posterior commissure nuclei, the PVN and, in some cases, the IGL (Korf and Wagner, 1980). These initial observations were confirmed by anterograde tracing from the PVN (Larsen et al., 1991), the lateral hypothalamus (Fink-Jensen and Moller, 1990), the habenular nuclei, and the IGL (Reuss and Moller, 1986; Mikkelsen and Moller, 1990; Mikkelsen et al., 1991) showing positive fibers in the proximal part of the pineal gland.

The neurotransmitters observed in these central fibers are mainly neuropeptides, especially vasopressin and oxytocin (PVN: Buijs and Pe´vet, 1980), sP (habenular nuclei: Ronnekleiv and Kelly, 1984), and NPY (IGL: Mikkelsen et al., 1991). In addition, histaminergic fibers originating in the tuberomammillary nucleus (Mikkelsen et al., 1992), 5-HTergic fibers originating in the dorsal raphe (Leander et al., 1998), and hypocretin (HCRT)-containing fibers originating in the lateral hypothalamus (Mikkelsen et al., 2001) were also demonstrated in the rodent pineal gland. The use of electrophysiological techniques has also confirmed the existence of pineal fibers of central origin. Stimulation of central structures such as the PVN, lateral hypothalamus, amygdala, hippocampus, and especially the habenular nuclei induced an electrophysiological response of the pinealocytes (Reuss et al., 1984, 1985). There are also immunohistochemical indications for the presence of cholinergic (Phansuwan-Pujito et al., 1991) and SP-immunoreactive central nerve fibers in the cow and the pig (Przybylska-Gornowicz et al., 2000). However, it is important to note that the central innervation in most rodents terminates in the deep pineal gland and the pineal stalk (Matsushima et al., 1999). In many primates, who only possess a deep

pineal, nerve fibers of the central innervation are reaching all parts of the gland (Moller and Baeres, 2002).

3. Parasympathetic Pathways

Numerous neuroanatomical tracing studies have consistently shown that pinealopetal nerve fibers also originate from parasympathetic ganglia and in the trigeminal ganglion. The strong anatomical evidence for the innervation of the pineal from parasympathetic ganglia has also been provided. Kenny (1961) carried out a model study in this field on the monkey. After cutting the greater petrosal nerve, he observed a degeneration of nerve fibres in the pineal gland. The greater petrosal nerve provides the spenopalatine ganglion with pre-ganglionic parasympathetic nerve fibers. Therefore, the degenerating nerve fibers in the pineal gland, observed after cutting of the greater petrosal nerve, are probably degenerating second order neurons in the parasympathetic nervous system (Moller and Liu, 1999). The presence of a parasympathetic innervation of the pineal gland has long been debated (Phansuwan-Pujito et al., 1999). However, localization of pinealopetal fibers originating in the pterygopalatine and the otic ganglia (Shiotani et al., 1986; Moller and Liu, 1999) together with the demonstration of pineal cholinergic fibers in the rat (Eranko et al., 1970), ferret (David and Herbert, 1973), rabbit (Romijn, 1973), monkey (David and Kumar, 1978), and cow (Phansuwan-Pujito et al., 1990, 1991) have demonstrated the occurrence of a parasympathetic input to the pineal gland. Besides ACh, VIPergic fibers originating from parasympathetic ganglia have also been observed in the rodent pineal gland. Immunohistochemical studies of neuropeptidergic fibres in the mammalian pineal have also pointed towards the sphenopalatine ganglion as the origin of pinealopetal nerve fibres (Moller et al., 1996). Other parasympathetic ganglia than the sphenopalatine ganglion also have to be considered with regards pineal innervation. Thus, the otic ganglion contains many neurons immunoreactive for VIP and PHI and is known to innervate brain vasculature (Moller and Baeres, 2002). In addition, demonstration of receptors and biochemical effects of cholinergic and VIPergic ligands in the pineal gland confirm the existence of parasympathetic control of pineal activity.

4. Pathways from Other Neural Structures

Retrograde tracing studies have demonstrated that the trigeminal ganglia project directly to the rodent pineal gland (Shiotani et al., 1986; Reuss et al., 1992a; Møller and Liu, 1999). These fibers contain sP, CGRP, and PACAP. The trigeminal input to the pineal gland is interesting because to date this ganglion has only been considered a sensory ganglion. New anatomical studies have shown that also perikarya in the trigeminal ganglion project to the pineal gland of mammals. The trigeminal ganglion is the classic sensory ganglion of the fifth cranial nerve. Thus, substance P (SP), calcitonin gene-related peptide (CGRP), and pituitary adenylate cyclase-activating peptide (PACAP) are present in cell bodies of the trigeminal ganglion. However, studies combining neuronal tracings and transmitter immunohistochemistry have clearly shown that SP-immunoreactive nerve fibers also originate from the trigeminal ganglion (Moller and Baeres, 2002). The peptide PACAP, belonging to the secretin family, has recently been demonstrated in intrapineal nerve fibers (Moller et al., 1999), and it stimulates melatonin secretion via specific receptors located on the pinealocyte cell membrane (Schomerus et al., 1996). Likewise, it has been demonstrated that PACAP elevates N-acetyltransferase (NAT) activities, cyclic AMP (cAMP), N-acetylserotonin in the rat pineal gland and pinealocytes (Chik and Ho, 1995, Simonneaux et al., 1993). The colocalization of PACAP with CGRP in the pinealopetal nerve fibers indicated that the majority of PACAP-immunoreactive nerve fibers might originate from the trigeminal ganglion (Moller et al., 1999).

Endocrine Inputs to the mammalian pineal gland

Because the pineal gland is outside the blood-brain barrier in most species, substances secreted into the bloodstream may affect pineal activity as long as receptors for those substances are present in the pineal gland (Moller and Baeres, 2002). For example, this has been shown for the pituitary peptides and gonadal hormones. Radioactive labeled peptides such as luteinizing hormone-releasing hormone (LHRH; Redding and Schally, 1973), melanin-stimulating hormone (Kastin et al., 1976), and delta-sleep inducing peptide (DSIP; Graf and Kastin, 1985) injected into the bloodstream accumulate in the pineal gland. VP and OT, released into the circulation

during osmotic regulation or during parturition and lactation, may also act on pineal activity. VP, for example, concentrates in the pineal gland (Zlokovic et al., 1991). Other circulating peptides such as natriuretic factors may also alter pineal activity since in vitro effects of these peptides have been observed. Some gonadal steroids also concentrate in the pineal gland, where they alter its activity (Nagle et al., 1974).

Paracrine Inputs to the mammalian pineal gland

In the pineal gland melatonin is synthesized from intracellular 5-HT, then released into the bloodstream. It has been reported that 5-HT and melatonin display additional autocrine/paracrine effects. Pineal cells also contain GABA (15% of bovine pineal cells: Rosenstein et al., 1989b), Glu (Mc-Nulty et al., 1992), aspartate (Imai et al., 1995), and taurine (LaBella et al., 1968), which are able to alter the metabolic activity of the pineal gland. Intrapineal neurons immunopositive for acetylcholinesterase have been identified in the pineal gland of several mammalian species (Romijn, 1975; Phansuwan-Pujito et al., 1999). Growth factors are present in the pineal gland (Garcia-Maurino et al., 1992) where they favor neurite development of the pinealocytes (McNulty et al., 1993). A particularity of the mammalian pineal gland is the ability to synthesize various peptides that are able to alter its metabolic activity. Cells containing Enk have been characterized in the rodent pineal gland (Aloyo, 1991; Coto-Montes et al., 1994). In the European hamster, Enk-containing cells display synaptic-like connections with other pineal cells (Coto-Montes et al., 1994), suggesting a paracrine function of this peptide. Pineal cells also contain LHRH and SOM (rat, Pe´vet et al., 1980), SP (cotton rat, Matsushima et al., 1994), C-type natriuretic peptide (CNP) (cytoplasmic vesicles of bovine pineal cells, Middendorff et al., 1996). The pineal gland of the Syrian hamster, but not the rat, displays a few cells containing secretoneurin (SN) (Simonneaux et al., 1997). The mRNA coding for VP has been detected in the pineal gland of rat, sheep (Matthews et al., 1993), and cow (Olcese et al., 1993), but no VP-IR cells have yet been observed. This suggests that VP is synthesized in low amounts in pineal cells, the mRNA is present but not translated, or the mRNA is present in VPergic neural fibers but not in pineal cells. In contrast to VP, the presence of a few OT-containing neuron-like cells in the bovine

pineal gland has been demonstrated both by ISH and immunocytochemistry (Badiu et al., 2001). The majority of peptide-containing cells are neuron-like or modified pinealocytes displaying synaptic contacts with the true pinealocytes. It is noteworthy, however, that the density of these peptidergic cells is usually very low. These active substances synthesized in the pineal gland may display auto/paracrine effects in the pineal gland because most of them are able to modify pineal metabolism in vitro. It is evident that some of these substances, in addition to melatonin, could have an endocrine function. However, currently there are no sufficient data on this subject (Simonneaux and Ribelayga, 2003).

The pineal gland is a junction of various neural inputs and the metabolic activity of the mammalian pineal gland is mainly under the control of the hypothalamic clock, its temporal message being delivered to the pineal gland by a polysynaptic pathway ending with sympathetic fibers. However, the various neuroanatomical and immunocytochemical data now have profoundly changed the former concept that the mammalian pineal gland is solely innervated by the sympathetic nervous system (Moller, 1999). Actually, the pineal gland is the target of several neurotransmitters of various origins that activate second messenger systems in the pinealocyte, thereby influencing the biochemistry of the pinealocyte. These findings have led to numerous biochemical studies to understand how, besides NE, these other pineal transmitters regulate the synthesis of melatonin.

Regulation of pineal function

Melatonin (*N*-acetyl-5-methoxytryptamine) was characterized after its isolation from bovine pineal tissue by Lerner and Lerner (1958), the only known hormonal output of the pineal, has various biological effects changing from light-dark entrainment of behavioral and physiological response to regulation of seasonal reproductive activity (Arendt, 1995). The main control of melatonin secretion is by a complex neural pathway starting from the retina to the pinealocytes via the suprachiasmatic nuclei, brain stem, spinal cord, superior cervical ganglia, and sympathetic nerves in the nervi conarii. Darkness or dim light stimulates fibers passing in the retinohypothalamic tracts to the suprachiasmatic nuclei in the lateral

hypothalamus. Secondary fibers then pass via the median forebrain bundle and largely uncharted routes in the brain stem to reach the intermediolateral columns of the spinal cord at the C_8 to T_5 levels. Sympathetic efferent fibers then go to the superior cervical ganglia and then to the tip of the pineal gland via the nervi conarii (Preslock, 1984).

A key function of the pineal gland is to transform information about environmental lighting into biological rhythms. In mammals, light information reaches the pineal gland via a circuitous route. There are specialized neurons which contain a unique photopigment in the retina that respond to light. This information is transduced into a neural message which is transferred to the anterior hypothalamus via axons of retinal ganglion cells in the optic nerve; this is part of the so-called retino-hypothalamic tract. In the hypothalamus, the axons from the retina terminate in the suprachiasmatic nuclei (SCN), a type of nucleus whose neurons exhibit inherent circadian electrical rhythms; these nuclei constitute the biological clock or the central circadian pacemaker (Cassone and Natesan, 1997). Between the SCN and the pineal gland, the neural pathways, at least centrally, are somewhat less defined but are believed to be as follows: SCN, paraventricular nuclei, intermediolated cell columns of the upper thoracic cord (preganglionic sympathetic neurons), superior cervical ganglia (postganglionic sympathetic neurons), pineal gland (Figure 3.3). This circuitous pathway conveys information about the light:dark environment to the pineal gland and thereby determines the melatonin synthesis cycle (Reiter, 2003).

The regulation of melatonin production in the pineal gland has been defined in significant detail (Reiter, 1991; Klein et al., 1997). The primary neurotransmitter released from the postganglionic sympathetic terminals that terminate in the pineal gland is norepinephrine (NE); during darkness at night, NE is discharged onto the pinealocytes, the endocrine cells of the gland, where it couples especially to beta-adrenergic receptors. This leads to a marked rise in intracellular cAMP levels, to de novo protein synthesis and eventually to the stimulation of the rate-limiting enzyme in melatonin production, arylalkylamine-N-acetyltransferase (AA-NAT). AA-NAT -N-acetylates serotonin to N- acetylserotonin (NAS), the immediate precursor of melatonin (Figure 3.4). Once generated, NAS is quickly *0*-methylated, a step catalyzed by hydroxyindole-*0*- methyltransferase (HIOMT); this reaction requires the transfer of a methyl group from 5-adenosylmethionine to the 5-hydroxy group of NAS. The

dramatic rise in AA- NAT drives melatonin synthesis and, consequently, the melatonin content of the pineal increases substantially at night (Reiter, 2003). Unlike other endocrine organs, the pineal does not store melatonin after it is synthesized but is secreted into the blood, where its half-life is 46 minutes. Melatonin quickly diffuses out of the pinealocytes into the rich capillary bed (Arendt, 2000) within the gland and possibly directly into the cerebrospinal fluid (CSF) of the third ventricle (Tricoire et al., 2002). It is excreted in the urine, partly in its original form, and mainly after catabolism to 6-hydroxymelatonin and conjugation by the liver (Preslock, 1984). As a result blood and CSF levels rise at night and the concentration of melatonin in these fluids is generally accepted as an index of its concurrent synthesis within the pineal gland; circulating nocturnal levels of melatonin are commonly 10-20 times higher than concentrations measured during the day. The observations that the mammalian pineal gland has a dense noradrenergic (NAergic) innervation and that SCGx suppresses nocturnal melatonin synthesis (Moore and Klein, 1974) were the origin of numerous pharmacological, biochemical, and molecular studies designed to delineate the effects of NE on the metabolic activity of the pineal gland. These experiments were performed mainly in the rat, although none of its physiological functions are known to vary according to the photoperiod (Klein et al., 1997).

In the rat, NE is the major neurotransmitter involved in the SCN clock control of the metabolic activity of the pineal gland. Rhythmic SCN activity is translated, via positive and negative outputs, as a nighttime stimulation of the SCG fibers (Kalsbeek et al., 1999a, 2000). The amount of NE released from the sympathetic fibers is approximately 100-fold higher during during the night than during the day (Drijfhout et al., 1996a). The pivotal role of NE in the control of rat pineal metabolic activity has been supported by several experiments: 1) intraperitoneal injections of an NAergic agonist during the day stimulate melatonin synthesis with a comparable amplitude to that of the endogenous nocturnal increase; 2) SCGx abolishes the nocturnal increase in *Aanat* mRNA, AA-NAT activity, and melatonin synthesis (Deguchi and Axelrod, 1972b; Roseboom et al., 1996); 3) electrical SCG stimulation during the day provokes an increase in melatonin synthesis in the pineal gland. 4) exogenous NAergic stimulation of the pineal gland in organ cultures or in perifusion or of pinealocytes in primary culture induces a large increase in AA-NAT activity and melatonin release; 5)

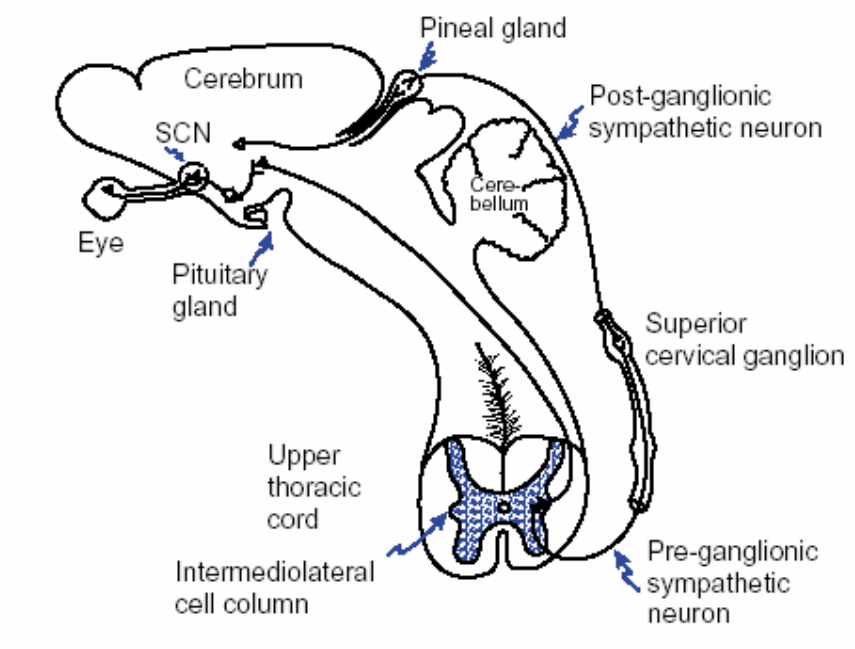


Figure 3.3 Circuitous neural connections between the eyes and pineal gland

(Reiter et al., 2003)

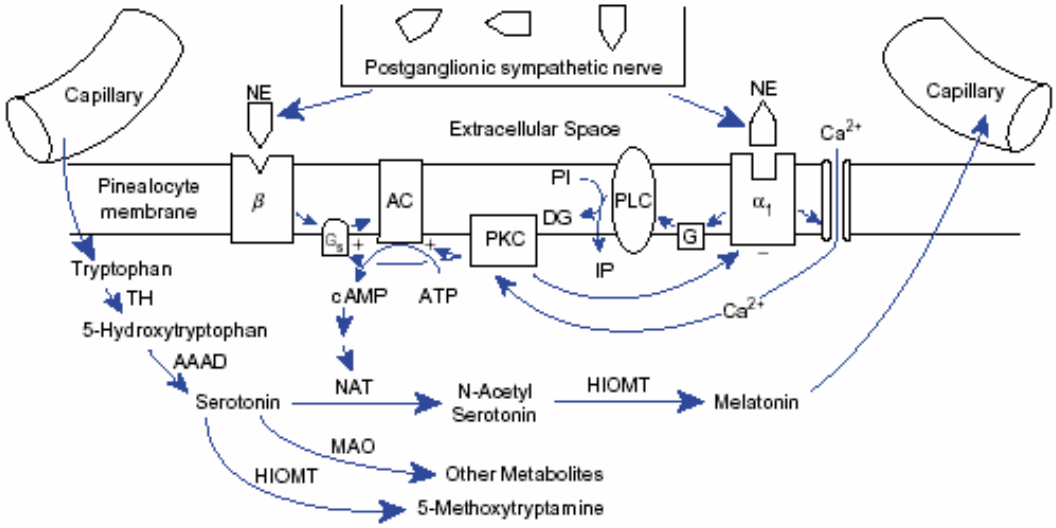


Figure 3.4 Interactions of NE (noradrenalin) released from postganglionic sympathetic fibres with beta-adrenergic receptors in the pinealocyte membrane. (Reiter et al., 2003)

the synthetic rate and renewal of NE in the pineal gland are higher at night than during the day and 6) the use of pineal microdialysis to study the in situ regulation of melatonin synthesis has demonstrated the positive coupling between the nighttime release of NE and stimulation of melatonin synthesis (Drijfhout et al., 1996b).

Role of cAMP and CREB in melatonin synthesis

Cyclic AMP (Adenosine 3', 5'-monophosphate) is an important intracellular messenger, known as a second messenger (the hormone being the primary messenger). The enzyme adenylate cyclase catalyzes the formation of cAMP from adenosine triphosphate (ATP). The increase in cAMP concentration may activate cAMP-dependent protein kinase, which regulates intracellular components by phosphorylation. Cyclic AMP is subsequently broken down by the enzyme phosphodiesterase, which terminates the effects of this second messenger (Figure 3.5). Cyclic AMP has been extensively documented to be an important mediator of intracellular responses to post-synaptic receptor stimulation (Wiener and Breslin, 1995).

The cAMP signal transduction pathway plays a key role in many biological processes. In the mammalian neuroendocrine system, it is central to the coordination of hormonal function. Cyclic AMP directs changes in gene expression and thereby effects long-term modulation. Intracellular levels of cAMP are regulated primarily by adenylyl cyclase. This enzyme is, in turn, modulated by various extracellular stimuli mediated by receptors and their interaction with G proteins. Increased cAMP levels directly affect the function of the tetrameric protein kinase A (PKA) complex. Binding of cAMP to two PKA regulatory subunits releases the catalytic subunits, enabling them to phosphorylate target proteins. In the nucleus, the phosphorylation state of transcription factors and related proteins appears to directly modulate their function and thus the expression of cAMP-inducible genes. Thus, there is a direct link between the activation of G-coupled membrane receptors and CRE-mediated gene expression (Sassone-Corsi, 1998).

CREB (cAMP responsive element binding protein) is a member of a large family (CREB/ATF) of structurally related transcription factors that bind to promoter

cAMP responsive element (CRE) sites on target genes. Although CREB was the first to be isolated, there are now at least 10 additional genes in the CREB family (Sassone-Corsi, 1995). This group of proteins shows many structural and functional variations, and they are expressed in a wide range of tissues and cell types. Most of the sequence homology among different members of the CREB family is restricted to the bZIP region (Hai et al., 1989). Based on the extent of homology at this region, members of the CREB family can be divided into the CREB, CREM (CRE modulator), and activating transcription factor (ATF) groups (Silva et al., 1998). Increases in the concentration of either calcium or cAMP can trigger the phosphorylation and activation of CREB. The crucial event in the activation of CREB is the phosphorylation of Ser 133 that is a key regulatory site controlling transcriptional activity (Brindle and Montminy, 1992, Sassone-Corsi, 1995). The transcription factor CREB binds the cyclic AMP response element (CRE) and activates transcription in response to a variety of extracellular signals including neurotransmitters, hormones, membrane depolarization, and growth and neurotrophic factors. In common with the inducible transcription factors fos and jun, the CREB/ATF proteins consist of three functional domains: a leucine- zipper domain that mediates dimerization, a basic DNA-binding domain and the transcriptional activation domain, which contains important phosphorylation sites that regulate the activity of CREB (Walton and Dragunow, 2000). Receptors that increase cAMP formation include β-adrenergic receptors and dopamine D_1 receptors. This in turn activates PKA by dissociating the regulatory from the catalytic subunits. Catalytic subunits can be translocated into the nucleus, where they can phosphorylate CREB. Activation of other receptors inhibits the activity of adenylate cyclase. These receptors include α-adrenergic receptors, dopamine D_2 receptors, and certain muscarinic and opiate receptors (Bacskai et al., 1993, Hagiwara et al., 1993).

The melatonin synthesis is controlled by the SCN, being elevated at night and low during the day. The cAMP-dependent signal transduction pathway serves as a relay to stimulate melatonin synthesis. Transcriptional regulation upon stimulation of this pathway is mediated by a family of cAMP-responsive nuclear factors. These factors contain the basic domain/leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). Stimulation of post-synaptic pineal *beta-receptors* both *in*

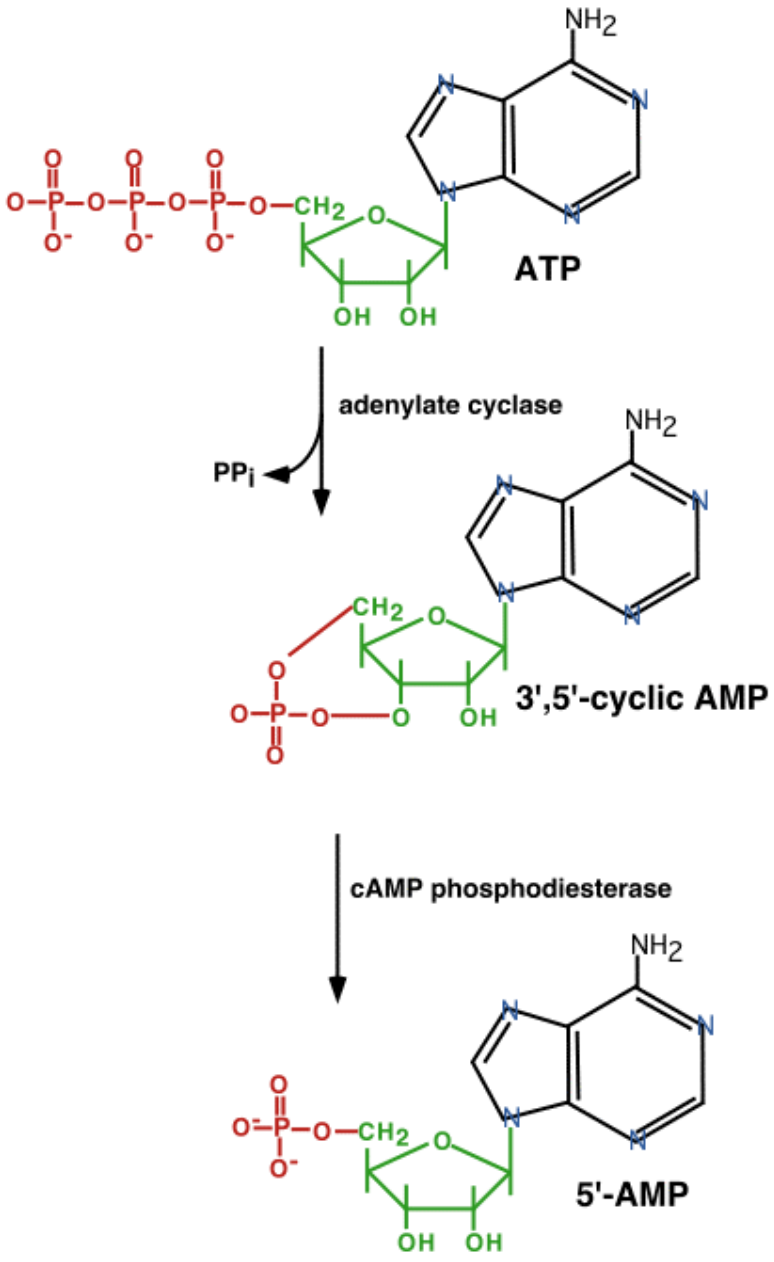


Figure 3.5 Synthesis of cAMP

vivo and *in vitro* is followed by a transient increase in cAMP and subsequently by elevated levels of acetyl coenzyme A: arylamine N-acetyltransferase (NAT) activity (Deguchi and Axelrod 1972a; Parfitt et al., 1976). While the exact sequence of events leading to NAT induction is still being investigated, it is generally accepted that the increase in cAMP content is causally related to the induction of NAT activity.

Work with dispersed pinealocytes in culture suggests that cAMP may also play an important role in the rapid pharmacologically mediated decrease in NAT activity. When *beta-recep*tors are blocked with propranolol, the subsequent decline in NAT activity is preceded by a significant decrease in cAMP levels. Stimulation of adrenergic pinealocyte receptors by the sympathetic nerves in the nervi conarii activates cyclase enzymes to produce cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) which in turn stimulate *N*-acetyltransferase to produce *N*-acetylserotonin, which is finally converted by hydroxyindole-*0*-methyltransferase to melatonin (Figure 3.4) (Erlich and Apuzzo, 1985).

The CREM gene has an alternative promoter that regulates the expression of ICER (Inducible cAMP Early Repressor), a strong CREB repressor (Stehle et al., 1993). The kinetic of ICER expression is characteristic of an early response gene (Sassone-Corsi, 1998). ICER is regulated in a circadian manner in the pineal gland, the site of the hormone melatonin production. This night–day oscillation is driven by the endogenous clock (located in the suprachiasmatic nucleus). The synthesis of melatonin is regulated by a rate-limiting enzyme, serotonin N-acetyltransferase (NAT). Analysis of the CREM-null mice and of the promoter of the NAT gene revealed that ICER controls the amplitude and rhythmicity of NAT, and thus the oscillation in the hormonal synthesis of melatonin (Sassone-Corsi, 1998). Analysis of CREM expression in the pineal gland has revealed a dramatic day–night regulation, with peak during the night. The CREM isoform in the pineal gland corresponds to ICER, the early response repressor known to be cAMP-inducible in endocrine cells (Stehle et al., 1993). The transcript shows a very characteristic and reproducible kinetic of expression. It appears likely that the autoregulatory loop shown to control ICER transient inducibility would also play a role in the day–night cyclic expression in the pineal gland. The mechanism controlling this pattern of ICER expression was determined and found to require clock-distal elements. Indeed, it is known that at

night, postganglionic fibers originating from the superior cervical ganglia (SCG) release norepinephrine, which in turn regulates melatonin synthesis via adrenergic receptors. These analyses have shown that signals from the SCN direct the induction of CREM expression (Stehle et al., 1993). The NAT promoter was shown to contain a CRE which binds ICER with high affinity. In addition, the amplitude of NAT oscillation in CREM-deficient mice was shown to be altered with respect to wild-type animals, demonstrating that NAT is a direct target of CREM (Foulkes et al., 1996). Another important finding concerning the role of CRE-binding factors in circadian rhythms concerns the cyclic phosphorylation of CREB in the suprachiasmatic nucleus (Ginty et al., 1993). During the night, upon light stimuli which phase-shift the clock, CREB appears to be efficiently phosphorylated by an SCN-endogenous kinase at the serine 133 residue. Phosphorylation at this site turns CREB into an activator and may be obtained by a number of kinases (Lalli and Sassone-Corsi, 1994).

In the rodent pineal gland, melatonin production is controlled via transcriptional mechanisms, norepinephrine (NE)-dependent activation of transcription factors is of central importance for the rhythmic production of melatonin. Night-time release of noradrenaline in the mammalian pineal gland activates adrenoceptors and thereby stimulates adenylate cyclase activity. The associated intracellular rise in cAMP is crucial in subsequent up-regulation of AANAT and melatonin synthesis (Sugden et al., 1985; Vanecek et al., 1985). Increases in intracellular cAMP levels lead to activation of cAMP-dependent protein kinase (PKA) and the transport of active catalytic subunits to the nucleus. CRE-binding protein (CREB) are phosphorylated by PKA and thereby converted into powerful transcriptional activators (Foulkes et al., 1996). Adrenergic stimulation at night drives CREB phosphorylation and the termination of adrenergic stimulation towards morning is associated with its dephosphorylation (Foulkes et al, 1996). There is abundant evidence to indicate that CREB phosphorylation involves PKA (Zatz and Romero, 1978) while the phosphatase that dephosphorylates CREB in the pineal gland has yet to be identified. Dephosphorylation of CREB and the instability of the ICER transcript causes icer mRNA levels to fall dramatically to low basal levels by the beginning of the day. In contrast, the ICER protein is more stable and therefore persists at elevated levels throughout the day (Foulkes et al., 1996). At variance with

rodents, NE regulates melatonin biosynthesis through post-translational mechanisms in ungulates.

In the cow, melatonin synthesis occurs rapidly following onset of night (Hedlund et al., 1977). In vitro experiments showed that β1-AR stimulation elevates cAMP level, activates AA-NAT via a type II PKA, and increases melatonin release (Ruppel and Olcese, 1991; Maronde et al., 1997; Schomerus et al., 2002). Similarly, in isolated bovine pinealocytes, immunoblots showed that the NE-induced immunoreaction was due to phosphorylation of the transcription factor CREB and another protein, presumably the activating transcription factor 1 (ATF-1) (Schomerus et al., 2003). Regulation of AA-NAT activity was therefore proposed to result from translational and post-translational mechanisms, which was thereafter confirmed (Schomerus et al., 2000). Following cloning of the gene coding for bovine AA-NAT, it was shown that pineal *Aa-nat* mRNA levels are high both during the day and night with only a small increase at night (Craft et al., 1999). Recently, it was proposed that during the day, in the absence of cAMP, AA-NAT protein is constantly translated but instantly degraded by proteosomal proteolysis; in contrast, during the night, β1-AR activation increases the levels of cAMP and PKA activity which, in turn, protects the protein from degradation and thereby enhances AA-NAT activity (Schomerus et al., 2000).

In humans and monkeys limited studies suggest a "sheep-like" regulation. There is an immediate increase in circulating melatonin at the onset of darkness (Reppert et al., 1979; Arendt, 1995). In rhesus monkey and human, the quantity of *Aa-nat* mRNA is high and displays no daily variations, while the enzyme activity increases by up to 10-fold at night (Coon et al., 1996, 2002). The mean daily level of pineal HIOMT activity is about 4.3 ± 0.1 nmol/h/mg protein in human (Bernard et al., 1995) and about 9 nmol/h/mg protein in rhesus monkey (Coon et al., 2002) with no significant day/night variation. Daytime β1-AR stimulation does not stimulate melatonin synthesis (Berlin et al., 1995), but its nocturnal synthesis can be inhibited by a β1-AR antagonist (Cowen et al., 1985). In humans, there is a large interindividual variability in the daily pattern of melatonin synthesis which also varies depending on age (Baskett et al., 2001).

In the pineal gland of most mammals, the nocturnal increase in melatonin synthesis and release is primarily driven by AA-NAT activity. Studies on the regulation of this enzyme in the rat have shown that the release of NE at the beginning of the night activates both β1- and α1-AR, resulting in a large increase in the intracellular levels of cAMP and PKA-induced phosphorylation of CREB into P-CREB. The latter transcription factor is thought to induce a massive expression of the gene coding for AA-NAT. The enzyme, rapidly synthesized/activated, catalyzes the synthesis of melatonin from 5-HT. NAergic stimulation also induces, but to a lesser degree, the expression of genes coding for tryptophan hydroxylase (TPOH) and hydroxyindole-*O*-methyltransferase (HIOMT), and other transcription factors that do not appear to be involved in the nocturnal stimulation of melatonin synthesis but rather in the modulation of this stimulation. Cessation of NE release at the end of the night or following a light exposure results in a rapid decrease in cAMP levels followed by post-translational inhibition of AA-NAT activity (destabilization/proteolysis).

In nonrodent species, nocturnal increase in the synthesis of melatonin appears to depend mainly on post-translational mechanisms (Stehle et al., 2001). The high level of *Aa-nat* mRNA throughout the 24-hr cycle allows a sustained synthesis of AA-NAT protein that is rapidly degraded by proteasome proteolysis during the day, whereas at night NE-induced cAMP accumulation inhibits AA-NAT proteolysis and allows rapid enzyme activation and melatonin synthesis. Besides *Aa-nat*, *Hiomt* mRNA is also regulated every night by the NE input, but with a different effect of time on HIOMT activity, due to the much higher stability of HIOMT protein compared to AA-NAT. Consequently, HIOMT activity displays a significant photoperiodic/seasonal variation in the pineal gland of several rodent species, with a higher activity under longer nights (Ribelayga et al., 1998, 1999).

The transmitter Receptor Sites in the Pineal Gland

In addition to NE, the metabolic activity of the pineal gland may be regulated by several other neurotransmitters and peptides that have been made the object of the reviews (Ebadi, 1980; Ebadi and Govitrapong, 1986; Simonneaux and Ribelayga, 2003).

1. Serotonergic recptor site

The pineal gland is characterized by high intracellular levels of 5-HT (5-hydroxytryptamine) stored, by vesicular monoamine type 1 transporter, in cytoplasmic vesicles in the long branching processes of pinealocytes (Hayashi et al., 1999). The 5-HT content in the pinealocytes has generally only been considered as cellular stock used as a substrate for the synthesis of melatonin (Mefford et al., 1983) because it exhibits a daily rhythm (90 ng/ gland during the day and 10 ng/gland at night in the rat; Quay, 1963) opposite to that of melatonin. While this function of 5-HT is important, it may not be its only role. Indeed, comparison of the daily melatonin and 5-HT rhythms shows, especially at the day/night and night/day transitions, that these two indoles do not vary in a strict opposition in the rat (McNulty et al., 1986), Syrian hamster (Miguez et al., 1995), Siberian hamster (Miguez et al., 1996), and European hamster (Pe´vet et al., 1989b). The concentration of 5-HT in the pineal gland decreases markedly at the beginning of the night before AA-NAT activation and melatonin release. In addition, in the rat pineal gland the nocturnal decrease in 5-HT (80 ng/gland) is far larger than the nocturnal increase in melatonin (1 ng/gland).

Several studies have reported that 5-HT is also a secretory product of the pinealocytes (Chuluyan et al., 1989; Miguez et al., 1997). Furthermore, using pineal microdialysis, it has been shown that 5-HT is released in the pineal extracellular medium during the day with a significant increase at the beginning of the night followed by a marked decrease later in the night (Sun et al., 2002). These observations, suggesting that pineal 5-HT may display auto/paracrine effects on pineal metabolism, have triggered several studies to elucidate the mechanisms regulating 5-HT release and the role of 5-HT in the rat pineal gland (Miguez et al., 1997). They have found that there is a high basal release of 5-HT compared to that of melatonin in cultured pineal cells. The in vitro release of 5-HT from stimulated rat pinealocytes depends on the metabolic orientation of 5-HT that depends on the level of AA-NAT activity: with moderate β_1-AR stimulation, the synthesis and release of both 5-HT and melatonin are increased; following strong β_1-AR stimulation, the intracellular levels and release of 5-HT are markedly decreased while melatonin synthesis and release are maximal (Miguez et al., 1997). These in vitro results are in agreement with the observations found using pineal microdialysis namely that extracellular 5-HT levels

are high during the day, further increased at the beginning of the night, and then markedly decreased during the night because of a major mobilization of 5-HT for melatonin synthesis. This triphasic rhythm in 5-HT release is circadian and depends on the NAergic input (Sun et al., 2002).

The putative role of extracellular 5-HT on pineal metabolic activity has been examined. Early studies showed that part of the 5-HT released into the extracellular medium was taken up by the sympathetic nerve endings to be oxidized into 5-HIAL (5-hydroxyindole acetaldehyde) and then metabolized into 5-MIAA (5-methoxyindole acetic acid) and 5-ML (5-methoxytryptophol) in the pinealocytes (Masson-Pe´vet and Pe´vet, 1989). This effect may be mediated by activation of 5-HT_2 receptors, although the 5-HT_2 agonist/antagonist concentrations used to obtain a significant effect were quite high (Miguez et al., 1997). This 5-HT_2 receptor was first characterized in the bovine pineal gland (Govitrapong et al., 1991) and was recently proposed to be of the $5HT_{2c}$ subtype in the rat pineal gland (Steardo et al., 2002).

2. Adrenergic Receptors

Several subtypes of adrenergic receptors (AR) are expressed in the rat pineal gland.

a. Subtype β_1 (β_1-AR)

This receptor is present at a very high density on the postsynaptic rat pineal membrane where it is positively coupled via a Gs protein to the membrane AC (Strada et al., 1972). Its physiological importance has been demonstrated by early in vivo experiments (Deguchi and Axelrod, 1972a). The effect of NE appears to be mainly mediated by this receptor subtype since in vivo injections of the β_1-AR agonist isoproterenol (ISO) during the day stimulates AA-NAT activity up to nighttime values (Deguchi and Axelrod, 1972a) while an in vivo injection of the β_1-AR antagonist propranolol strongly inhibits the nocturnal increase in AANAT activity (Deguchi and Axelrod, 1972b). Studies by Craft et al. (1985) showed that superior cervical ganglionectomy in rat increased the B_{max} of β-adrenergic receptors from 596 to 816 fmol/mg protein, while the KD value remained unchanged. In the hamster, superior cervical ganglionectomy did not alter either the KD or the B_{max} values. The density of the β_1-AR displays a circadian and daily variation, with the highest density observed at the end of the day/beginning of the night. The mRNA expression of β_1-AR displays an

opposite circadian rhythm, with nighttime values being 2-fold higher than the daytime values (Moller et al., 1997).

b. Subtype α_1 (α_1-AR)

This receptor is localized postsynaptically in the pineal gland (180 fmol/mg protein in the rat; Sugden and Klein, 1984) where it is coupled to the phospholipase C (PLC) transduction system involving IP_3, Ca^{2+}, and DAG (Klein, 1985). The mRNA coding for both types 1A and 1B is expressed in the rat pinealocytes, but only the protein of the 1B subtype appears to be present (Sugden and Klein, 1984). The mRNA expression of these receptors displays a circadian and daily variation, with higher values at night. The receptor density, however, shows no daily variation, but increases 2-fold after 3 weeks in L/L or after superior cervical ganglionectomy (SCGx) (Sugden and Klein, 1985) suggesting a slow turnover of the receptor protein.

c. Subtype α2 (α2-AR)

This receptor has been characterized pharmacologically as the α_2-ARA/D subtype (70 fmol/mg protein in the bovine and rat pineal glands, Simonneaux et al., 1991). Several in vivo and in vitro studies have shown that this receptor is localized on the presynaptic NAergic terminals, where it inhibits NE release (Simonneaux et al., 1994). Other studies, however, have shown that this receptor is also present on the pinealocyte membranes, where it indirectly activates a guanylate cyclase (GC) and stimulates AA-NAT activity and melatonin release (Mustanoja et al., 1999).

2. Dopaminergic Receptors

Some observations indicate that DA is not only the precursor of NE, but also a true pineal neurotransmitter. TH-IR and DA β-hydroxylase immunonegative fibers exist in the pineal gland (Jin et al., 1988). In addition, DA concentrations display a marked daily rhythm with higher nocturnal values in the rat, cow, Siberian hamster, and Syrian hamster (Fujiwara et al., 1980; Govitrapong et al., 1989; Miguez et al., 1995, 1996). Furthermore, after SCGx, TH activity and DA are still detectable in the rat pineal gland (Hernandez et al., 1994). In isolated membranes of the bovine pineal gland, a high density of typical subtype 1 DA receptor (D_1-R) (positively coupled to AC) has been characterized (Simonneaux et al., 1990). The bovine pineal gland also contains typical subtype 2 DA receptors (D_2-R) (negatively coupled to AC) although

with a lower density (Govitrapong et al., 1984). In this species the density of D_1-R is markedly higher (6- to 20-fold) than the density of β_1-AR, α_1-AR, and D_2-R (Simonneaux et al., 1991), suggesting an important role for DA in the regulation of pineal metabolic activity. Biochemical studies performed in cultured rat pineal glands have shown that DA displays an inhibitory effect at low concentrations (0.1 μM) and a stimulatory effect at high concentrations (10 μM) on AA-NAT activity and melatonin release (Axelrod et al., 1969; Govitrapong et al., 1989), probably related to the presence of the two subtypes of DA receptors. A recent report shows that DA may interfere with β_1-AR to induce Ca^{2+} signaling in the rat pineal gland (Rey et al., 2001). The presence of DAcontaining fibers, the identification of specific DA receptors, and the demonstration of biochemical effects of DA suggest that DA may be a pineal neurotransmitter whose physiological role remains to be established.

Govitrapong et al. (1984) using [^{3}H]-spiroperiodol and other specific dopaminergic receptor site agonists and antagonists, characterized two [^{3}H]-spiroperidol binding sites in the pineal gland, with K_d values of 0.18 and 2.1 nM and B_{max} values of 36.8.and 630 fmol/mg protein. Dopamine may have a synergistic effect with that of norepinephrine in stimulating the activity of serotonin-*N*-acetyl-transferase (Axelrod et al., 1969; Deguchi and Axelrod, 1972a, 1972b; Lynch et al., 1973). Moreover, Govitrapong et al. (2003) using [^{3}H] GBR 12935 investigated the existence of dopamine transporter in the bovine pineal gland. The result of this study indicated that dopamine transporters were present in the bovine pineal gland.

3. Muscarinic Cholinergic Receptors

Cholinergic fibers have been identified in the pineal gland of several mammals (Romijn, 1973; David and Kumar, 1978; Phansuwan- Pujito et al., 1990, 1991, 1999). The origin of these pineal cholinergic fibers may be the habenular nucleus or peripheral parasympathetic (pterygopalatine or otic) ganglia. In addition, some cells of the pineal gland (nervous cells and/or pinealocytes) synthesize ACh (Romijn, 1975; Phansuwan- Pujito et al., 1999). The ACh content of the pineal gland exhibits a marked daily rhythm with nighttime values being 10- to 20- fold higher than daytime value. The characterization of cholinergic receptors in the pineal gland of some mammals strengthens the idea of parasympathetic modulation of pineal metabolic

activity. High-affinity muscarinic receptors (mACh-R) have been characterized in the pineal gland of the rat, sheep, and cow (Taylor et al., 1980; Finocchiaro et al., 1989; Govitrapong et al., 1989).

The existence and subunit identification of the nicotinic cholinergic receptors in the rat pineal gland were examined by autoradiography (Stankov et al., 1993), using [^{3}H]cytisine and [^{125}I]alpha-bungarotoxin as labelled ligands. The experiments suggested that the nicotinic cholinergic receptor could be involved in the functional regulation of the gland. The presence of nicotinic receptors (nACh-R) has also been demonstrated by immunocytochemistry (in 25% of pineal cells: Reuss et al., 1992b), and by in situ hybridization (Yeh et al., 2001). The effects of chronic oral nicotine administration on the pineal melatonin and brain transmitter monoamines were studied in male mice, which possessed a clear daily rhythm of melatonin secretion (Gaddnas et al., 2002). The results suggested that chronic nicotine treatment slightly suppressed the melatonin production but did not alter the daily rhythm of pineal melatonin in mice maintained on a light–dark cycle. However, the results indicated that nicotinic receptors might be involved in the regulation of pineal function.

The main effect of mACh-R activation, however, probably occurs at the presynaptic level. A presynaptic effect was first postulated following the observation of an effect of ACh on the whole pineal gland but not on cultured pinealocytes (Laitinen et al., 1995). This hypothesis has now been confirmed by pineal microdialysis showing that carbachol inhibits the production of NAS and melatonin release via presynaptic inhibition of NE (Drijfhout et al., 1996a). Activation of the postsynaptic nACh-R induces, in a large majority of rat pinealocytes, Ca^{2+} influx via L-type Ca^{2+} channels following membrane depolarization (Schomerus et al., 1995).

In addition, it has been shown that nicotine has no effect by itself but inhibits NE-induced melatonin secretion (Stankov et al., 1993). It has been proposed that nACh-R-induced cell depolarization leads to the release of Glu from pineal microvesicles (MV), which in turn inhibits the secretion of melatonin (Yamada et al., 1998). Interestingly, recent studies reported a developmental switch from rat pineal mAChR to nAChR around the third week of life with the parallel appearance of L-type Ca^{2+} channels (Schomerus and Korf, 1999; Wagner et al., 2000). In adult bovine pineal cells activation of either nACH-R or mACh-R induces an increase in

intracellular level of Ca^{2+}, but with no apparent effect of basal or NE-induced AA-NAT activity and melatonin synthesis (Schomerus et al., 2002). In summary, parasympathetic input would therefore exert a tonic inhibition on pineal activity, on the one hand via presynaptic inhibition of NE release and, on the other hand, via postsynaptic activation of the inhibitory intrapineal Gluergic system.

4. Glutamate receptor

Glutamate (Glu), usually considered to be an excitatory amino acid, is present in the pineal gland at high concentrations (1.2 mg/g rat pineal). It is mainly localized in pinealocytes, associated with MV (the endocrine counterpart of synaptic vesicles), but it has also been found in glial cells and fibers whose origin is unknown (McNulty et al., 1992). It has been suggested that the pineal Glu concentration is partly controlled by NE (McNulty et al., 1992). The transport of Glu in MV and its effect on pineal metabolic activity has been well studied in several mammals (Govitrapong and Ebadi, 1988; McNulty et al., 1992; Yamada et al., 1996, 1998). Glu is taken up into both pinealocytes and interstitial cells (Redecker and Pabst, 2000) mainly via a type 1 Na^{+}-dependent Glu transporter and then stored in MV via the synaptic vesicle protein of type 2 (SV2B, Hayashi et al., 1998). Following cell depolarization, Glu is released by exocytosis via Ca^{2+}-dependent mechanisms. The endogenous transmitter responsible for depolarization-induced Glu release could be ACh acting via nACh-R. Extracellular Glu inhibits AA-NAT activity and melatonin secretion induced by NAergic stimulation. In the rat pineal gland the binding site for Glu is a class II metabotropic Glu receptor of type 3 (mGluR3) coupled to a Gi protein responsible for the cAMP-dependent decrease in AANAT activity and melatonin synthesis (Yamada et al., 1998). Other ionotropic Glu receptors have also been reported in the pineal glands of several species (Govitrapong et al., 1986; Yatsushiro et al., 2000). In the rat pineal gland GluR1 is functionally expressed in pinealocytes and may participate in a Ca^{2+}-signaling cascade that enhances and expands the Gluergic signal throughout the pineal gland (Yatsushiro et al., 2000). Glu has also been proposed to inhibit HIOMT activity, but not HIOMT mRNA (Ishio et al., 1999). It is interesting to note that Glu also activates NOS in several tissues and could therefore be involved in cGMP synthesis. Apart from Glu, L-aspartate is present in high concentrations in the rat

pineal gland; it is released together with Glu during exocytosis and inhibits the NE-induced increase in AA-NAT activity and melatonin synthesis (Yatsushiro et al., 1997). Of any mammalian tissue, the highest concentrations of D-aspartate occur in the pineal gland (Schell et al., 1997). D-aspartate is actively taken up by the pineal cells and then released upon NE-stimulation, where it strongly inhibits the NE-induced increase in AA-NAT activity and melatonin synthesis (Takigawa et al., 1998). In addition to Glu and aspartate, cultured pinealocytes also release glycine upon stimulation with depolarizing concentrations of KCl (Redecker et al., 2001). Furthermore, the immunohistochemical study was performed on the human pineal glands by using antiserum against glutamate as probes. Only a few Glu-IR nerve fibers but many Glu-IR cell bodies were demonstrated in the human pineal gland. They were arranged with unstained cells into clusters and some of them appeared to be neuron-like cells. These data show that the amino acid Glu (and possibly aspartate) is probably an important auto/paracrine transmitter involved in the regulation of melatonin synthesis in the pineal gland. In vitro, it appears to be released upon ACh stimulation and inhibits NE-induced melatonin synthesis. In addition, the glutamatergic communication in the pineal gland may enable paracrine cross-talk among pinealocytes as well as interactions between pinealocytes and interstitial cells. Additional in vivo experiments are now needed to clarify the exact role of this amino acid negative loop in the regulation of melatonin synthesis (Simonneaux and Ribelayga, 2003).

5. GABAergic receptor

GABA, an inhibitory neurotransmitter, is present in the pineal gland of several mammals where it is considered to be an intrapineal transmitter with paracrine effects (Ebadi and Chan, 1980; Ebadi and Govitrapong, 1986; Rosenstein et al., 1989, 1990). The immunodetection of GABA transporters (GAT 1–3) in pinealocytes, and to a lesser extent in interstitial cells, together with the GABA synthesizing enzyme confirms the paracrine function of GABA in the gerbil pineal gland (Redecker, 1999). GABA has also been observed in the pinealopetal fibers that remain after SCGx and seen passing through the posterior and habenular commissures and the deep pineal gland, both observations indicating a central origin of this innervation (Sakai et al.,

2001). Typical A-type (GABAA-R) and B-type (GABAB-R) GABA receptors have been identified in the pineal gland. In the rat pineal gland, GABA inhibits NE-induced melatonin synthesis via GABAA-R and inhibits the NE release via GABAB-R (Rosenstein et al., 1989, 1990). In the bovine pineal gland GABA decreases NAergic stimulation of AA-NAT activity, increases Cl^- flux, and decreases 5-HT release (Ebadi and Chan, 1980). In the sheep pineal gland, GABA also inhibits the NE-induced increase of AA-NAT activity (Foldes et al., 1984). The quantity of GABA in the pineal gland of the rat (Waniewski and Suria, 1977) and Syrian hamster (Kanterewicz et al., 1993) exhibits a daily variation with higher nighttime values.

6. Histaminergic receptor

Pineal gland of various mammalian species contains histamine. The rat pinealgl and is moderately innervated by histaminergic fibers of central origin (Mikkelsen et al., 1992). Histaminergic neurons of the tuberomammillary nucleus of the posterior hypothalamus project via the posterior commissure to the deep pineal gland, the pineal stalk, then to the proximal part of the superficial pineal gland. In the chicken, histamine is a powerful stimulator of cAMP (Nowak et al., 1997), but in the rat no effect has been observed on AA-NAT activity or the metabolism of PI (Muraki, 1972). The possibility that a metabolite of histamine may have an effect needs to be considered, since enzymes involved in the metabolism of this amine are present at high concentration in the pineal gland. In addition, a presynaptic effect of histamine on the release of a neurotransmitter, especially NE, is possible (Yamazaki et al., 2001).

7. Nitric Oxide

NO is a diffusible neurotransmitter implicated in a variety of neuroendocrine processes. Three isoforms of the synthesizing enzyme NOS have been described: type I is neuronal, Ca^{2+}-dependent, and not inducible; type II is Ca^{2+}-independent and inducible; type III is the endothelial isoform (Jacobs et al., 1999). NO is synthesized in the sympathetic fibers innervating the pineal gland of sheep (Lopez-Figueroa et al., 1996) and rat (Lopez-Figueroa and Moller, 1996). NO is also synthesized in nonsympathetic (VIPergic) fibers of the sheep and rat pineal gland (Lopez-Figueroa et al., 1997). In addition, neuronal NOS present in the rat (Lopez- Figueroa and Moller,

1996) and cow (Maronde et al., 1995) pinealocytes. Although NO appears to be synthesized only in a small subpopulation of pineal cells, it is thought to be an intercellular messenger acting on all pineal cells (Spessert et al., 1998). Pineal NOS expression and activity are regulated by NE in the long-term/ photoperiodic range (Spessert and Rapp, 2001). NO could also be involved in the regulation of pineal blood flow (most VIPergic fibers end in pineal perivascular spaces; NO is known to display vasorelaxant effects, similar to VIP; and NOS activity has been measured in the endothelium of blood vessels in the sheep and rat pineal gland). Finally, exogenous NO is reported to be a powerful inhibitor of melatonin synthesis in the rat and bovine pinealocytes (Maronde et al., 1995) via cGMP-independent mechanisms that remain to be determined.

8. Benzodiazepine Receptors

Benzodiazepine receptors have been characterized in the bovine gland by Lowenstein and Cardinali (1983), in the human pineal gland by Lowenstein et al. (1984), and in the rat pineal gland by Weissman et al. (1984). Using [^{3}H] flunitrazepam and [^{3}H]-ethyl-β-carboline-3-carboxylate ester as ligands, Lowenstein and Cardinali (1983) identified a single population of binding sites with a K_d value of 3.4 nM and a B_{max} value of 55.6 fmol/mg protein. Studies by Weissman et al. (1984) have shown that the density of "peripheral type" binding sites for benzodiazepines in the rat pineal gland, but not the cerebral cortex, was reduced by 50 three weeks after superior cervical ganglionectomy or exposure to constant light.

9. Opioid receptor

Opiates stem from three precursor families: proopiomelanocortin (proopiomelanocortin, giving the β-endorphins; MSH; and corticotrophin), pro-Enk (giving Leu-Enk and Met-Enk), and prodynorphins (giving the dynorphins A and B, and neoendorphins). The pineal gland of several species contains fibers and cells IR to various opiates. Fibers containing some opiates, especially Leu-Enk and Met-Enk, β-endorphins, and dynorphin, have been observed in the pineal gland of the guinea pig (Schroder et al., 1988), human (Moore and Sibony, 1988), cow (Cherdchu et al., 1989; Moller et al., 1991), European hamster (Coto-Montes et al., 1994), and tree shrew (Phansuwan-Pujito et al., 1998). The origin of these fibers is not known but could be

the SCG, habenular nuclei, trigeminal ganglia, or parasympathetic ganglia. In several species, namely the guinea pig (Schroder et al., 1988), rat (Aloyo, 1991), and European hamster (Coto-Montes et al., 1994), cells of the pineal gland have been shown to synthesize opiates, especially Enk. In the bovine pineal gland most of opiate receptors are of the δ-type and fewer of the μ subtype (Govitrapong et al., 1992, 2002; Aloyo and Pazdalski, 1995). In mice, high levels of mRNA coding for δ opiate receptors have been observed in the pineal and pituitary gland (Bzdega et al., 1993). In contrast, only low levels of δ and μ opiate receptor mRNA expression were found by RT-PCR in the rat pineal gland (Chetsawang et al., 1999).

Most endorphins and Enk display a stimulatory effect on melatonin synthesis in vivo and ex vivo (Govitrapong et al., 1992). One study, however, has demonstrated a positive effect of high concentrations of morphine on AA-NAT activity and melatonin production in the bovine pineal gland (Govitrapong et al., 1992, 1998). The opiates are also considered to be the endogenous ligands for the σ receptors. These receptors were characterized in the rat (with a high density: Jansen et al., 1990) and sheep (Abreu and Sugden, 1990) pineal gland. Two studies have shown contradictory results on the effect of activation of these receptors on melatonin synthesis. One study has reported that the haloperidol inhibits NE-induced melatonin release via inhibition of cAMP production and PI turnover (Olcese, 1995). An association between melatonin/opiates/analgesia, especially the possibility that nocturnal endogenous melatonin has analgesic and hypnotic properties, has been discussed extensively by Ebadi et al. (1998).

Several lines of evidence have reported that there are opioidergic nerve fibers innervating the pineal gland via opioid receptors. By using a nonselective radio-ligand, [^{3}H]-Diprenorphine revealed the existence of opioid receptors on bovine pinealocyte membranes. Thus, the results indicated that a majority of opioid receptor sites are delta opioid receptors, with a minority being mu opioid receptor sites and revealed an interaction among opioids and pineal gland (Govitrapong et al., 2002).

10. Neuropeptides

Neuropeptides are true pineal transmitters. Since the 1980s, most studies on pineal peptides have focused either on the immunocytochemical demonstration of their

presence and origin in the pineal gland or on their biochemical effects on melatonin synthesis. All the preceding studies have shown that several peptides of the pineal gland bind to specific receptors to regulate some metabolic pathways, especially synthesis of melatonin. The precise physiological role of these pineal peptides in the regulation of melatonin rhythmicity, however, remains to be determined. The observations of daily and seasonal variations in their pineal content associated with specific daily and seasonal modulation of pineal metabolism (for example, the associated variations in NPY content and HIOMT activity: Moller et al., 1998; Ribelayga et al., 1998) support a physiological function of these neuropeptides in the expression of the daily and annual melatonin rhythms. To evaluate their function in the pineal physiology, it will be necessary to make timed correlations between the presence/absence/variations of each peptide with a particular situation of pineal metabolism and/or an associated physiological function, and then to prove causality. This will definitely require an expansion of studies to other species, especially those with marked seasonal rhythms. For example, in the European hamster, the seasonal variations in pineal NPY-IR are associated in time with those of pineal HIOMT activity and melatonin and 5-ML (5-methoxytryptophol) concentrations (Moller et al., 1998; Ribelayga et al., 1998). These in vivo results are very important because for the first time in a possible physiological function of a neuropeptide in the mammalian pineal gland. In addition, in vivo microdialysis experiments with local pineal infusion of neuropeptide agonists/antagonists or antisense molecules for neuropeptide receptors should be continued to investigate the in vivo effect of neuropeptides in physiological conditions. It will be necessary to determine the nature of the information brought to the pineal gland by the peptides. It is thus possible that some of the peptides present in the pineal gland might represent the anatomical and functional way by which nonphotic stimuli reach and are integrated by the pineal gland (Pe´vet et al., 1989a).

An Overview of Dopamine Systems

The neurotransmitter dopamine belongs to the catecholamine group that, in turn, belongs to the wider group of neurotransmitters: the monoamines. A monoamine is any molecule that contains a single amine group ($-NH_2$). Therefore, all

catecholamines will contain one amine group. The unifying characteristic of catecholamines that chemically differentiating them from other monoamines is clear that they all have a similar “base” known as the “cathechol nucleus” which is composed of a benzene ring with two adjacent hydroxyl groups (-OH).

Dopamine can have either an excitatory or inhibitory effect on the postsynaptic potential. In other words, when dopamine leaves the presynaptic neuron and goes into the synapse, it can then bind to receptors on the postsynaptic neuron. After dopamine is bound to the postsynaptic cell, it can either facilitate an action potential or inhibit it. Neurotransmitters are the messengers within the central nervous system. They are responsible for communicating with other neurons either through chemical or electrical signals. The precursor for the synthesis of DA is the aromatic amino acid tyrosine (Figure 3.6). Hydroxylation of the amino acid tyrosine by tyrosine hydroxylase to produce L-dihydroxyphenylalanine (L-dopa) is the rate limiting step in the synthesis of all major catecholamines. The L-dopa is converted to dopamine by aromatic L-amino acid decarboxylase. L-dopa crosses the blood-brain barrier, but dopamine cannot. Dopamine is converted to norepinephrine by dopamine β-hydroxylase, which is the only catecholamine-synthesizing enzyme that is located inside the neurotransmitter vesicles. The rest of the enzymes are located in the cytoplasm (Wiener and Breslin, 1995).

DA constitutes about 80% of the catecholamine content in the brain (Vallone et al., 2000). The application of in situ hybridization and polymerase chain reaction (PCR) with the newly cloned receptor probes made it possible to localize DA receptors to specific brain regions or peripheral tissues even where they had not been anticipated before. The function of many of these receptors, however, is still completely unknown, thus highlighting a serious gap between the molecular biology and the functional approaches. Pharmacological manipulations have, in fact, partially clarified the role of D_1 and D_2 receptors in the control of various functions as well as the interaction of DA with other neurotransmitter systems (Missale et al., 1998).

Classification of Dopamine Receptors

The first evidence for the existence of DA receptors in the CNS came in 1972 from biochemical studies showing that DA was able to stimulate adenylyl cyclase (AC). In 1978, DA receptors were first proposed, on the basis of pharmacological and biochemical evidence, to exist as two discrete populations, one positively coupled to AC and the other one independent of the adenosine 3', 5'-cyclic monophosphate (cAMP) generating system (Spano et al., 1978). In 1979 it was clearly recognized that the actions of dopamine are mediated by more than one receptor subtype.

Two dopamine receptors, termed type 1 (D_1) and type 2 (D_2) were distinguished on the basis of differential binding affinities of a series of agonists and antagonists, distinct effector mechanisms, and distinct distribution patterns within the CNS (Kebabian and Calne, 1979). It was subsequently found that therapeutic efficacy of antipsychotic drugs correlated strongly with their affinities for the D_2 receptor, implicating this subtype as an important site for the action of antipsychotic drugs. Three additional dopamine receptor genes have been identified, encoding the D_3, D_4, and D_5 dopamine receptors. Based on their regional brain distributions and primary effector mechanisms, the D_3 and D_4 receptors are considered to be D_2-like, and the D_5 receptor is considered to be D_1-like (Missale et al., 1998).

All DA receptor subtypes are based on structural, pharmacological, and biochemical studies (Table 3.1). The D_1 receptor was initially distinguished from the D_2 subtype by its high affinity for the antagonist SCH 23390 and its relatively low affinity for butyrophenoens such as haloperidol. Where as D_1 receptor activation stimulates cAMP formation, D_2 receptor stimulation produces the opposite effect on cAMP formation. In addition to the stimulation of adenylate cyclase, D_1 receptors may also stimulate phosphoinositide turnover. D_1 receptor mRNA is expressed in the terminal fields of the nigrostriatal and mesocorticolimbic pathways, with high levels in the dorsal striatum, nucleus accumbens, and amygdala.

In contrast, little D_1 mRNA expression is found in dopamine cell body regions such as the sunstantia nigra pars compacta and the ventral tegmental area. This finding, and the persistence of D_1 receptor binding following lesions of dopaminergic

neurons, suggests that this receptor subtype is not found on dopaminergic neurons and is therefore not an autoreceptor.

In addition, the initial distinction between D_1 and D_2 receptors in terms of signaling events, that is, positive and negative coupling to AC, appears to apply to the members of the DA receptor family, the D_5/D_1b receptor being coupled to stimulation of AC (Dearry et al., 1990) and the D_2/D_3 (Robinson and Caron, 1996) and D_4 receptors (Chio et al., 1994; Cohen et al., 1992) to inhibition of cAMP formation. In intact tissue D_2 receptors are 2-3 orders of magnitude more sensitive to dopamine than D_1 receptors (Civelli et al., 1993; Kebabian and Calne 1979; Seeman and Van Tol 1994). Finally, one D_2 receptor subtype functions as a presynaptic autoreceptor whose activation inhibits dopamine release (Starke et al., 1989). Both D_1 and D_2 receptors are present in the vertebrate retina (Schorderet and Nowak, 1990). The activation of D_2 autoreceptors on dopaminergic cells inhibits dopamine release in the fish retina (Wang et al., 1997).

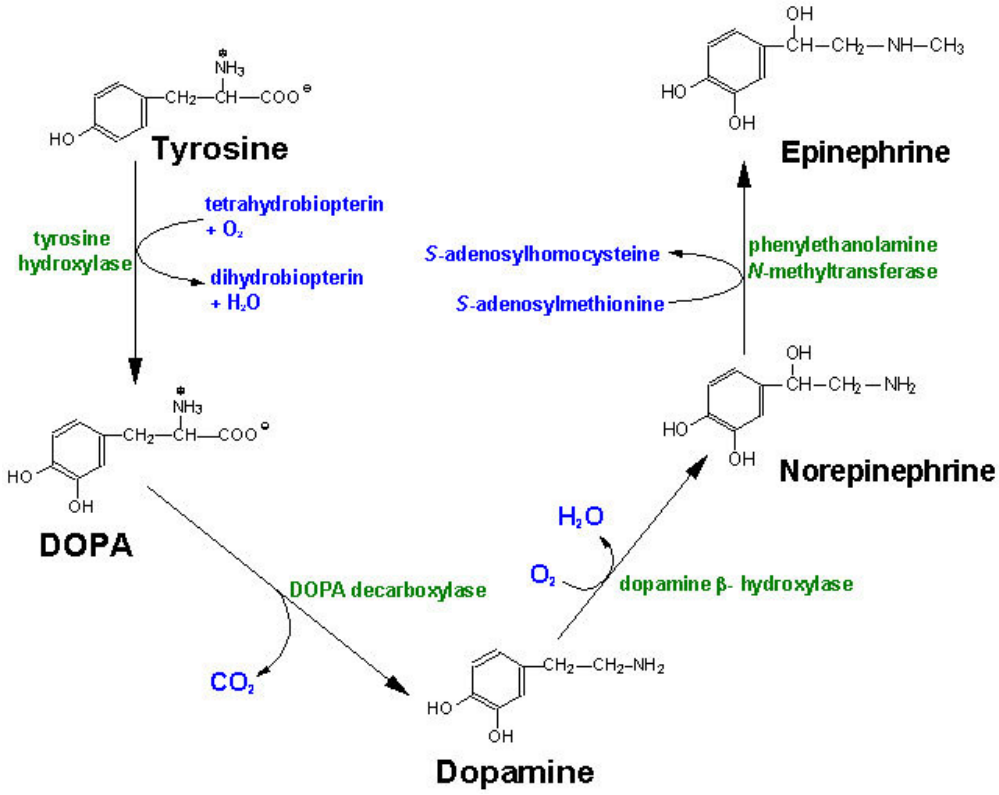


Figure 3.6 Dopamine synthesis pathways

Table 3.1 Dopamine Receptor Pharmacology

D_1-like receptor	D_1	D_5
Effector	↑ Adenylate cyclase	↑ Adenylate cyclase
G protein	Gs	Gs
Localisation	Caudate/putamen Nucleus accumbens Olfactory tubercle	Hippocampus Hypothalamus
Example agonists	SKF 38393 CY 208-243	SKF 38393 CY 208-243
Example antagonists	SCH 23390	SCH 23390

D_2-like receptor	D_2	D_3	D_4
Effector	↓ Adenylate cyclase	↓ Adenylate cyclase	↓ Adenylate cyclase
G protein	Gi/o	Gi/o	Gi/o
Localisation	Caudate/putamen Nucleus accumbens Olfactory tubercle	Nucleus accumbens Olfactory tubercle Island of Calleja	Frontal cortex Amygdala Cardiovascular system
Example agonists	Bromocriptine Quinpirole	Bromocriptine Quinpirole	PD 168077 Quinpirole
Example antagonists	L-741,626	GR 103691	L-741,742

Distribution of Dopamine Receptors

A neuron that classically releases dopamine is called a dopaminergic neuron. A series of neurons that are connected by synapses forms a pathway called a dopaminergic pathway (Figure 3.7).

There are three dopaminergic pathways:

1. Nigrostriatal System
2. Mesolimbic System
3. Mesocortical System

The nigrostriatal pathway originates in the most rostral division of the brain stem known as the midbrain, and its projections terminate in the striatum of the brain. The striatum is composed of the caudate nucleus and the putamen and is a key component of the basal ganglia, a region associated with the control of normal voluntary movement. This pathway is thought to be greatly altered in patients with Parkinson's disease, as evidenced by the severe motor disturbances connected to it. The mesocortical pathway originates in the ventral tegmental area where the cell bodies are located and its projections (axons) extend all the way to the frontal cortex. They have an excitatory effect, playing a role in the formation of short-term memories, planning, strategy, problem solving, as well as reinforcement in learning. The cell bodies of the mesolimbinc pathway also originate in the ventral tegmental area while their axons project to areas of the limbic system including the nucleus accumbens, amygdala, and hippocampus. This pathway is an integral part of the limbic system and also plays a major role in reinforcing behavior (Wiener and Breslin, 1995; Sadock and Sadock, 2000).

The tuberohypophyseal system consists of dopaminergic neurons in the hypothalamic arcuate and periventricular nuclei, and their projections to the pituitary gland. These projections provide inhibitory regulation of prolactin release. The administration of dopamine receptor antagonist antipsychotic drugs may lead to a disinhibition of release in galactorrhea (Sadock and Sadock, 2000).

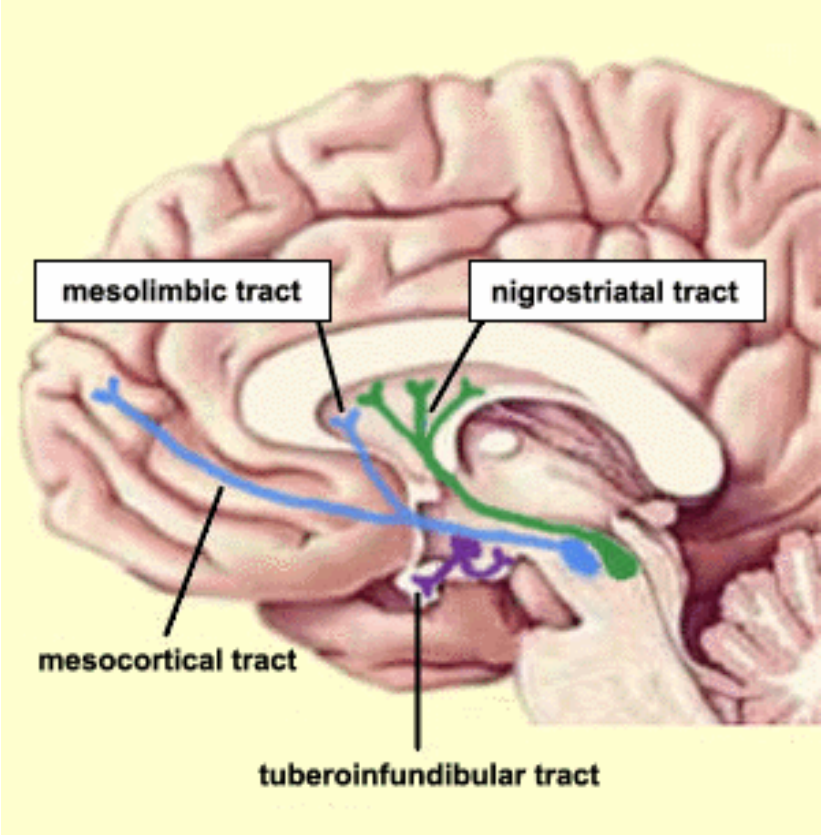


Figure 3.7 Dopaminergic pathways

The D_1 receptor is the most widespread DA receptor and is expressed at higher levels than any other DA receptor (Dearry et al., 1990; Weiner et al., 1991). D_1 mRNA has been found in the striatum, the nucleus accumbens, and the olfactory tubercle. In addition, D_1 receptors have been detected in the limbic system, hypothalamus, and thalamus. On the other hand, in other areas where the D_1 receptor protein is highly expressed such as the entopeducular nucleus and the substantia nigra pars reticulata, no mRNA has been detected (Dearry et al., 1990; Weiner et al., 1991). D_5 receptor mRNA has been found in several rostral forebrain regions including cerebral cortex, lateral thalamus, diagonal band area, striatum, and, to a lesser extent, substantia nigra, medial thalamus, and hippocampus (Rappaport et al., 1993). Ultrastructural analysis suggested that both D_1 and D_5 receptors are at postsynaptic densities of small synapses characteristics of DA terminals, and that presynaptic D_1 and D_5 receptors are on axons forming asymmetrical synapses (Bergson et al., 1995).

The D_2 receptor may have either a postsynaptic function or an autoreceptor function. D_2 autoreceptors may be found on dopaminergic terminals or on the cell bodies and dendrites of dopaminergic neurons. The D_2 receptor has been found mainly in the striatum, in the olfactory tubercle, in the core of nucleus accumbens. It is also found in the hypothalamus, in the substantia nigra pars compacta, and in the ventral tegmental area, where it is expressed by dopaminergic neurons (Bouthenet et al., 1991). The D_2 receptors are present in perikarya and dendrites within the substantia nigra pars compacta and are much more concentrated in the external segment of the globus pallidus than in other striatal projections (Levey et al., 1993). The D_3 receptor has a specific distribution to limbic areas (Landwehrmeyer et al., 1993) the olfactory tubercle, and the islands of Calleja (Bouthenet et al., 1991). D_4 receptor appears to be highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus, and mesencephalon (Van Tol et al., 1991). Significant levels of D_4 mRNA were also found in the retina (Cohen et al., 1992).

Structure of Dopamine Receptors

Analysis of the primary structure of the cloned DA receptors revealed that they are members of the seven transmembranes (TM) domain G protein-coupled receptor

families and share most of their structural characteristics. D_2-like receptors are characterized by a shorter COOH-terminal tail and by a bigger 3rd intracellular loop. Residues involved in dopamine binding are highlighted in transmembrane domains. Potential phosphorylation sites are represented on 3rd intracellular loop (I3) and on COOH terminus (Figure 3.8). Members of this family display considerable amino acid sequence conservation within TM domains (Missale et al., 1998). The NH_2-terminal stretch has a similar number of amino acids in all the receptor subtypes and carries a variable number of consensus N-glycosylation sites. The D_1 and D_5 receptors possess two such sites, one in the NH_2 terminal and the other one in the second extracellular loop. The D_2 receptor has four potential glycosylation sites, the D_3 has three, and the D_4 possesses only one (Civelli et al., 1993; Gingrich and Caron, 1993; Jackson and Westlind-Danielsson, 1994). The COOH terminal is about seven times longer for the D_1-like receptors than for the D_2-like receptors, is rich in serine and threonine residues, and contains a cysteine residue that is conserved in all G protein-coupled receptors (O'Dowd et al., 1989). In the D_1-like receptors, this cysteine residue is located near the beginning of the COOH terminus, whereas in the D_2-like receptors, the COOH terminus ends with this cysteine residue.

The D_2-like receptors have a long third intracellular loop, a feature which is common to receptors interacting with Gi proteins to inhibit AC, whereas the D_1-like receptors are characterized by a short third loop as in many receptors coupled to Gs protein (Civelli et al., 1993). The coupling of DA receptors to second messenger pathways (Figure 3.9) has been a subject of intense interest ever since their existence was recognized. The existence of a D_1 receptor-stimulated AC was recognized in most dopaminergic brain regions, such as striatum, nucleus accumbens, and olfactory tubercle (Memo et al., 1986). In a variety of cell culture lines, it was shown that the D_1 receptor robustly stimulated cAMP accumulation (Dearry et al., 1990). Upon the cloning of the second D_1-like receptor, the D_5 was also found to be coupled to stimulation of AC, as was predicted from its structural similarity to the D_1 receptor (Sunahara et al., 1991; Weiner et al., 1991).

Recent cloning of two more nonmammalian D_1-like receptor subtypes has indicated that these subtypes also stimulate cAMP accumulation in COS-7 cells (Demchyshyn et al., 1995). Thus activation of AC seems to be a general property of all

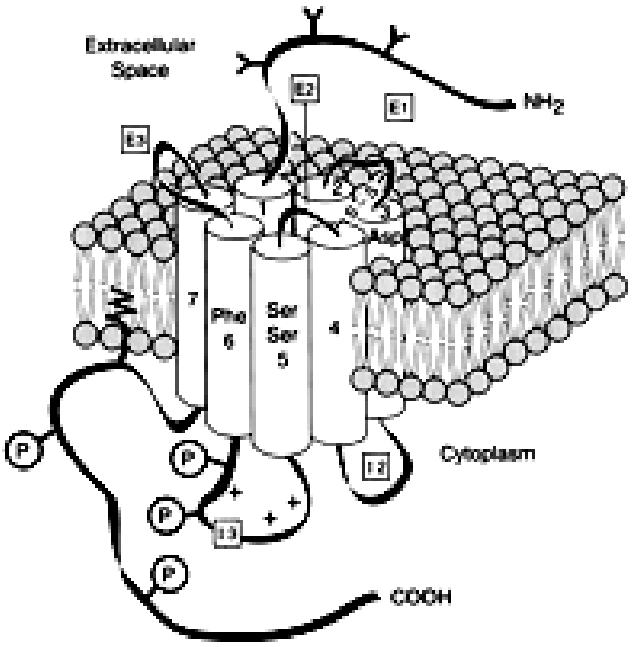


Figure 3.8 Dopamine receptor structure

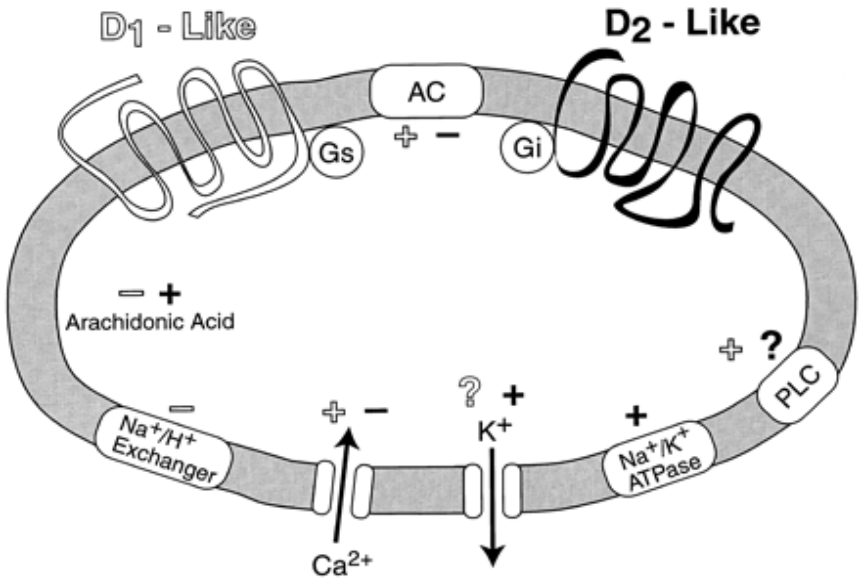


Figure 3.9 Signal transduction of dopamine receptor

(AC = adenylate cyclase, PLC = phospholipase C)

D_1-like receptors. It is generally assumed that the activation of AC by D_1-like receptors is mediated by the Gs subunit of G proteins. The cAMP-dependent PKA pathway is positively coupled to D_1-like receptors and negatively coupled to D_2-like receptors (Kebabian and Calne, 1979). D_2 receptor can inhibit AC was shown in the CNS (Onali et al., 1985). It has been shown that the D_3 receptor does weakly inhibit AC in some cell lines (Robinson et al., 1996). On the other hand, that the D_4 receptor can inhibit cAMP accumulation was reported in retina (Cohen et al., 1992) and a variety of cell culture lines (Chio et al., 1994). Thus inhibition of AC seems to be a general property of the D_2-like receptors.

As D_1 receptors may be coupled to different intracellular transduction systems, such as cAMP/PKA (Stoof and Kebabian, 1981), phospholipase C/PKC (Undie and Friedman, 1990). Phosphorylation of CREB following D_1 receptor stimulation has already been reported to occur in primary dissociation cultures of embryonic rat striatum and in organotypic slice cultures (Liu and Graybiel, 1996). Moreover, DA as well as D_1 receptor stimulation drastically increased the number of pCREB-IR cells, which could be abolished by inhibition of PKA (Schmidt et al., 1998).

The D_1-like receptors appear to modulate intracellular calcium levels by a variety of mechanisms. One mechanism is via the stimulation of phosphatidylinositol (PI) hydrolysis by phospholipase C (PLC), resulting in the production of inositol 1,4,5-trisphosphate, which mobilizes intracellular calcium stores. It has been shown that D_1-like receptor agonists cause increases in PI metabolism in various brain regions (Undie et al., 1990). On the other hand, the D_1 receptor appears to stimulate release of intracellular calcium stores via a mechanism other than stimulation of PI turnover. D_1 receptor- induced increase in intracellular calcium levels in 293 cells is mimicked, in fact, by other means of increasing cAMP levels (Lin et al., 1995), and thus is probably the result of activation of protein kinase A (PKA).

D_2-like receptors can also cause a decrease in intracellular calcium levels by inhibition of inward calcium currents (Seabrook et al., 1994). D_3 receptors also inhibit calcium currents in differentiated NG108-15 cells, whereas D_4 receptors have this effect in GH_4C1 cells (Seabrook et al., 1994). Thus, similar to the D_1-like receptors, the D_2 receptor seems to alter intracellular calcium levels through multiple

mechanisms, whereas to date, the D_3 and D_4 receptors have only been shown to inhibit calcium currents.

Function of Brain Dopamine Receptors

DA plays an important role in motor regulation, reinforcement, olfaction, mood, concentration, hormone control, and hypoxic drive. Dopamine pathways project from the substantia nigra in the midbrain to the basal ganglia and from the midbrain to the limbic cortex and other limbic structures. A third pathway (tuberoinfundibular) is involved in regulating prolactin release. The nigrostriatal pathway is concerned with modulating the control of voluntary movement and its degeneration results in Parkinsonism. The mesolimbic pathway is overactive in Schizophrenia, but it is not known why. Dopamine agonists are used in the treatment of Parkinsonism and antagonists (neuroleptics) are used in schizophrenia (Neal, 1992).

In the early 1960s, analysis of brains of patients dying with Parkinsonism revealed greatly decreased levels of dopamine in the basal ganglia (caudate nucleus, putamen, globus pallidus). Parkinsonism is a disease of the basal ganglia and is characterized by a poverty of movement, rigidity and tremor. Parkinsonism thus became the first disease to be associated with a specific transmitter abnormality in the brain. The main pathology in Parkinsonism is extensive degeneration of the dopaminergic nigrostriatal tract. The cell bodies of this tract are localized in the substantia nigra in the midcrain, and it seems that frank symptoms of Parkinsonism only appear when more than 80% of these neurons have degenerated. About one-third of patients with Parkinsonism eventually develop dementia (Neal, 1992).

Schizophrenia is a syndrome characterized by specific psychological manisestations. These include auditory hallucinations, delusions, thought disorders and behavioral disturbances. Neuroleptic drugs control many of the symptoms of schizophrenia. They have most effect on the positive symptoms such as hallucinations and delusion. Negative symptoms such as social withdrawal and emotional apathy are less affected by neuroleptic drugs. The neuroleptics are all antagonists at dopamine receptors, suggesting that schizophrenia is associateed with increased activity in the dopaminergic mesolimbic and/or mesocortical pathway (Neal, 1992).

The effects of both D_1 and D_2 receptor were attenuated in these animals; moreover, these mice were resistant to the hyperlocomotor effects of cocaine, indicating that D_1 receptors contribute significantly to the effects of cocaine on the CNS. A great deal of attention has focused on the clinical correlates of D_2 receptor function. It has been proposed that the brains of untreated schizophrenia patients have elevations in D_2 receptor density. Furthermore, radioligand binding studies have revealed a correlation between the clinical efficacy of antipsychotic drugs and their antagonist affinities for this receptor subtype. This finding has contributed significantly to the "dopamine hypothesis" of schizophrenia. The extrapyramidal reverse effects of antipsychotic drugs, such as dystonia and Parkinson-like symptoms, have been attributed to the blockade of striatal D_2 receptors. A significant contribution of D_2 receptors to the dopaminergic regulation of motor function is further highlighted by a Parkinson-like movement disorder observed in a mutant mouse strain that lacks this receptor subtype (Missale et al., 1998).

The effects of DA on motor activity have been extensively investigated. The degree of forward locomotion is primarily controlled by the ventral striatum through activation of D_1, D_2, and D_3 receptors. The D_3 and D_4 receptors are considered to be D_2-like on the basis of similarities in their gene structures. Activation of D_2 autoreceptors, which results in decreased DA release, has been shown to decrease locomotor activity, whereas activation of postsynaptic D_2 receptors slightly increases locomotion. The D_3 receptor, which has been shown to be mainly postsynaptically located in the nucleus accumbens, seems to play an inhibitory role on locomotion. D3-preferring agonists inhibit, in fact, locomotor activity, whereas D_3-preferring antagonists evoke motor activation. The opposing effects of D_2 and D_3 receptors on locomotor activity may find a neurochemical correlate in their opposite effects on neurotensin gene expression in the nucleus accumbens (Diaz et al., 1994). The attention has been paid to a potential role of D_4 receptors in schizophrenia. On postmortem, elevated D_4 receptor levels have been found in the brains of patients with schizophrenia. Moreover, the atypical antipsychotic drug clozapine has a high affinity for the D_4 receptor; this receptor is highly polymorphic in humans and at least 25 distinct alleles have been identified. Studies are therefore under way to determine whether particular D_4 alleles are associated with psychotic disorders or with

responsiveness to antipsychotic drugs. Activation of D_1 receptors has little or no effect on locomotor activity. However, it is now clear that there is synergistic interaction between D_1 and D_2 receptors in determining forward locomotion so that concomitant stimulation of D_1 receptors is essential for D_2 agonists to produce maximal locomotor stimulation (Waddington and O'boyle, 1989).

Although some inconsistencies are present in the literature, there is a general agreement that mesolimbocortical DA plays a role in learning and memory. In the monkey, DA neurons in the A10 area have been reported to be involved with transient changes of impulsive activity in basic attention and motivational processes underlying learning and cognitive behavior. Activation of both D_1 and D_2 receptors in the hippocampus improves acquisition and retention of different working memory tasks in the rat. In the monkey, activation of both D_1 and D_2 receptors in the prefrontal cortex has been reported to improve performance in a working memory task. Because of the lack of true agonists and antagonists discriminating among D_1-like and D_2-like receptors, the role of DA receptor subtypes in learning and memory has not been investigated. However, it is worth noting that although the D_1 receptor is poorly expressed in the hippocampal formation, the D_5 receptor is highly expressed in this area so that the D_5, more than the D_1 receptor, is likely to mediate the effects of D_1 agonists on learning and memory. Similarly, D_3 and D_4 receptors are expressed in the hippocampus, and D_3 receptors are present in the septal area, suggesting a possible contribution of these receptor subtypes to the behavioral effects of D_2 agonists. In contrast, because of their distribution at the cortical level, a central role of D_1 and D_2 receptors can be proposed in the prefrontal cortex-mediated behaviors (Missale et al., 1998).

Pharmacological studies have shown that both D_1 and D_2 receptors mediate the effects of DA on learning and memory. In particular, DA acting on D_1 receptors has been shown to exert dual actions on these types of behaviors. Thus, D_1 agonist administration into the PFC of rats with poor performance on attentional function tasks significantly improved their performance in rats that had higher baseline attentional skills (Granon et al., 2000). This is consistent with studies suggesting that optimal DA levels are required to maintain function in the PFC, with too high or low D_1 stimulation leading to impaired working memory function (Murphy et al., 1996).

Several studies have shown that the DA system is activated by rewarding stimuli, such as food (Taber and Fibiger, 1997). However, it is becoming evident that DA is not the reward signal per se, but instead is necessary for the acquisition of reinforcing stimuli. Mesolimbocortical DA is implicated in reward and reinforcement mechanisms as shown by the observation that administration of psychostimulants and drugs of abuse elicits an increase of DA release in the mesolimbic areas, whereas withdrawal of these drugs results in a reduction of dopaminergic transmission. Various experimental models have been developed such as intracranial self-stimulation and drug self-administration. In the intracranial self-stimulation paradigm, rats work to obtain electrical stimulation that has rewarding properties and results in DA release in the prefrontal cortex and nucleus accumbens. Pharmacological studies clearly show that both D_1 and D_2 receptors are involved in this behavior, with agonists at both receptors stimulating and antagonist inhibiting the behavior (Franklin and Vaccarino, 1983).

In some cases, DA has been described as a type of error signal (Hollerman and Schultz, 1998), in which the predicted occurrence of reward does not correlate with the behavioral response emitted to generate this reward. Thus, when a task is well learned, DA neuron firing no longer is a necessary correlate of the reward signal. But if reward is absent, DA neuron firing appears to decrease. Studies of DA overflow in the nucleus accumbens show that DA is released when the DA cell bodies are stimulated electrically. However, when the stimulation is contingent on a bar press by the rat, the DA overflow does not occur even in the presence of the electrical stimulus (Garris et al., 1999). These data suggest that the lack of DA system activation during a well-learned contigent reinforcement task is not simply a failure to activate DA neuron firing, but instead may represent an offsetting inhibitory influence over the DA sytem, either at the level of the DA cell body or the terminal.

In the case of drug self-administration, it has been shown that both D_1 and D_2 receptors are involved in the reinforcing properties of different drugs of abuse, with D_2 receptors mediating the stimulant drug reinforcement and D_1 receptors playing a permissive role (Self et al., 1996). Stimulation of D_1 receptors by endogenous DA is thus required for the expression of D_2 receptor-mediated behaviors and gene regulation. A recent study suggested that although D_1-like and D_2-like receptor

agonists are themselves reinforcing and can both substitute for cocaine in drug discrimination tests, they nevertheless may mediate qualitatively different aspects of the reinforcing stimulus produced by cocaine. In particular, activation of D_2-like receptors has been shown to mediate the incentive to seek further cocaine reinforcement in an animal model of cocaine-seeking behavior. In contrast, D_1-like receptors appear to mediate a reduction in the drive to seek further cocaine reinforcement (Self et al., 1996). Agonists of D_1-like receptors may thus be evaluated as a possible therapy of cocaine addiction. Recently, it has been shown that D_3 receptor stimulation inhibits cocaine self-administration in the rat in a way indicating an enhancement of cocaine reinforcement. Indeed, the reports of an anticipatory increase in extracellular DA in the accumbens prior to self-administration of a DA drug such as cocaine (Gratton and Wise, 1994) could potentially increase extracellular DA sufficiently to inhibit phasic DA release occurring via stimulation of the DA cell bodies. Overall, studies support the suggestion that DA actions in the PFC may have a greater involvement in the regulation of novel circumstances, with the striatum involved more in expression of learned behaviors (Willkinson et al, 1998).

The role of D_3 and D_4 receptors in the physiology of dopaminergic system is still mostly unknown. They are specifically expressed in limbic and cortical regions involved in the control of cognition and emotion and, to a lesser extent in the dorsal striatum, and this makes them attractive and promising targets for new generations of antipsychotic drugs with low incidence of extrapyramidal side effects (Missale et al., 1998).

Dopamine and dopamine receptors in pineal gland

The mammalian pineal glands contain dopamine and its metabolite dihydroxyphenylacetic acid. The origin of dopamine fibers found in pineal glands is not definitely established. The presence of a dopaminergic function in the pineal gland was first suspected by Axelrod et al. (1969), who demonstrated that in addition to L-norepineprine, dopamine also stimulated the production of [^{14}C] melatonin in the cultured pineal gland. Furthermore, the subcutaneous administration of 150 mg/kg L-dopa increased the activity of NAT whereas this activation was not seen in rats

pretreated with MK-486, an inhibitor of aromatic L-amino acid decarboxylase, suggesting that the L-dopa-mediated stimulation of NAT was because of the formation of a catecholamine. In addition, following superior cervical ganglionectomy, which denervated the peripheral innervation of pineal gland, the L-dopa-mediated stimulation of NAT became potentiated (Degushi and Axelrod, 1972a,b). Moreover, studies by Lynch et al. (1973) showed that the administration of L-dopa increased the concentration of melatonin, and this effect was potentiated by pretreatment with 6-hydroxydopamine, which is known to destroy catecholaminergic nerve terminals (Tranzer and Thoenen, 1967, 1968).

Catecholamine control of melatonin synthesis and release has been studied primarily in the pineal gland of rat (Ebadi, 1980). In addition to beta-adrenergic receptor agonists, L-dopa and dopamine have been also shown to activate the production of melatonin and its synthesizing enzyme, serotonin N-acetyltransferase. In an attempt to characterize dopaminergic receptor sites (Govitrapong et al., 1984), bovine pineal synaptosomes were prepared by differential centrifugation techniques. Washed disrupted synaptic membranes were used to study ^{3}H-spiroperidol binding, using standard membrane-binding techniques. Association of ^{3}H-spiroperidol to pineal membranes was very rapid, reaching equilibrium within 2 min and remaining stable for 20 min. Dissociation was also rapid with at 1/2 of 3 min. Analysis of saturation studies using the LIGAND program indicated the presence of two binding sites with KDS (dissociation equilibrium constant) of 0.18 nM and 2.1 nM. The Bmax (receptor density) of the sites were 37 and 630 fmoles/mg protein respectively. This result has shown that the bovine pineal gland possesses dopamine D_2-like receptors with a B_{max} value of 37 fmol/mg protein (Govitrapong et al., 1984).

Furthermore, by using the rat pineal gland in culture, Govitrapong et al. (1989) showed that bovine pineal glands not only contain D_2 dopamine receptors, but also that dopamine has dual effects on the activity of pineal serotonin N-acetyltransferase, inhibiting the basal activity at 0.1 microM and stimulating it at 10 microM. Furthermore, the magnitude of activity of the dopamine stimulated NAT (> 10 μM) was attenuated significantly but not completely by preincubation of the pineal glands with propranolol. In addition, the D_2 dopamine receptor agonists such as bromocriptine and quinpirole partially attenuated the norepinephrine-induced

stimulation of N-acetyltransferase, and these attenuating effects were reversed by D_2-dopaminergic antagonists such as haloperidol (10 microM) or domperidone (10 microM). The results of these studies are interpreted to indicate that for the synthesis of melatonin, the pineal D_2-dopaminergic receptors may function independently from those of the beta 1-adrenergic receptor sites. Furthermore, the administration of haloperidol increased the melatonin level and activity of NAT. The D_2 dopaminergic receptor mediated inhibition of NAT activity like mentioned above, whereas chronic treatment with bromocriptine did not alter NAT activity (Govitrapong et al., 1989).

Simonneaux et al. (1990) reported that the bovine pineal gland also possesses D_1 dopamine receptors, which were characterized by using [^{3}H]SCH 23390, the selective D_1 dopamine receptor antagonist. SCH 23390 bound to bovine pineal membranes in a stereoselective, saturable and reversible manner. The receptor density (B_{max}) was 974 fmol/mg protein and the dissociation constant (KD), as determined by saturation studies, was 0.56 nM, which is in good agreement with the KD calculated from kinetic studies (0.40 nM). Dopaminergic receptor agonists and antagonists inhibited [^{3}H]SCH 23390 binding in a concentration-dependent and stereoselective fashion, demonstrating a pharmacology characteristic of D_1 dopamine receptors. The results of this study are interpreted to indicate that the effects of dopamine in the pineal gland are modulated through mechanisms that involve both D_1 and D_2 dopamine receptors (Simonneaux et al., 1990).

In bovine pineal gland, D_1- and D_2-dopaminergic and alpha 1-adrenergic receptors have been characterized pharmacologically in several laboratories, while beta 1-adrenergic receptors have been studied using physiological technique. The study presents a quantitative autoradiographic analysis of these four dopaminergic and noradrenergic receptors in bovine pineal gland (Simonneaux et al., 1991). The density order of the receptors is D_1 greater than alpha 1 greater than D_2 greater than or equal to beta 1. The Bmax of dopamine D_1 receptors is about 5 to 6 times higher than the B_{max} for alpha 1-adrenergic receptors and about 20 times higher than the Bmax values for beta 1-adrenergic and D_2-dopaminergic receptors. Dopamine D_1 receptors are significantly denser in the pineal cortex than in the medulla. Both dopamine receptors are more concentrated in the distal area than in the proximal area (close to the habenula), whereas both noradrenergic receptors are homogeneously distributed along

the longitudinal axis. Only D_1-dopaminergic receptors display a heterogeneous distribution between the superior and the inferior areas, being denser in the inferior area. Analysis of autoradiographic section of bovine pineal indicated that dopaminergic and noradrenergic receptors are not homogeneously distributed through out the gland (Simonneaux., et al 1991). D_1-dopaminergic receptors are more concentrated in the proximal area than in the distal area whereas D_2-dopaminergic receptors are more concentrated in the distal area.The observation of a much higher concentration of D_1-dopaminergic receptors relative to the other receptors suggests an important role for dopamine in the regulation of bovine pineal. Consequently, a through pharmacological study is required to establish the transducing system linked to D_1 and D_2 dopamine receptors in mammalian pineal glands (Simonneaux et al., 1991).

The concentrations of dopamine in the rat retina (Wirz-Justice et al., 1984) and rat pineal gland (Saavedra et al., 1982) follow a circadian pattern. However, in the rat pineal gland, the peak concentration of dopamine in the dark (10 ng/mg protein) is considerably lower than the peak concentration of norepinephrine in the dark (40 ng/mg protein) (Saavedra et al., 1982). Moreover, Vanderheyden et al (1986) and Brainard and Morgan (1987) have shown that the light stimulates not only the release of dopamine but also the synthesis of dopamine, and increase the activity of dopaminergic neurons in the retina by increasing increments of white light. Studies related to retina have shown that melatonin may modulate the functions associated with dopamine.

Dopamine Transporter (DAT)

Dopamine neurotransmission is initiated by the presynaptic release of dopamine and terminated largely by its reuptake through a specific, sodium-dependent dopamine transporter (DAT). The dopamine transporter is a member of the family of $Na^{+,}$ CI^{-} dependent substrate specific neuronal membrane transporters, which includes transportets for norepinephrine, serotonin, γ-aminobutyric acid, glycine, taurine, proline, betaine and creatine. The accepted mode of the structure of the dopamine transporter cDNA codes for 620 amino acids, and hydropathy analysis suggests the

presence of 12 transmembrane domains, with both the N^- and C^- terminus locates in the cytosol (Shimada et al., 1991). The dopamine transporter or carrier located on the plasma membrane of nerve terminals, transports dopamine across the membrane. By taking up synaptic dopamine into neurons, it plays a critical role in terminating dopamine neurotransmission and in maintaining dopamine homeostasis in the central nervous system (Giros et al., 1996). Furthermore, through this mechanism, DAT may be critically involved in certain pharmacological or pathological conditions (Brian et al., 1995).

DAT is densely concentrated in the substantia nigra, pars compacta (SNc) and scattered throughout the ventral tegmental area (VTA) (Freed et al., 1995). Both regions contain high concentrations of DAT mRNA. The relative abundance of DAT mRNA varies across subregions: it is highest in SNc, medium in the parabrachial pigmentosis and lowest in the intrafascicular nucleus (Burchett and Bannon, 1997). Low levels of DAT mRNA are present in the central gray and hypothalamus. In addition, DAT mRNA can be detected in the amygdala, caudate-putamen, nucleus accumbens (NAcc), pons/medulla, globus pallidus (GP), pituitary and frontal cortex (Maggos et al., 1997).

Govitrapong et al. (2003) found the existence of a dopamine transporter in the bovine pineal gland by using a radioligand binding technique. As a matter of fact, the density of D_1 dopamine receptors in the pineal gland is higher than that of corpus striatum, suggesting that this organ must possess a high affinity dopamine transporter, which has been identified in this study by using [^{3}H]GBR 12935 as a radiological ligand. The saturation analysis of [^{3}H] GBR 12935 revealed the dissociation equilibrium constant (K_d) of 6.02 ± 0.92 nM and the receptor density (B_{max}) of 6.93 ± 0.29 pmol/mg protein, which were comparable with those values obtained from bovine striatum and frontal cortex (Table 3.2). The K_d and Bmax values were 5.32 ± 0.7 nM and 7.05 ± 0.28 fmol/mg protein respectively. The K_d and B_{max} values in the bovine pineal gland obtained from both non-specific binding ligands (GBR 12909, nomifensine) were approximately similar.

Table 3.2 Comparison of Kd and Bmax values of [^{3}H]GBR 12935 saturation studies in various bovine tissues (Govitrapong et al., 2003)

Tissue	Non-specific ligand used	^{K}d $^{(nM)}$	B_{max} (pmol/mg protein)
Bovine pineal gland	GBR 12909	6.0 ±0.9	6.9 ±0.3
Bovine pineal gland	nomifensine	5.3 ±0.7	7.1 ±0.3
Bovine striatum	nomifensine	5.0 ±0.3	6.5 ±0.1
Bovine frontal cortex	nomifensine	7.6 ±0.1	7.3 ±0.8
Bovine cerebellum	nomifensine	undetectable	undetectable

These results show that drugs capable of blocking dopamine transporters were effective in displacing [^{3}H]GBR binding; whereas specific norepinephrine and serotonin transporter inhibitors were less effective or ineffective. Interestingly, the K_d and B_{max} values obtained from bovine pineal were closer to those values obtained from bovine striatum and frontal cortex. These results indicate that a high affinity dopamine transporter exists in the bovine pineal, which may exhibit circadian periodicity, and whose physiological functions need to be delineated and characterized in future investigations.

Furthermore, the previous study of dopamine D_1 and D_2 receptors in the bovine pineal gland (Govitrapong et al., 1984; Simonneaux et al., 1990, 1991), the involvement of dopamine in regulating melatonin synthesis in rat pineal glands (Govitrapong et al., 1989), were caused to elucidate the gene expression, function, and mechanism of dopamine D_1 and D_2 receptors on NAT activity, melatonin release, cAMP accumulation and CREB phosphorylation in the bovine pineal gland.

CHAPTER 4
MATERIALS AND METHODS

Chemicals

All primers used in this study were purchased from the Bioservice Unit (BSU) of the National Service and Technology Development Agency (NSTDA), Bangkok, Thailand. The primer sequences for PCR and sequencing are listed in Table 1. TRIzol reagent was purchased from GIBGO-BRL (Bethesda, USA). Taq DNA polymerase and avian myeloblastosis virus reverse transcriptase were obtained from Promega (Wisconsin, USA). [^{3}H]-acetyl coenzyme A (specific activity 2.83 Ci/mmol) and [^{3}H]-melatonin were purchased from New England Nuclear (Bost, MA). Antisera against melatonin (G/S/704-6483) were purchased from Stockgrand Ltd., Guildford Surrey, UK. The following drugs were obtained from the respective companies: (±)SKF-38393 [(±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride] and R(+)-SCH- 23390 [R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride] domperidone, haloperidol, quinpirole, and spiperone from Sigma-RBI (St. Louis, MO, USA), Cy 208-203 [(-)- (6aR)-4, 6, 6a, 7, 8,12b-Hexahydro-7-methylindolo (4,3-a) phenanthridin] and Dihidrexidine hydrocholride from Tocris (Tocris Cookson, Ltd., Bristol, UK). Fetal calf serum was purchased from Seromed (Berlin, Germany). Dulbecco's Modified Eagle Medium and other chemicals relate to cultures were purchased from Gibco, (Grand Island, NY). Bovine serum albumin and tryptamine hydrochloride were purchased from Sigma (St. Louis, MO). All other chemicals and the reagents were of the highest commercially available purity, purchased mainly from Sigma (St. Louis, MO), E. Merck (Darmstadt, Germany), and May and Baker, Ltd. (Dagenham, UK). All solutions containing drugs were prepared freshly for each experiment.

[^{125}I]cAMP (a commercial RIA) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA), (Catalog Number: NEK033, 200 Tubes). Primary

antibodies specific for either Ser-133 phosphorylated CREB (p-CREB) or total CREB (T-CREB) and secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase were purchased from New England BioLabs (Beverly, MA, USA), The following drugs were obtained from the respective companies: (±)SKF-38393 [(±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride], R(+)SCH-23390 [R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride], quinpirole, spiperone, and phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) from Sigma-RBI (St. Louis, MO, USA). Fetal calf serum was purchased from Seromed (Berlin, Germany). Dulbecco's Modified Eagle Medium (DMEM) and other chemicals related to cultures were purchased from Gibco-BRL, (Grand Island, NY, USA). Bovine serum albumin, norepinephrine, isoproterenol were purchased from Sigma-RBI (St. Louis, MO, USA). Trans-Blot nitrocellulose membrane was purchased from Biorad (Hercules, CA, USA). Hyperfilm ECL was purchased from Amersham Pharmacia Biotech. Developer solution and Fixative solution were purchased from Kodak Co, Ltd. (Thailand). All other chemicals and the reagents were of the highest commercially available purity, purchased mainly from Sigma (St. Louis, MO), E. Merck (Darmstadt, Germany), and May and Baker, Ltd. (Dagenham, UK). All solutions containing drugs were prepared freshly for each experiment.

To determine the gene expression of corresponding D_1, D_2 dopamine receptors and dopamine transporter in bovine pineal glands

Tissue preparation

Fresh bovine pineal glands were dissected from brain of 6-12 months cow (*Bos taurus*) between 10.00-11.00 AM from a local slaughterhouse immediately after animals were euthanized and kept in ice- cold PBS solution for RT-PCR technique. Glands (weighed between 100-250 mg/ gland) were dissected free from connective tissues, blood vessels and pineal stalks. Furthermore, the glands were sliced and kept in ice-cold Dulbecco's Eagle Medium (DMEM) containing 1% fetal calf serum (FCS) (pH 7.4), for NAT and melatonin assays.

RNA isolation

Total RNAs were extracted from bovine pineal glands using TRIzol™ Reagent using the protocol recommended by the manufacturer. The bovine pineal gland in 0.5 ml of TRIzol Reagent was homogenized and then incubated at room temperature for 5 min before the addition of 0.1 ml chloroform. The sample were mixed vigorously and centrifuged at 12,000 x g at 4°C for 15 min. The RNA was collected from the aqueous phase and precipitated by the addition of 0.25 ml isopropanol. After incubation at room temperature for 10 min, the mixture was centrifuged at 12,000 x g at 4°C for 10 min. The RNA pellet was collected and washed with 75% ethanol and air-dried. Finally, the RNA was resuspended in 30-40 μl DEPC-treated water and stored at -80°C.

RNA quantification

The amount of RNA was quantified by measuring with a UV spectrophotometer at wavelength 260 nm (1 O.D. equal to 40 μg/ml of single strand RNA), and the quality of RNA was determined by ascertaining that the absorbancy ratio of A260/A280 lay between 1.7- 2.0.

Reverse transcriptase-polymerase chain reaction amplification for dopamine receptors

PCR primers sets for dopamine receptors were designed against the nucleotide sequences of the bovine dopamine receptors cDNA which are available in the Genbank. The primers were designed by the Oligo 4 computer program. All the primer sequences are shown in Table 4.1. The total mRNAs isolated from the bovine pineal tissues were reverse transcribed and amplified by PCR in a single tube using the protocol as described in our previous study (Chetsawang et al., 1999). Two PCR reactions were initially performed per each RNA sample to generate the fragments designated as D_1- and D_2-receptors by two pairs of primers, named DAR1 (sense: 5′-TCC ATC CTC AAT CTC TGT GT-3′) / DAR2 (antisense: 5′-ACA GTT GTT GAT GGT CTT GC-3′) and DAR3 (sense: 5′-CCC GCC TTC GTG GTC TAC TC-3′) / DAR4 (antisense: 5′-GGG GCT GTA TCG GGT CCT CT-3′), respectively. The total volume of 25 μl reaction mixture was composed of 0.2 mM of dNTPs (dATP, dCTP, dGTP, and dTTP), 2 mM of $MgCl_2$, 20 pmol of each oligonucleotide primer, 1 unit of Taq DNA

polymerase and 4 units of avian myeloblastosis virus reverse transcriptase, in buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1% TritonX-100. The reaction mixtures were initially incubated in a thermocycler for 30 min at 42^{o}C to reverse transcribe RNA into cDNA then followed by 35 cycles of thermal cycling for amplification to generate fragments of D_1- or D_2-receptor. The PCR profiles for amplification of D_1-receptor was: 95^{o}C for 30 second, 56^{o}C for 45 second and 72^{o}C for 45 second. The PCR profiles for D_2-receptor was: 95^{o}C for 30 second, 54^{o}C for 45 second and 72^{o}C for 45 second. The final cycle in each case was followed by a 7-min extension step at 72^{o}C before reducing the temperature to 14^{o}C for storage. The amplified products were kept at 4^{o}C until used.

Reverse transcriptase-polymerase chain reaction amplification for dopamine transporter

PCR primers sets for dopamine receptors were designed against the nucleotide sequences of the bovine dopamine receptors cDNA which are available in the Genbank. The primers were designed by the Oligo 4 computer program. All the primer sequences are shown in Table 4.2.

The total mRNAs isolated from the bovine pineal tissues were reverse transcribed and amplified by PCR in a single tube using the protocol as described in our previous study (Chetsawang et al., 1999). Two PCR reactions were initially performed per each RNA sample to generate the fragments designated as dopamine transporter by one pair of primers, named DAT1 (sense: 5′-CGT GGT GTA GGC AGA TGG AG -3′) / DAT2 (antisense: 5′-TTA ATG GGG GCA AAA CAA AG -3′). The total volume of 25 µl reaction mixture was composed of 0.2 mM of dNTPs (dATP, dCTP, dGTP, and dTTP), 2 mM of $MgCl_2$, 20 pmol of each oligonucleotide primer, 1 unit of Taq DNA polymerase and 4 units of avian myeloblastosis virus reverse transcriptase, in buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1% TritonX-100. The reaction mixtures were initially incubated in a thermocycler for 30 min at 42^{o}C to reverse transcribe RNA into cDNA then followed by 40 cycles of thermal cycling for amplification to generate fragments of dopamine transporter. The PCR profile for amplification of dopamine transporter was: 95^{o}C for 1 min, 58^{o}C

for 1.5 min and 72°C for 1.5 min. The final cycle in each case was followed by a 7-min extension step at 72°C before reducing the temperature to 14°C for storage. The amplified products were kept at 4°C until used.

Restriction endonuclease enzyme digestion

To verify the identity of DAR fragments obtained from the mRNA of bovine pineal gland, the amplification product containing the sequences derived from cDNA of dopamine D_1 and D_2 receptors were further digested by the specific restriction endonuclease enzymes. The types of restriction enzymes were selected by using the MacVector program. The DAR1/DAR2 and DAR3/DAR4 fragments were separately digested with *Pvu II* and *BspH I* respectively. The digestive reaction was performed according to the protocol recommended by the manufacturer. The digestive reaction was performed in a 20 μl final volume containing 5 μl of amplified product and 10 units of restriction enzyme in an appropriate buffer. The reaction was incubated for 1 hr at 37°C for *Pvu II*, or 1 hr incubation at 37°C and followed with 65°C, 20 min for *BspH I*, then the digested DNA was analyzed by 2% agarose gel electrophoresis.

Gel electrophoresis

Agarose gel with concentration of 1.5% was used to analyze the PCR products after amplifications. The gel was prepared by completely dissolving agarose powder upon heating in 1X TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) and it was allowed to cool to around 50°C before pouring into an electrophoresis tray with comb inserted. The DNA samples were mixed with 1/3 volume of loading dye (25% glycerol, 60 mM EDTA, 0.25% bromophenol blue) and loaded into the gel slots in the submarine condition. Electrophoresis was carried out at 80 volts for 90 min. The gel was stained with 0.1% ethidium bromide solution for 5 min and destained with distrilled water. The DNA bands were visualized under UV light exposure and scanned by using the gel documentation system, GelDoc 2000 (BioRad, Hercule, USA).

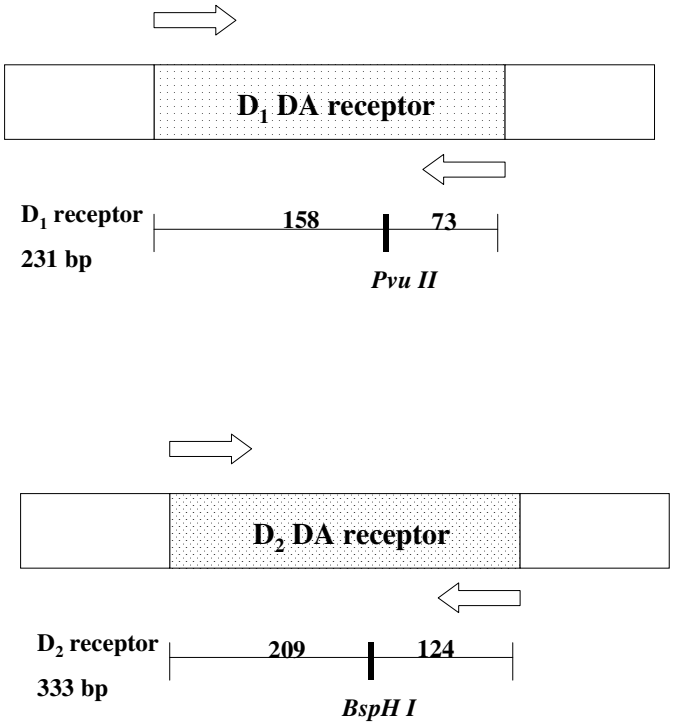
Table 4.1 The primer names, sequences, PCR product names and PCR product sizes of D_1 and D_2 dopamine receptors.

Primer names	*Sequences*	*PCR product name/size (bp)*
DAR1	**5'TCC ATC CTC AAT CTC TGT GT 3'**	
		D_1 DA receptor/231
DAR2	**5'ACA GTT GTT GAT GGT CTT GC3'**	
DAR3	**5'CCC GCC TTC GTG GTC TAC TC3'**	
		D_2 DA receptor/333
DAR4	**5'GGG GCT GTA TCG GGT CCT CT3'**	

Table 4.2 The primer names, sequences, PCR product names and PCR product sizes of dopamine transporter.

Primer names	*Sequences*	*PCR product name/size (bp)*
DAT1	**5′ CGT GGT GTA GGC AGA TGG AG 3′**	**Dopamine transporter/405**
DAT2	**5′ TTA ATG GGG GCA AAA CAA AG 3′**	

Figure 4.1 The schematic illustrated the D_1 and D_2 DA receptors primers and the predicted sizes of the respective RT-PCR products and the predicted fragments after the restriction endonuclease enzyme reactions



To study the status of D_1, D_2 dopamine receptors and its possible involvement in the pineal function, particularly on melatonin synthesis

Cell culture

Fresh bovine pineal glands were dissected from the brains of 6-12-month-old cows (*Bos taurus*) between 10.00-11.00 AM from a local slaughterhouse, within 15 min after animals were killed. The glands were sliced and kept in ice-cold DMEM containing 10% fetal calf serum (FCS) (pH 7.4), while being transported to the laboratory.

The preparation of pinealocytes was performed according to our previous procedure (Govitrapong, et al., 1998) as follows: fresh bovine pineal glands were dissected free of adhering connective tissues, blood vessels, and the pineal stalks. The pineal glands were minced with a sterile razor blade for 15 min, suspended in DMEM and then triturated with a pasteur pipette for 15 min. All steps were carried on ice. The minced tissues were resuspended with DMEM and precipitated on ice for 7 min. The suspensions were then filtered through a circle nylon mesh (pore size 41 μm, diameter 13 cm). Then the cell suspensions were centrifuged at 800 x g at 4°C for 5 min and were collected. Cell numbers were then counted.

The pinealocytes were plated into 24-well culture dishes (Nunc, Germany; ~ 5 x 10^6 cells per well). The viability of the adherent cells, assessed by trypan blue exclusion, was found to be ~95%. Immobilized pinealocytes were incubated in DMEM/F12 growth medium containing fetal calf serum (1%), penicillin (250 U/ml), streptomycin (25 μg/ml) and amphotericin B (1 μg/ml) under 95% air/5% CO_2 at 37°C. For routine procedures, the pinealocytes were acclimatized by preincubating them for 4 hr prior to the onset of the experiments. The pinealocytes were incubated for 30 min in the presence of antagonist prior to the addition of agonist. The period of incubation for testing the effects of dopamine receptor agonists was 2 hr.

At the end of the treatment, all pinealocytes were collected in 1.5 ml microcentrifuge tubes and centrifuged at 4,000 x g, 4°C for 5 min. The pellets were collected and frozen immediately on dry ice for assay of *N*-acetyltransferase (NAT) activity and the cultured media were collected and stored at –80°C for melatonin determination. For analysis of cAMP accumulation and phosphorylation of CREB, at

the end of the treatment period, cells were collected by centrifugation (2 min, 10,000 x *g*). The supernatant was aspirated and the tube was placed on solid CO_2. The frozen cell pellets were stored at -20°C until analysis. The experiments were performed throughout the year; there was no evidence for a seasonal variation in the responses studied.

_N_-acetyltransferase (NAT) activity assay

The activity of *N*-acetyltransferase (NAT) in bovine pinealocyte was determined according to a technique of Deguchi and Axelrod (1972a) as adapted by Govitrapong et al (1984). NAT activity was measured in homogenates from cells treated for 2 h with the indicated drugs. The pinealocytes were homogenized in 40 µl of 0.1 M sodium phosphate buffer (pH 6.5) containing [^{3}H]-acetyl CoA (specific activity 2.83 Ci/mmol, 0.1-1.0 mCi/assay tube). An aliquot of 30 µl of the homogenate was transferred into a test tube, which had already contained 10 µl of tryptamine in 0.1 M sodium phosphate buffer (pH 6.5), giving a final concentration of 10 mM in the reaction mixture. The mixture was then incubated at 37^{o}C for 20 min and stopped by adding 1 ml of water-saturated chloroform. Thereafter, the solution was vortexed for 15 sec and centrifuged at 1,000 rpm for 5 min. The aqueous layer was aspirated and the organic phase containing radiolabelled *N*-[^{3}H]acetyltryptamine was washed twice with 0.2 ml of 0.1 M sodium phosphate buffer, pH 6.5. Finally, a 0.5 ml sample of the chloroform phase was taken to dryness, and radioactivity was determined by a liquid scintillation spectrometer. The activity of NAT was expressed as pmol of *N*-[^{3}H]-acetyltryptamine formed/h/mg protein. Activity values were normalized against the amount of total protein determined using the method of Bradford (1976).

Melatonin determination

Melatonin level in cultured media was determined by radioimmunoassay (Fraser et al., 1983). Cultured media were diluted with assay buffer (0.1 M tricine containing 0.9% NaCl and 0.1% gelatin). Samples were incubated with a specific antisera against melatonin (G/S/704-6483) at a final dilution of 1:4,000 and the [^{3}H]melatonin, for 18 h at 4^{o}C. Dextran-coated charcoal was used to separate bound from free tracer. The reaction mixture was centrifuged at 3,000 rpm for 15 min at 4^{o}C.

Seven hundred microliters of the supernatant was aliquoted into a scintillation vial that contained scintillation fluid. Radioactivity was counted by a liquid scintillation spectrometer. The amount of melatonin was then calculated. Activity values were normalized against the amount of total protein determined using the method of Bradford (1976).

To study the influence of dopaminergic stimuli on cAMP in the bovine pinealocyte

Cyclic AMP measurement (cAMP [^{125}I] RIA Kit)

Cyclic AMP was measured as follows: cell pellets were homogenized in a 150 µl assay buffer (50 mM Tris and 4 mM EDTA) then heated for 5 min in a boiling water bath to coagulate the proteins. To remove precipitates, samples were centrifuged at 10,000 x g at 4 ^{o}C for 10 min then the supernatant was collected. The protein pellets were assayed using the Bradford method. Cyclic AMP in the supernatant was acetylated and the amount determined by use of a [^{125}I]cAMP commercial RIA kit obtained from Perkin Elmer and used according to the manufacturer's instructions. Radioactivity was measured in a gamma counter. In order to prevent the degradation of cAMP, cells were incubated in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) and then stimulated with the indicated drugs. The amount of intracellular cAMP was expressed as pmol/mg protein. All values were normalized against the amount of total pinealocyte protein.

To study the influence of dopaminergic stimuli on CREB phosphorylation in the bovine pinealocyte

Western blot analysis

Pinealocytes were lysed in a sodium dodecylsulfate (SDS) sample buffer and then centrifuged at 10,000 x g at 4 ^{o}C for 10 min. The concentrations of protein in the homogenates were measured according to established procedures (Bradford et al., 1976) using bovine serum albumin as a standard protein. Approximately 20 µg of the protein was loaded per lane. The proteins were then separated by electrophoresis using

10% SDS-polyacrylamide gels and finally electroblotted onto a Trans-Blot nitrocellulose (0.2 μm) membrane in a Mini Trans-Blot Electrophoresis Transfer Cell (Bio Rad, Hercules, CA). The process of transferring proteins to the membranes was performed using a 48 mM Tris buffer (pH 9.2) containing 39 mM glycine and 20% methanol at 400 mA, and lasted for 60 min. Nonspecific binding sites on the membranes were blocked with 5% (w/v) skimmed milk in Tris buffered saline (0.9% NaCl, 20 mM Tris-HCl, pH 7.8) supplemented with Tween-20 (0.1%). The membranes were incubated for 1 h at room temperature with the primary antibodies specific for either Ser-133 phosphorylated CREB (p-CREB) at 1: 1000 dilution or total CREB (T-CREB) at 1:1000 dilution, recognizing both phospho- and dephospho-CREB at 1:1000 dilution. The blots were washed in a PBS containing 0.1% Tween-20 and then incubated in the blocking solution with the secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (New England BioLabs, Beverly, MA) at 1: 2000 dilution, for 60 min. The membranes were extensively washed and immunolabeling was detected by enhanced chemiluminescence's (ECL, Amersham Biosciences Bangkok, Thailand) according to the manufacturer's instructions. Immunoreactivity was quantified using densitometry analysis. Analysis of the protein concentration in the cell extracts (Bradford et al., 1976) indicated that equal amounts of protein were loaded in each lane. Equal loading was ensured by measuring the amount of total CREB which remained constant under the various treatment conditions.

Data analysis

To minimize the effects of inter-individual variation, pineal glands from at least six animals were pooled for each cell preparation, and all data presented are based upon at least six different preparations. Data is expressed as mean ± S.E.M. values derived from at least six individual experiments. Data was statistically analyzed using an ANOVA with subsequent Tukey-Kramer multiple comparison test with $p < 0.05$ as the criterion of significance.

CHAPTER 5
RESULTS

Determination of the gene expression of corresponding D_1, D_2 dopamine receptors and dopamine transporter in bovine pineal glands

Total RNAs were prepared from the bovine pineal gland. Total cellular RNAs were isolated by a rapid single step method using TRIzol^{TM} reagent or the guanidinium-thiocyanate-phenol-chloroform extraction method. All RNA samples used in this study had an A260/A280 absorbancy ratio between 1.7-2.0, indicating acceptable purity or quality of the RNA samples.

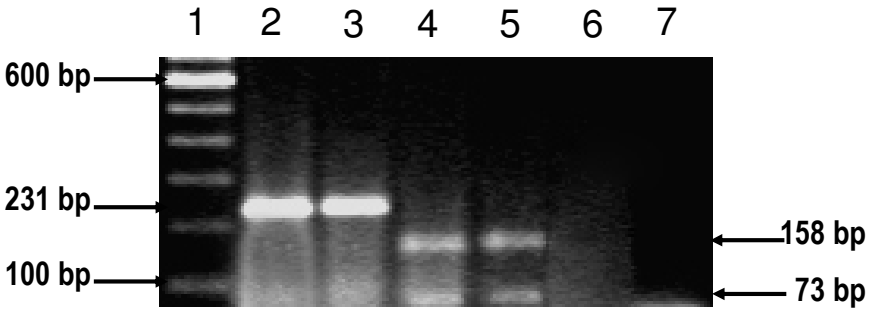
D_1-dopamine receptor gene expression in bovine pineal gland

The single-tube RT-PCR technique was designed to yield qualification of RNA from bovine pineal tissue samples. The amplification results are presented in figure 5.1. With the one set of pairs of D_1-dopamine receptor primers, an amplification product of the predicted size, 231 bp, was obtained with cDNA from the bovine pineal (Figure 5.1, lane 2) and from the bovine striatum as the positive control (Figure 5.1, lane 3).

Identity of the D_1-PCR products of the pineal and bovine striatum was confirmed by digestion with the restriction endonuclease *Pvu II* to yield the expected fragments of 158 and 73 bp (Figure 5.1, lanes 4 and 5).

In order to confirm that the PCR products that did not express the amplification of contaminating DNA, the reverse transcriptase enzyme was omitted in the negative control PCR reactions, and these yielded no detectable product (lane 6 of figure 5.1).

To detect any PCR reagent contamination the RNA was substituted by diethyl pyrocarbonate-treated water (DEPC-H_2O). These also yielded no detectable product (lane 7 of figure 5.1).



Dopamine-D1 receptor mRNA

Figure 5.1 Gel electrophoresis of RT-PCR products from bovine pineal D_1 dopamine receptor mRNAs, using one set of a pair of specific primers. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. Lane 1: 100 bp DNA marker, lanes 2, 3: PCR products of D_1 dopamine receptors in bovine pineal and striatum, respectively, lanes 4, 5: PCR products of D_1 dopamine receptors in bovine pineal and striatum, digested with *Pvu II* and lanes 6, 7: the negative and the reaction controls, respectively.

D_2-dopamine receptor gene expression in bovine pineal gland

The single-tube RT-PCR technique was designed to yield qualification of RNA from bovine pineal tissue samples. The amplification results are presented in figure 5.2. The D_2-dopamine receptor mRNA was also expressed in the bovine pineal tissues, and the specific D_2-dopamine primers were used to yield the expected PCR product at 333 bp from the bovine pineal (Figure 5.2, lane 2) and from the bovine striatum as the positive control (Figure 5.2, lane 3).

Identity of the D_2-dopamine PCR products of the pineal and striatum was confirmed by digestion with the restriction endonuclease *BspH I* to yield the expected fragments of 209 and 124 bp (Figure 5.2, lanes 4 and 5).

In order to confirm that the PCR products that did not express the amplification of contaminating DNA, the reverse transcriptase enzyme was omitted in the negative control PCR reactions, and these yielded no detectable product (lane 6 of figure 5.2).

To detect any PCR reagent contamination the RNA was substituted by diethyl pyrocarbonate-treated water (DEPC-H_2O). These also yielded no detectable product (lane 7 of figure 5.2).

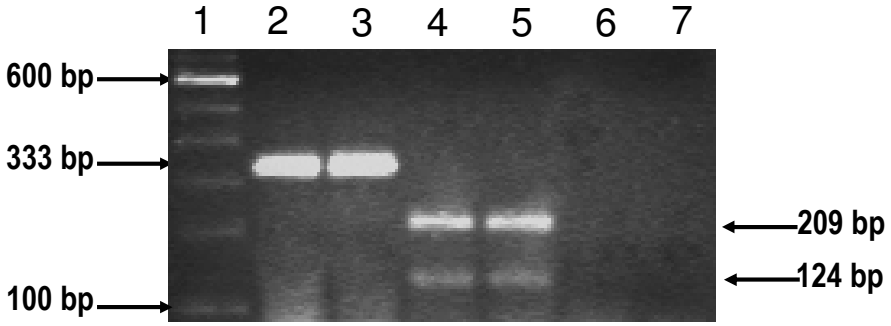


Figure 5.2 Gel electrophoresis of RT-PCR products from bovine pineal D_2 dopamine receptor mRNAs, using one set of a pair of specific primers. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. Lane 1: 100 bp DNA marker, lanes 2, 3: PCR products of D_2 dopamine receptors in bovine pineal and striatum, respectively, lanes 4, 5: PCR products of D_2 dopamine receptors in bovine pineal and striatum, digested with *BspH I,* respectively, and lanes 6, 7: the negative and the reaction controls, respectively.

The status of D_1, D_2 dopamine receptors and its possible involvement in the pineal function, particularly on melatonin synthesis

Effect of D_1-dopamine agonists in various doses on NAT activity in cultured bovine pinealocytes

Different selective D_1-agonists (SKF 38393, CY 208-203, Dihidrexidine) were evaluated on the bovine pinealocyte melatonin synthesis in various concentrations. Various concentrations (1-100 μM) of all D_1-agonists in this study stimulated NAT activity in a concentration-dependent manner on bovine pinealocytes (Table 5.1, Figure 5.3).

Effect of D_1-dopamine agonists in various doses on melatonin secretion in cultured bovine pinealocytes

Different selective D_1-agonists (SKF 38393, CY 208-203, dihidrexidine) were evaluated on the bovine pinealocyte melatonin synthesis in various concentrations. Various concentrations (1-100 μM) of all D_1-agonists in this study stimulated melatonin production in a concentration-dependent manner on bovine pinealocytes (Table 5.2, Figure 5.4).

Table 5.1 Effect of D_1-dopamine agonists in various doses on NAT activity in cultured bovine pinealocytes

Treatment	*N*-acetyltransferase activity (pmol/mg protein/h)	Control (%)
Control	6.33 ± 0.68	100
1μM SKF 38393	8.80 ± 1.29	139
10 μM SKF 38393	11.37 ± 0.80	179
100 μM SKF 38393	13.18 ± 0.82	208
1 μM CY 208-203	7.47 ± 1.98	117
10 μM CY 208-203	13.28 ± 2.49	209
100 μM CY 208-203	13.76 ± 1.16	216
1 μM Dihidrexidine	7.14 ± 1.10	112
10 μM Dihidrexidine	11.61 ± 1.32	183
100 μM Dihidrexidine	13.05 ± 2.30	206

The bovine pinealocytes were preincubated at 37^{o}C for 2 hr in a 5% CO_2 incubation chamber prior to addition of drugs. All pinealocytes were collected and centrifuged at 4,000 x g, 4^{o}C, 5 min. The pellets were collected by frozen immediately on dry ice for assayed of NAT activity and stored at –80^{o}C until assays according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six separate experiments.

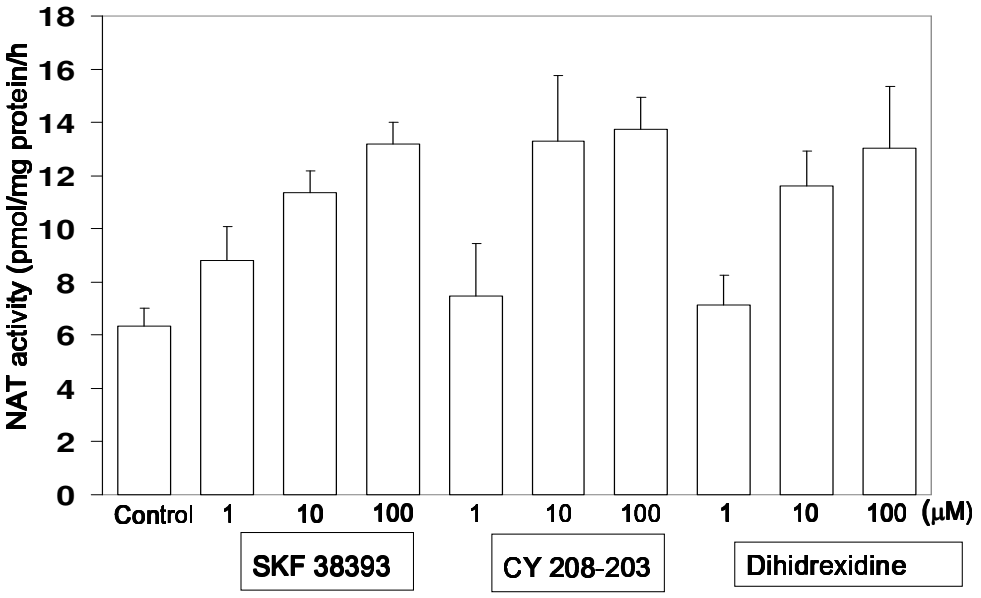


Figure 5.3 Various doses of D_1-dopamine agonists on NAT activity in cultured bovine pinealocytes treated with 1, 10, 100 μM SKF 38393, CY 208-203 and Dihidrexidine.

Table 5.2 Effect of D_1-dopamine agonists in various doses on melatonin secretion in cultured bovine pinealocytes

Treatment	Melatonin (ng/mg protein)	Control (%)
Control	11.44 ± 0.48	100
1μM SKF 38393	13.43 ± 2.57	117
10 μM SKF 38393	19.20 ± 3.95	167
100 μM SKF 38393	22.04 ± 5.87	192
1 μM CY 208-203	14.49 ± 1.67	126
10 μM CY 208-203	14.74 ± 1.47	129
100 μM CY 208-203	19.61 ± 3.96	171
1 μM Dihidrexidine	16.26 ± 2.84	142
10 μM Dihidrexidine	17.37 ± 3.01	151
100 μM Dihidrexidine	20.84 ± 4.78	182

The bovine pinealocytes were preincubated at 37^{o}C for 2 hr in a 5% CO_2 incubation chamber prior to addition of drugs. All pinealocytes were collected and centrifuged at 4,000 x g, 4^{o}C, 5 min. The cultured media were kept and stored at –80^{o}C until assays for melatonin level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six separate experiments.

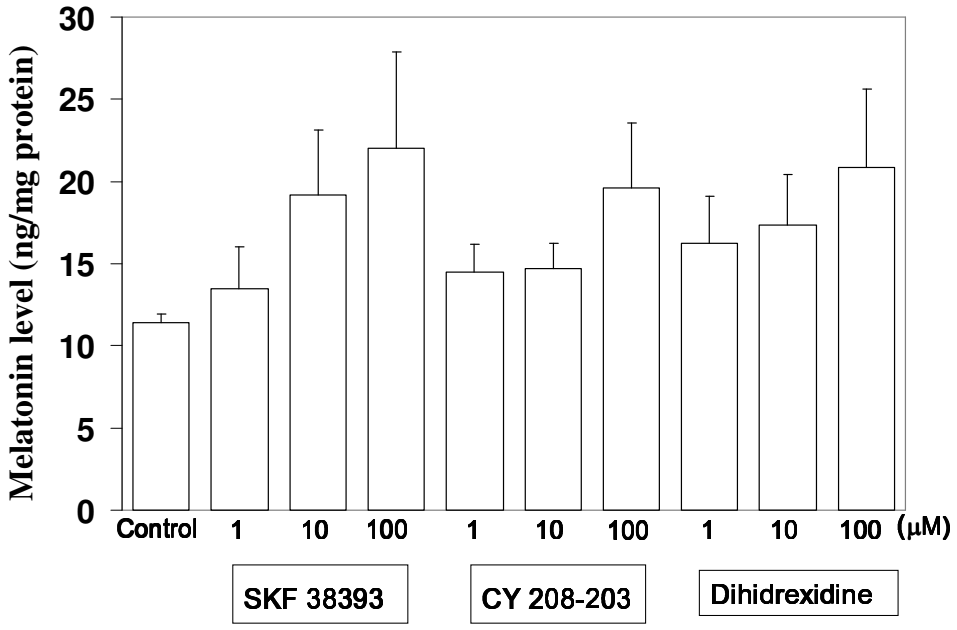


Figure 5.4 Various doses of D_1-dopamine agonists on melatonin synthesis in cultured bovine pinealocytes treated with 1, 10, 100 μM SKF 38393, CY 208-203 and Dihidrexidine.

Effect of norepinephrine and D_1-dopamine agonist on NAT activity in cultured bovine pinealocytes

In order to compare the stimulatory effect of D_1-dopamine agonist with norepinephrine, the same concentration of β-adrenergic agonists, norepinephrine and isoproterenol and D_1-dopamine receptor agonist, SKF 38393 were added into the cultured bovine pinealocytes. Ten micromolar of isoproterenol, norepinephrine and SKF 38393 significantly ($p < 0.05$) increased (1.96, 1.71, 1.7 folds, respectively) in NAT activity. When SKF 38393 and norepinephrine were added simultaneously to cultured bovine pinealocytes, the stimulatory effect on NAT (1.72 fold increase) did not differ from each individual effect (Table 5.3).

Effect of norepinephrine and D_1-dopamine agonist on melatonin secretion in cultured bovine pinealocytes

In order to compare the stimulatory effect of D_1-dopamine agonist with norepinephrine, the same concentration of β-adrenergic agonists, norepinephrine and isoproterenol and D_1-dopamine receptor agonist, SKF 38393 were added into the cultured bovine pinealocytes. Ten micromolar of isoproterenol, norepinephrine and SKF 38393 significantly ($p < 0.05$) increased (2.02, 1.59, 1.62 folds, respectively) in melatonin synthesis. When SKF 38393 and norepinephrine were added simultaneously to cultured bovine pinealocytes, the stimulatory effect on melatonin synthesis (2.31 fold increase) differs from each individual effect (Table 5.4).

Table 5.3 Effect of norepinephrine and D_1-dopamine agonist on NAT activity in cultured bovine pinealocytes

Treatment	N-acetyltransferase activity (pmol/mg protein/h)	Control (%)
Control	5.26 ± 0.42	100
10 µM Isoproterenol	10.34 ± 0.86[a]	196
10 µM Norepinephrine	8.99 ± 0.69[a]	171
10 µM SKF 38393	8.93 ± 0.95[b]	170
10 µM Norepinephrine + 10 µM SKF 38393	9.06 ± 0.94[a]	172

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. All pinealocytes were collected and centrifuged at 4,000 x g, 4°C, 5 min. The pellets were collected by frozen immediately on dry ice for assayed of NAT activity and stored at –80°C until assays according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.01$ when compared with control

[b] $p < 0.05$ when compared with control

Table 5.4 Effect of norepinephrine and D_1-dopamine agonist on melatonin secretion in cultured bovine pinealocytes

Treatment	Melatonin (ng/mg protein)	Control (%)
Control	11.52 ± 1.39	100
10 µM Isoproterenol	23.29 ± 5.09[a]	202
10 µM Norepinephrine	18.35 ± 2.09[a]	159
10 µM SKF 38393	18.68 ± 3.12[b]	162
10 µM Norepinephrine + 10 µM SKF 38393	26.63 ± 6.95[b]	231

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. All pinealocytes were collected and centrifuged at 4,000 x g, 4°C, 5 min. The cultured media were kept and stored at –80°C until assays for melatonin level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.01$ when compared with control

[b] $p < 0.05$ when compared with control

Effect of D_1-dopamine antagonist on NAT activity in cultured bovine pinealocytes

Ten micromolar of SKF 38393 (D_1-agonist) had a significant effect on melatonin synthesis when it was added to incubation medium containing bovine pinealocytes (Table 5.5). It caused significant ($p < 0.05$) increase (2.4 folds) in the NAT activity in the cultured bovine pinealocytes, and this stimulatory effect was partially blocked by SCH 23390, a selective D_1-dopamine antagonist (Table 5.5, Figure 5.5).

Effect of D_1-dopamine antagonist on melatonin synthesis in cultured bovine pinealocytes

Ten micromolar of SKF 38393 (D_1-agonist) had a significant effect on melatonin synthesis when it was added to incubation medium containing bovine pinealocytes. It caused significantly ($p < 0.01$) increased (1.8 folds) melatonin production in cultured bovine pinealocytes, this effect was completely blocked by SCH 23390 (Table 5.6, Figure 5.6).

Table 5.5 Effect of D_1-dopamine agonist and antagonist on NAT activity in cultured bovine pinealocytes

Treatment	N-acetyltransferase activity (pmol/mg protein/h)	Control (%)
Control	4.43 ± 1.02	100
10 μM SKF 38393	10.57 ± 1.64[a]	239
10 μM SCH 23390	6.67 ± 0.98	150
10 μM SKF 38393 + 10 μM SCH 23390	7.99 ± 1.45[b]	180

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of 10 μM SCH 23390 prior to the addition of 10 μM SKF 38393 and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 4,000 x g, 4°C, 5 min. The pellets were collected by frozen immediately on dry ice for assayed of NAT activity and stored at –80°C until assays according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.05$ when compared with control

[b] $p < 0.05$ when compared with 10 μM SKF 38393

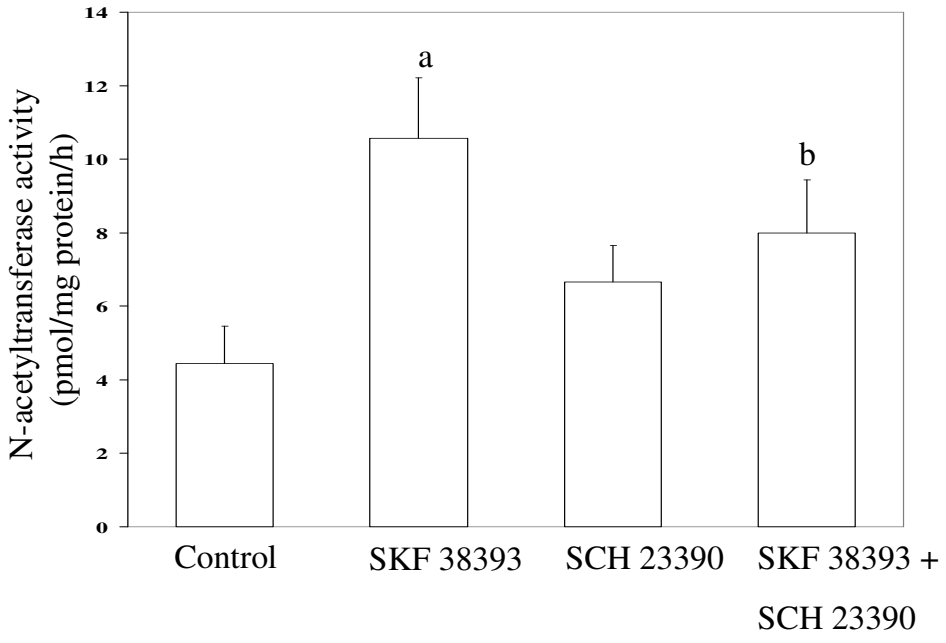


Figure 5.5 NAT activity of D_1-dopamine receptor in cultured bovine pinealocytes treated with 10 μM SKF 38393 (D_1-dopamine agonist), and 10 μM SCH 23390 (D_1-dopamine antagonist).

[a] $p< 0.05$ when compared with control

[b] $p < 0.05$ when compared with 10 μM SKF 38393

Table 5.6 Effect of D_1-dopamine agonist and antagonist on melatonin secretion in cultured bovine pinealocytes

Treatment	Melatonin (ng/mg protein)	Control (%)
Control	10.16 ± 1.06	100
10 μM SKF 38393	18.57 ± 1.07[a]	183
10 μM SCH 23390	9.25 ± 1.06	91
10 μM SKF 38393 + 10 μM SCH 23390	9.08 ± 1.51[b]	89

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of 10 μM SCH 23390 prior to the addition of 10 μM SKF 38393 and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 4,000 x g, 4°C, 5 min. The cultured media were kept and stored at –80°C until assays for melatonin level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.01$ when compared with control

[b] $p < 0.001$ when compared with 10 μM SKF 38393

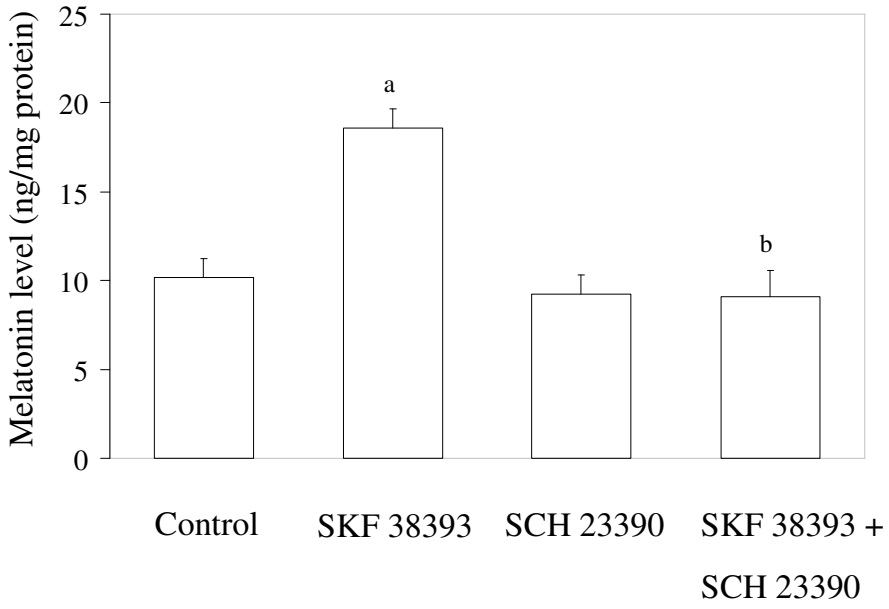


Figure 5.6 Melatonin level of D_1-dopamine receptor in cultured bovine pinealocytes treated with 10 μM SKF 38393 (D_1-dopamine agonist), and 10 μM SCH 23390 (D_1-dopamine antagonist).

[a] $p < 0.01$ when compared with control

[b] $p < 0.001$ when compared with 10 μM SKF 38393

Effect of D_2-dopamine agonist and antagonists on NAT activity in cultured bovine pinealocytes

D_2-dopamine agonist (quinpirole) and antagonists (spiperone, haloperidol and domperidone) had significant effect on NAT activity when they were added to incubation medium containing bovine pinealocytes. Quinpirole significantly reduced the basal NAT activity in a concentration-dependent fashion in the cultured bovine pinealocytes. D_2-dopamine antagonists (spiperone, haloperidol and domperidone) significantly increased the basal activity of NAT. The inhibitory effect of quinpirole was prevented by D_2-dopamine antagonists, spiperone, haloperidol and domperidone (Table 5.7, Figure 5.7).

Effect of D_2-dopamine agonist and antagonists on melatonin synthesis in cultured bovine pinealocytes

The bovine pinealocytes are incubated with a specific antiserum to melatonin raised and trace amounts of tritiated melatonin are then added. The free and antibody bound fractions of melatonin are separated using a dextran coated charcoal suspension. The free melatonin fraction is precipitated with the charcoal by centrifugation and the radioactivity of the antibody bound fraction in the supernatant counted in a scintillation counter. As standard curve is constructed at the same time from standards made up in melatonin free plasma. The assay can conveniently be set up in the afternoon and incubated overnight. The following morning the assay is separated and counted.

In addition, D_2-dopamine agonist (quinpirole) and antagonists (spiperone, haloperidol and domperidone) had significant effects on melatonin production in cultured bovine pinealocyte (Table 5.9). Quinpirole significantly ($p<0.05$) reduced the melatonin level in a dose-related manner, in the cultured bovine pinealocytes whereas D_2-dopamine antagonists (spiperone, haloperidol and domperidone) significantly induced the basal level of melatonin and this stimulatory effect was abolished in the presence of D_2-dopamine agonist, quinpirole (Table 5.8, Figure 5.8).

Table 5.7 Effect of D_2-dopamine agonists and antagonists on NAT activity in cultured bovine pinealocytes

Treatment	N-acetyltransferase activity (pmol/mg protein/h)	Control (%)
Control	6.67 ± 0.47	100
10 μM Quinpirole	4.89 ± 0.65[a]	73
100 μM Quinpirole	3.78 ± 0.62[b]	57
10 μM Domperidone	8.40 ± 0.99[a]	126
10 μM Haloperidol	11.21 ± 1.05[b]	168
10 μM Spiperone	12.14 ± 1.29[b]	182
10 μM Quinpirole + 10 μM Domperidone	6.15 ± 0.89	92
10 μM Quinpirole + 10 μM Haloperidol	7.59 ± 0.59[c]	114
10 μM Quinpirole + 10 μM Spiperone	10.13 ± 1.28[d]	152

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of 10, 100 μM quinpirole prior to the addition of 10 μM domperidone, 10 μM haloperidol, and 10 μM spiperone and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 4,000 x g, 4°C, 5 min. The pellets were collected by frozen immediately on dry ice for assayed of NAT activity according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.05$ when compared with control

[b] $p < 0.01$ when compared with control

[c] $p < 0.05$ when compared with 10 μM quinpirole

[d] $p < 0.01$ when compared with 10 μM quinpirole

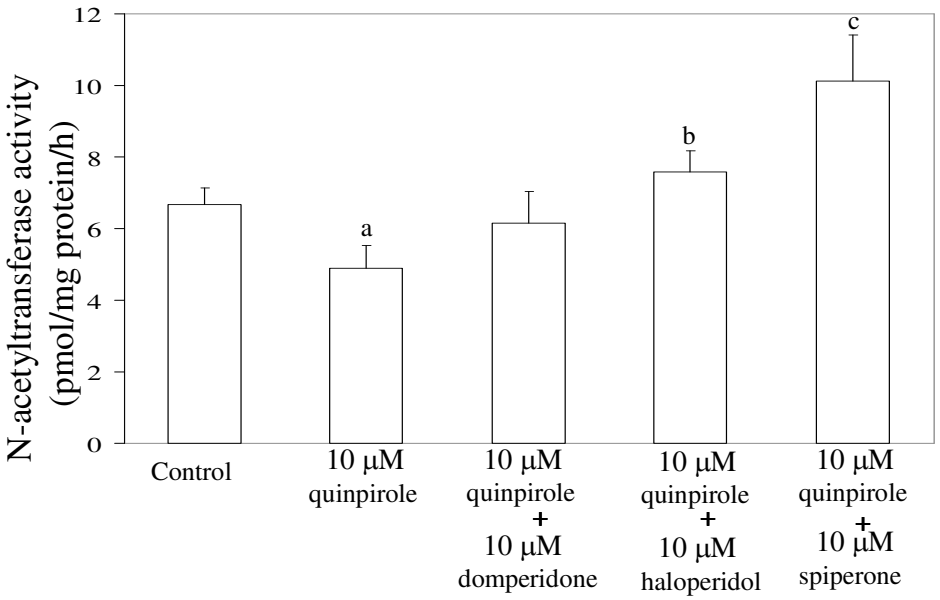


Figure 5.7 NAT activity of D_2-dopamine receptor in cultured bovine pinealocytes treated with D_2-dopamine agonist (10 μM Quinpirole), and D_2-dopamine antagonist (10 μM Domperidone, 10 μM haloperidol, and 10 μM Spiperone).

[a] $p < 0.05$ when compared with control

[b] $p < 0.05$ when compared with 10 μM quinpirole

[c] $p < 0.01$ when compared with 10 μM quinpirole

Table 5.8 Effect of D_2-dopamine agonists and antagonists on melatonin secretion in cultured bovine pinealocytes

Treatment	Melatonin (ng/mg protein)	Control (%)
Control	12.67 ± 0.89	100
10 µM Quinpirole	9.34 ± 0.75[a]	74
100 µM Quinpirole	8.42 ± 0.72[b]	66
10 µM Domperidone	16.00 ± 0.95[a]	126
10 µM Haloperidol	19.11 ± 1.09[b]	150
10 µM Spiperone	21.69 ± 1.67[b]	171
10 µM Quinpirole + 10 µM Domperidone	12.76 ± 1.33	101
10 µM Quinpirole + 10 µM Haloperidol	14.53 ± 0.92[c]	115
10 µM Quinpirole + 10 µM Spiperone	15.30 ± 1.03[c]	121

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of 10, 100 µM quinpirole prior to the addition of 10 µM domperidone, 10 µM haloperidol, and 10 µM spiperone and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 4,000 x g, 4°C, 5 min. The cultured media were kept and stored at –80°C until assays for melatonin level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.05$ when compared with control

[b] $p < 0.01$ when compared with control

[c] $p < 0.01$ when compared with 10 µM quinpirole

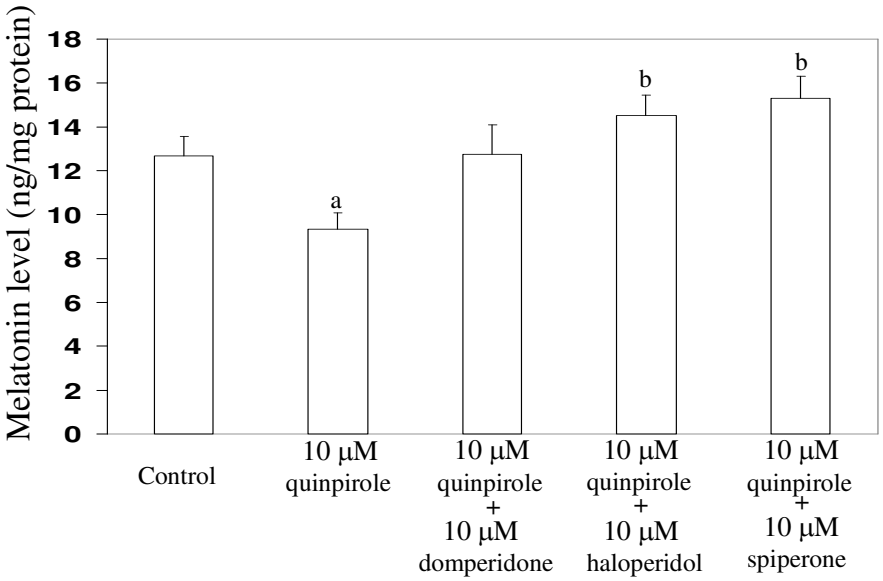


Figure 5.8 Melatonin synthesis of D_2-dopamine receptor in cultured bovine pinealocytes treated with D_2-dopamine agonist (10 μM Quinpirole), and D_2-dopamine antagonist (10 μM Domperidone, 10 μM haloperidol, and 10 μM Spiperone).

[a] $p < 0.05$ when compared with control

[b] $p < 0.01$ when compared with 10 μM quinpirole

The influence of dopaminergic stimuli on cAMP in the bovine pinealocyte

Effect of norepinephrine and D_1-dopamine agonist on cAMP in cultured bovine pinealocytes

In order to compare the stimulatory effect of D_1-dopamine on cAMP production, with norepinephrine, the same concentration of β-adrenergic agonists, norepinephrine and isoproterenol and D_1-dopamine receptor agonist, SKF 38393 were added into the cultured bovine pinealocytes respectively. Intracellular cAMP accumulation was measured in cells treated with dopamine agonist. Treatment with 10 μM of isoproterenol, norepinephrine, and 100 μM of SKF 38393 significantly ($p< 0.001$) increased (4.6, 3.47, 3.81 folds, respectively) in cAMP accumulation over control values (Table 5.9, Figure 5.9).

Effect of D_1-dopamine agonist on cAMP in cultured bovine pinealocytes

One hundred micromolar of SKF 38393 caused significantly ($p < 0.001$) increased (3.01 folds) in the cAMP accumulation in the cultured bovine pinealocytes, and this stimulatory effect was completely blocked by SCH 23390, a selective D_1-dopamine antagonist (Table 5.10, Figure 5.10).

Effect of D_2-dopamine agonist on cAMP in cultured bovine pinealocytes

One hundred micromolar of quinpirole significantly ($p < 0.01$) reduced level of cAMP in a concentration-dependent fashion in the cultured bovine pinealocytes whereas forskolin and spiperone significantly induced the cAMP accumulation and this stimulatory effect was abolished in the presence of D_2-dopamine agonist, quinpirole (Table 5.11, Figure 5.11).

Table 5.9 Effect of norepinephrine and D_1-dopamine agonist on cAMP in cultured bovine pinealocytes

Treatment	cAMP (pmol/mg protein)	Control (%)
Control	16.57 ± 2.53	100
10 µM Isoproterenol	76.25 ± 6.60[a]	460
10 µM Norepinephrine	57.56 ± 4.72[a]	347
10 µM SKF 38393	32.88 ± 3.68[b]	198
100 µM SKF 38393	63.17 ± 5.71[a]	381

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. After addition of different drugs, pinealocytes were further incubated for 2 hr. All pinealocytes were collected and centrifuged at 10,000 x g, 4°C, 5 min. The pellets were frozen immediately on dry ice and stored at –80°C until assays for cAMP level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.001$ when compared with control

[b] $p < 0.05$ when compared with control

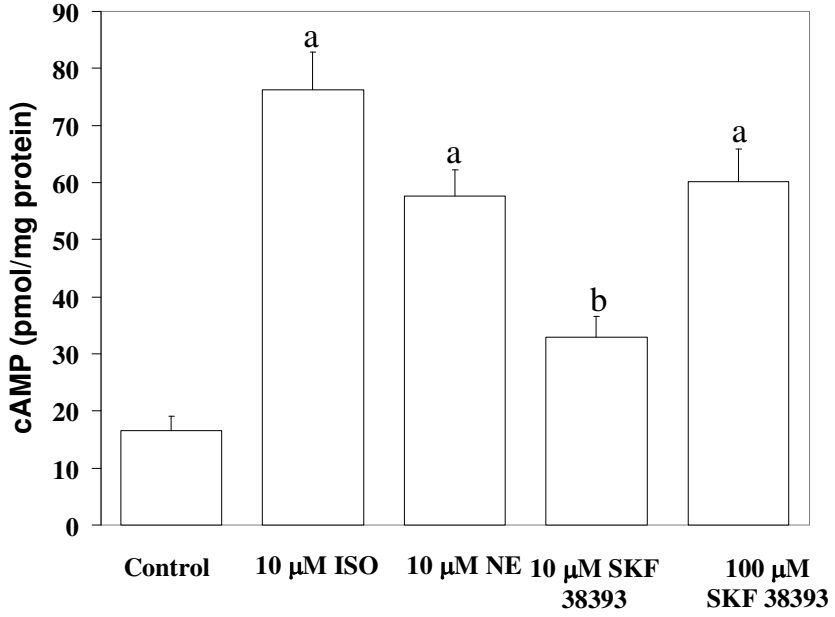


Figure 5.9 cAMP accumulation of D_1-dopamine agonists (10 µM, 100 µM SKF 38393) in cultured bovine pinealocytes compared with 10 µM Isoproterenol and 10 µM Norepinephrine.

[a] $p < 0.001$ when compared with control

[b] $p < 0.05$ when compared with control

Table 5.10 Effect of D_1-dopamine agonist and antagonist on cAMP in cultured bovine pinealocytes.

Treatment	cAMP (pmol/mg protein)	Control (%)
Control	19.13 ± 1.87	100
100 µM SKF 38393	57.56 ± 5.31[a]	301
100 µM SCH 23390	22.13 ± 1.75[b]	115
100 µM SKF 38393 + 100 µM SCH 23390	27.63 ± 2.37[b]	144

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of SCH 23390 prior to the addition of SKF 38393 and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 10,000 x g, 4°C, 5 min. The pellets were frozen immediately on dry ice and stored at –80°C until assays for cAMP level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.001$ when compared with control

[b] $p < 0.001$ when compared with SKF 38393 treated group

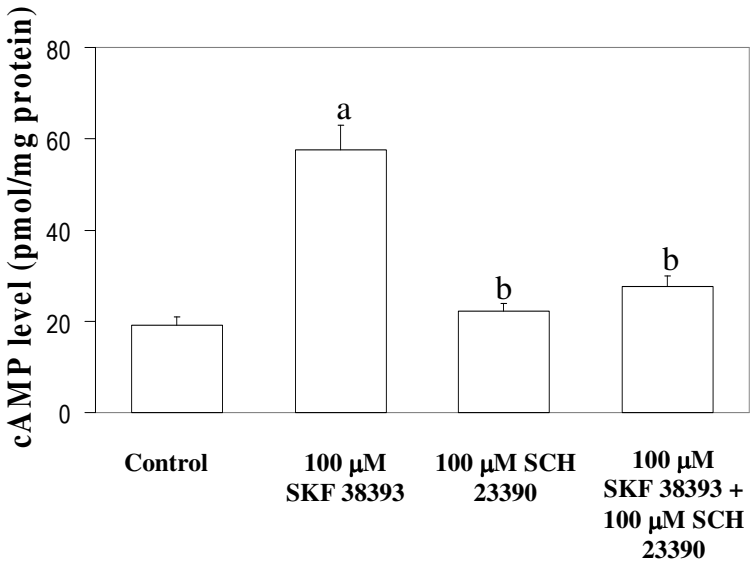


Figure 5.10 cAMP accumulation of D_1-dopamine receptor in cultured bovine pinealocytes treated with 100 µM SKF 38393 (D_1-dopamine agonist) and SCH 23390 (D_1-dopamine antagonist).

[a] $p < 0.001$ when compared with control

[b] $p < 0.001$ when compared with SKF 38393 treated group

Table 5.11 Effect of D_2-dopamine agonist on the level of cAMP in cultured bovine pinealocytes

Treatment	cAMP (pmol/mg protein)	Control (%)
Control	19.68 ± 1.02	100
10 μM Quinpirole	19.41 ± 0.70	98
100 μM Quinpirole	12.98 ± 0.83[a]	65
100 μM Forskolin	56.44 ± 4.97[b]	287
10 μM Spiperone	38.23 ± 1.15[b]	194
100 μM Forskolin + 100 μM Quinpirole	29.60 ± 2.06[c]	150
10 μM Spiperone + 100 μM Quinpirole	23.62 ± 0.59[d]	120

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of either forsoholin or spiperone prior to the addition of D_2-dopamine agonist and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 10,000 x g, 4°C, 5 min. The pellets were frozen immediately on dry and stored at –80°C until assays for cAMP level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.01$ when compared with control

[b] $p < 0.001$ when compared with control

[c] $p < 0.001$ when compared with forskolin treated group

[d] $p < 0.01$ when compared with spiperone treated group

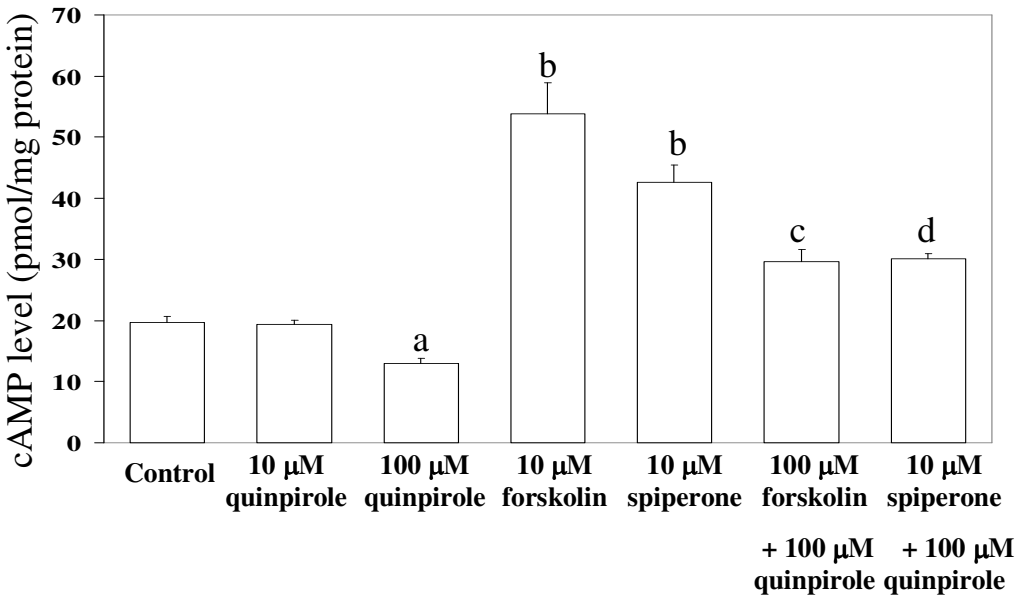


Figure 5.11 cAMP accumulation of D_2-dopamine receptor in cultured bovine pinealocytes treated with 10, 100 μM Quinpirole (D_2-dopamine agonist), 10 μM Forskolin, and 10 μM Spiperone (D_2-dopamine antagonist).

[a] $p < 0.01$ when compared with control

[b] $p < 0.001$ when compared with control

[c] $p < 0.001$ when compared with forskolin treated group

[d] $p < 0.01$ when compared with spiperone treated group

Effect of D_1-dopamine agonist on phosphorylation of CREB in cultured bovine pinealocytes

In order to investigate the D_1-dopamine-dependent phosphorylation of the transcription factors treatment with β-adrenergic agonists, norepinephrine and isoproterenol, D_1-dopamine receptor agonist, SKF 38393 and D_1-dopamine receptor antagonist SCH 23390 were added into the cultured bovine pinealocytes, cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) and phosphorylated CREB (pCREB) were determined. Immunoblots showed that a protein of 43 kDa corresponding to CREB accounts for the pCREB and confirmed that SKF-38393, a selective D_1 agonist is effective in inducing CREB phosphorylation (pCREB) like norepinephrine. The amount of total (phosphorylated and unphosphorylated) CREB was not changed upon stimulation of the cells with NE or SKF 38393. This stimulatory effect was blocked by SCH 23390, a D_1-dopamine antagonist (Table 5.12, Figure 5.12, 5.13).

Effect of D_2-dopamine agonist on phosphorylation of CREB in cultured bovine pinealocytes

Quinpirole, a selective D_2 agonist significantly inhibited the basal level and forskolin stimulated CREB phosphorylation (pCREB) whereas D_2-dopamine antagonists (spiperone) significantly induced the phosphorylation of CREB and this stimulatory effect was abolished by quinpirole (Table 5.13, Figure 5.14, 5.15).

Table 5.12 Effect of D_1-dopamine agonist on phosphorylation of CREB in cultured bovine pinealocytes

Treatment	Level of P-CREB	Control (%)
Control	30.79 ± 8.26	100
10 µM Isoproterenol	115.38 ± 9.61[a]	383
100 µM norepinephrine	79.54 ± 7.45[a]	265
100 µM SKF 38393	61.91 ± 6.34[b]	206
100 µM SCH 23390	38.19 ± 4.89	127
100 µM SKF 38390 + 100 µM SCH 23390	41.84 ± 14.80[c]	139

The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of SCH 23390 prior to the addition of SKF 38393 and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 10,000 x g, 4°C, 5 min. The pellets were frozen immediately on dry ice and stored at –80°C until assays for pCREB level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p< 0.01$ when compared with control

[b] $p < 0.05$ when compared with control

[c] $p < 0.05$ when compared with SKF 38393 treated group

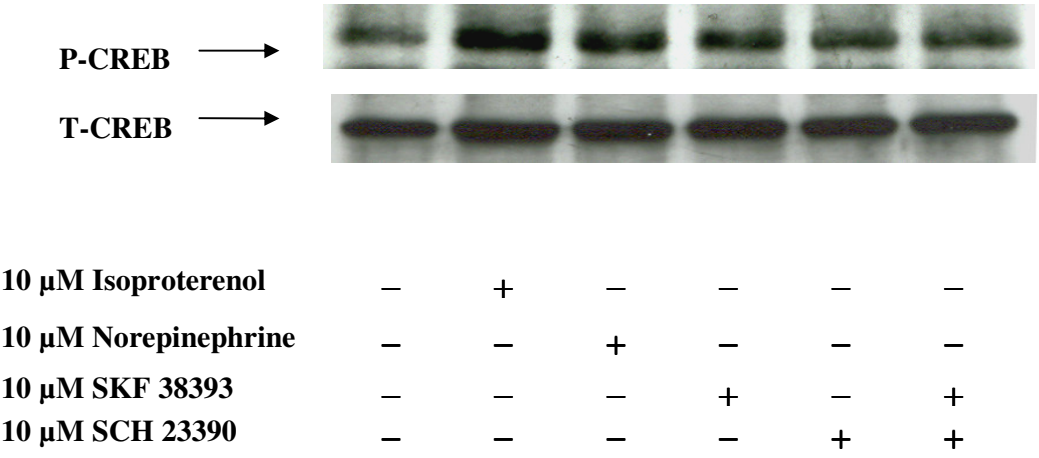


Figure 5.12 Treatment with D_1-dopamine agonist induced CREB phosphorylation in cultured bovine pinealocytes. The pinealocytes were pre-incubated for 2 hr and treated with the drugs indicated for 2 hr. The cell extracts (20 µg protein) were separated on a 10% SDS-PAGE. The immunoblotting was performed using a polyclonal antibody which recognizes pCREB or CREB and was conducted as described in Materials and Methods. The upper panel shows the representative immunoblots of CREB and pCREB obtained from unstimulated (control) and stimulated pinealocyte groups. Intensity of immunoreactivity was quantified and expressed as percentage of the maximum.

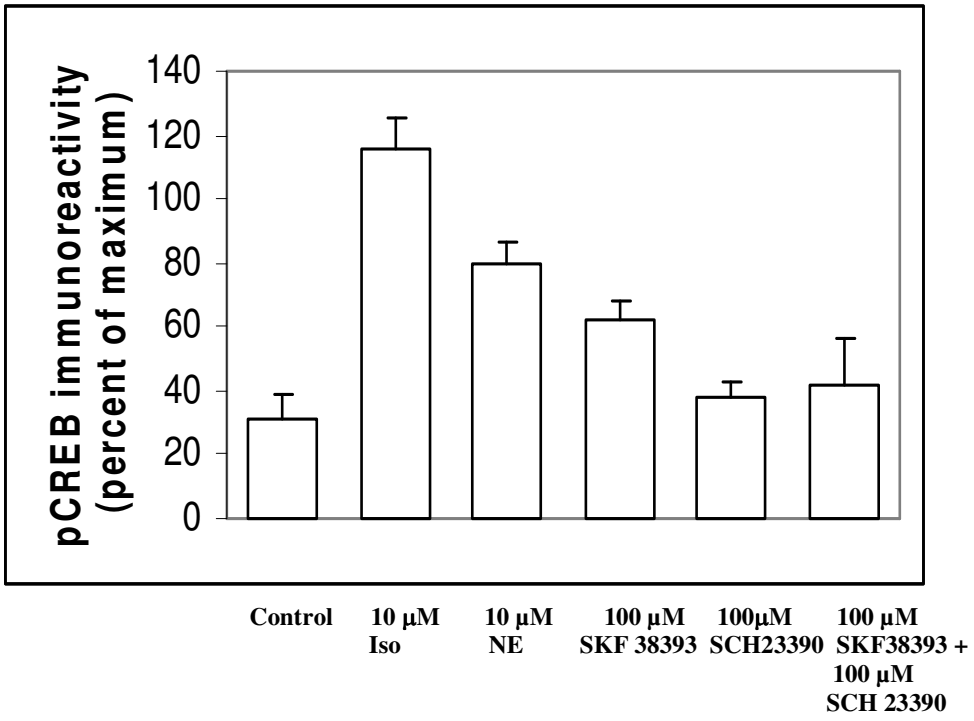


Figure 5.13 CREB phosphorylation of D_1-dopamine receptor in cultured bovine pinealocytes treated with 10 µM Isoproterenol, 10 µM Norepinephrine, 10 µM SKF 38393, and 10 µM SCH 23390 compared as a percentage of control.

Table 5.14 Effect of D_2-dopamine agonist on phosphorylation of CREB in cultured bovine pinealocytes.

Treatment	Level of P-CREB	Control (%)
Control	34.85 ± 6.34	100
100 μM forskolin	83.76 ± 6.15[b]	240
100 μM quinpirole	22.70 ± 4.09[a]	65
10 μM Spiperone	53.35 ± 6.35	153
100 μM forskolin + 100 μM quinpirole	39.13 ± 4.29[c]	112
10 μM Spiperone + 100 μM quinpirole	35.64 ± 1.16[d]	102

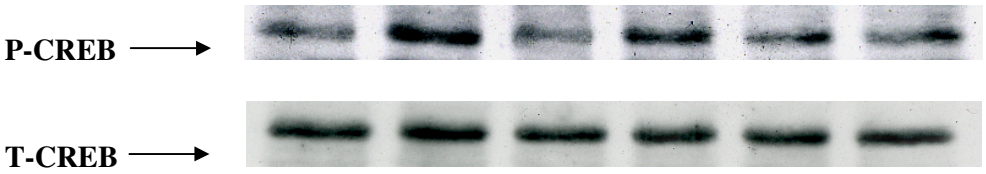
The bovine pinealocytes were preincubated at 37°C for 2 hr in a 5% CO_2 incubator prior to addition of drugs. The bovine pinealocytes were preincubated for 30 min in the presence of either forsoholin or spiperone prior to the addition of D_2-dopamine agonist and further incubated for 2 hr. All pinealocytes were collected and centrifuged at 10,000 x g, 4°C, 5 min. The pellets were frozen immediately on dry and stored at –80°C until assays for pCREB level according to the method described in "Materials and Methods". Each value represents the mean ± SEM from at least six individual experiments.

[a] $p < 0.05$ when compared with control

[b] $p < 0.01$ when compared with control

[c] $p < 0.01$ when compared with forskolin treated group

[d] $p < 0.001$ when compared with spiperone treated group



100 µM Quinpirole	–	–	+	–	+	+
100 µM Forskolin	–	+	–	–	+	–
10 µM Spiperone	–	–	–	+	–	+

Figure 5.14 Treatment with D_2-dopamine agonist decreased forskolin-induced CREB phosphorylation in cultured bovine pinealocytes. The pinealocytes were pre-incubated for 2 hr and treated with the drugs (quinpirole, forskolin, and spiperone) indicated for 2 hr. The cell extracts (20 µg protein) were separated on a 10% SDS-PAGE. The immunoblotting was performed using a polyclonal antibody which recognizes pCREB or CREB and was conducted as described in Materials and Methods. The upper panel shows the representative immunoblots of CREB and pCREB obtained from unstimulated (control) and stimulated pinealocyte groups. Intensity of immunoreactivity was quantified and expressed as percentage of the maximum.

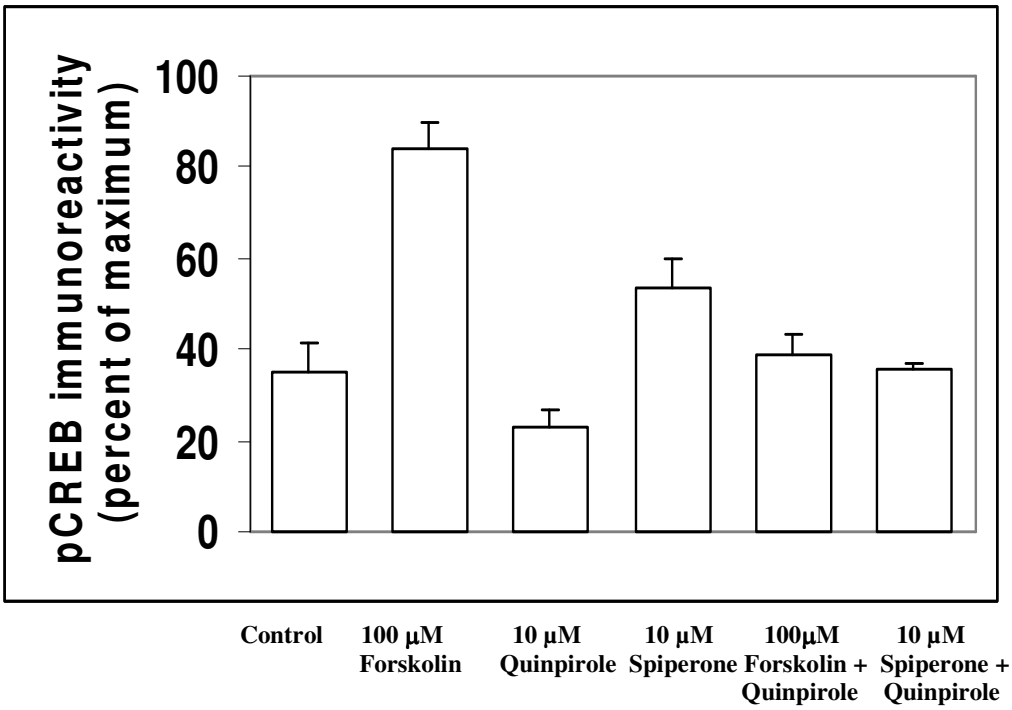


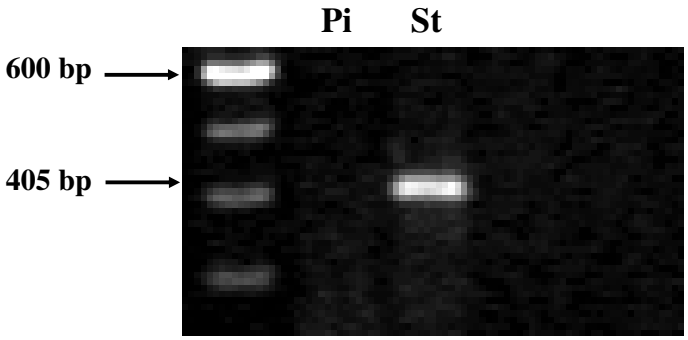
Figure 5.15 CREB phosphorylation of D_2-dopamine receptor in cultured bovine pinealocytes treated with 100 µM Quinpirole, 100 µM Forskolin, 10 µM Spiperone compared as a percentage of control.

Determination of the gene expression of dopamine transporter in bovine pineal glands

Total RNAs were prepared from the bovine pineal gland. Total cellular RNAs were isolated by a rapid single step method using TRIzol^{TM} reagent or the guanidinium-thiocyanate-phenol-chloroform extraction method. All RNA samples used in this study had an A260/A280 absorbancy ratio between 1.7-2.0, indicating acceptable purity or quality of the RNA samples.

Dopamine transporter gene expression in bovine pineal gland

The single-tube RT-PCR technique was designed to yield qualification of RNA from bovine pineal tissue samples. The amplification results are presented in figure 5.3. The dopamine transporter mRNA was expressed in the bovine striatum tissues but not in bovine pineal gland. The specific dopamine transporter primers were used to yield the expected PCR product at 405 bp from the bovine striatum. (Figure 5.16, lane 3).



Dopamine transporter mRNA in striatum

Figure 5.16 Gel electrophoresis of RT-PCR products from bovine pineal dopamine transporter mRNAs, using one set of a pair of specific primers. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. Lane 1: 100 bp DNA marker, lane 3: PCR products of dopamine transporter in bovine striatum.

CHAPTER 6
DISCUSSION

The aims of this study were to determine the gene expression of corresponding D_1, D_2 dopamine receptors in bovine pineal glands and to study the status of D_1, D_2 dopamine receptors and its possible involvement in the pineal function, particularly on melatonin synthesis and to clarify how melatonin production was regulated in the vertebrate pineal gland by the study of influence of dopaminergic stimuli on cAMP accumulation and CREB phosphorylation in the bovine pinealocyte.

The gene expression of corresponding D_1 and D_2 dopamine receptors in bovine pineal glands

In this study, the expressions of D_1- and D_2-dopamine receptor mRNAs were demonstrated in bovine pineal tissue. This supports the previous studies that the bovine pineal gland possesses both dopamine D_1-like receptors with a B_{max} value of 974 fmol/mg protein (Simonneaux et al., 1990) and dopamine D_2-like receptors with a B_{max} value of 37 fmol/mg protein (Govitrapong et al., 1984). Furthermore, dopamine has biphasic effects on the activity of NAT, inhibiting the basal activity at low concentration (0.1 μM) while stimulating it at high concentration (10 μM) and this study demonstrated that acute administration of haloperidol, a dopamine receptor antagonist, increased the concentration of melatonin and NAT activity (Govitrapong et al., 1984).

It is well established that both pineal gland and retina, extracellular control of melatonin synthesis is performed mainly by catecholamines. Further support for this mechanism comes from the findings that melatonin and dopamine systems in the retina may be functionally interrelated, controlling each other’s activity in an

inhibitory manner (Witkovsky et al., 1988; Dubocovich et al., 1989; Nowak et al., 1990; Zawilska and Nowak, 1992). However, the receptor implicated is species-and tissue-dependent. In addition, activation of postsynaptic D_1 receptors increases cAMP production, whereas activation of D_2 receptors either inhibits or has no effect on adenylate cyclase activity (Kebabian and Calne, 1979; Stoof and Kebabian, 1981). In addition, D_1 and D_2 receptors can also be distinguished by their interactions with selective pharmacological agonists and antagonists, as well as by their affinity for dopamine. That is, intact tissue D_2 receptors are 2-3 orders of magnitude more sensitive to dopamine than D_1 receptors (Kebabian and Calne, 1979; Civelli et al., 1993; Seeman and Van Tol, 1994). Finally, one of D_2 receptor subtype functions is a presynaptic autoreceptor whose activation inhibits dopamine release (Starke et al., 1989).

The status of D_1, D_2 dopamine receptors and its possible involvement in the pineal function, particularly on melatonin synthesis

The present results indicate that various concentrations of D_1-dopamine agonist from low to high increase NAT activity and melatonin release, supporting that NAT activity and melatonin release were stimulated in a concentration-dependent manner. In addition, this reports revealed that both D_1 and D_2-dopamine receptors are directly involved in the regulation of NAT activity and melatonin synthesis in the bovine pineal glands. The data showed that SKF-38393, a selective D_1 agonist, enhanced the basal activity of NAT and increased the basal level of melatonin, and the stimulatory effect was blocked by SCH-23390, a D_1 selective antagonist, although SCH-23390 incompletely blocked the effect of NAT activity. The stimulatory effects of D_1-dopamine receptor on NAT activity and melatonin secretion were comparable with those of norepinephrine. When D_1-dopamine agonist and norepinephrine were added simultaneously to cultured bovine pinealocyte, the stimulatory effect on NAT did not differ from each individual effect; however, the increase in melatonin level was closed to the additive level. According to autoradiographic localization of dopaminergic and noradrenergic receptors in the bovine pineal gland, Simonneaux et al. (1991) have

shown that the density order of the receptors was dopamine D_1 > α_1 adrenergic > dopamine D_2 > β_1 adrenergic receptors. This dopaminergic control has been also demonstrated in other tissue, for example, SKF-38393, a D_1 receptor agonist stimulated adenylate cyclase activity in coated vesicles isolated from bovine striatal tissues and inhibited by dopamine D_2 receptor agonists (Ozaki et al., 1994). Furthermore, functional interaction between melatonin and dopamine D_1- receptors was reported in the retina (Iuvone and Gan, 1995), which indicated a link through the intracellular cyclic AMP system. According to Dubocovich and Weiner (1985) who suggested that a receptor linked to the dopamine stimulate adenylate cyclase of the rabbit retina possesses the characteristics of a D_1-receptor.

Traditionally, the D_1-dopamine receptor has been positively linked to adenylate cyclase. Maternal pinealectomy changed D_1-dopamine receptor binding activity exclusively in the suprachiasmatic nucleus. In rat pups, the influence of maternal pinealectomy on the D_1-dopamine receptors of suprachiasmatic nucleus was prolonged, although the binding in the maternal suprachiasmatic nucleus was affected. These indicated that functional interactions between melatonin and D_1-dopamine receptors in the retina were linked through the intracellular cyclic AMP system (Naitoh et al., 1998). Data from the present study suggested that each system, D_1-dopamine or norepinephrine receptor contributes about the same. Dopamine has a distinct effect on pineal function. Both systems act independently through a common pathway, perhaps the adenylate cyclase, other signal transduction systems, or some transcription factors, competitively that lead to the stimulatory effect on NAT activity.

The inhibitory effect of the D_2-agonist, quinpirole, on NAT activity and melatonin release after 2 hr of culture indicated the role of D_2-receptors, while D_2-antagonists, spiperone, haloperidol, and domperidone antagonized these actions. Likewise, this dopaminergic control has also been described in the previous study in rat pineal tissue, quinpirole partially attenuated the norepinephrine-induced stimulation of NAT, and these attenuating effects were reversed by D_2-dopaminergic antagonists such as haloperidol or domperidone (Govitrapong et al., 1989). Binding studies confirmed the existence of D_1 and D_2-like receptors (Govitrapong et al., 1984;

Simonneaux et al., 1990). In functional studies, the selective action of dopamine D_2-receptors rapidly decreases the nocturnal increase or pharmacologically caused increase in the activity of a key regulatory enzyme in melatonin biosynthesis and melatonin levels, in vertebrate retina *in vitro* and *in vivo* (Nowak et al., 1990; Iuvone and Besharse, 1986; Zawilska and Iuvone, 1989). Iuvone et al. (1990) suggested that dopamine acted on D_2-like receptors on photoreceptors. The receptors appear to be coupled to adenylate cyclase through an inhibitory GTP-binding protein and to mediate inhibition of cyclic AMP synthesis and consequently inhibited NAT activity. Furthermore, dopamine metabolism, which during the light period is high, and NAT activity, which in animals kept under constant lighting conditions, seems to be tonically inhibited by endogenously released dopamine (Stoof and Kebabian, 1981).

Increased dopaminergic turnover during the night enabled the regulation of melatonin synthesis and the synchronization of the pineal function (Miguez et al., 1996). In addition, the reduction of NAT activity, which may be linked to impaired pineal catecholaminergic neurotransmission, was suggested (Miguez et al., 1998). Similarly, Iuvone (1986) and Iuvone and Besharse (1986) showed that in frog retina, dopamine, bromocriptine, apomorphine, and quinpirole but not alpha flupenthixol (a mixed D_1- and D_2-dopamine antagonist) inhibited NAT activity specifically in the dark through a mechanism that resulted in a reduction in the formation of cyclic-AMP. Furthermore, Iuvone and Besharse (1986) demonstrated that benztropine (a dopamine reuptake blocker), but not atropine, also produced a dose-dependent inhibition in the NAT activity in the dark.

The current study showed that the increase in both the basal activity of NAT and the basal level of melatonin were induced by spiperone, haloperidol, and domperidone, all D_2-antagonists, supporting the fact that NAT activity and melatonin content might be suppressed by endogenous dopamine in the bovine pineal glands. The previous study (Govitrapong et al., 1989) showed that the concentration of dopamine (6 μg/gm wet tissue) exceeded that of norepinephrine (2 μg/gm wet tissue) in bovine pineal glands. The bovine pineal glands also contained dopamine metabolites. The concentration of dopamine in rat pineal glands followed a circadian pattern. However, in the rat pineal glands, the peak concentration of dopamine in the

dark (10 ng/mg protein) is considerably lower than the peak concentration of norepinephrine in the dark (40 ng/mg protein) (Saavedra, 1982).

This study revealed that dopamine D_1-receptors stimulate the activity of NAT and, hence, enhances the formation of melatonin, and that the said action is blocked by SCH-23390 whereas dopamine D_2-receptors inhibit NAT activity and melatonin level, and the inhibitory effect is blocked by D_2-antagonists. Taken together, these results directly demonstrate for the first time that in the mammalian pineal gland dopamine stimulates NAT activity and enhances melatonin level, and that stimulation is mediated by D_1-dopaminergic receptors.

The influence of dopaminergic stimuli on cAMP in the bovine pinealocyte

This study determined the intracellular level of cAMP after a two hour incubation of drug in cultured bovine pinealocyte. In order to compare the stimulatory effect of D_1-dopamine on the intracellular level of cAMP to that of norepinephrine, separate experiments using equal concentrations of the β-adrenergic agonists, norepinephrine or isoproterenol, or the D_1-dopamine receptor agonist, SKF 38393, added to cultured bovine pinealocytes were conducted. The intracellular level of cAMP was then measured for each experiment. Treatment with 10 μM of isoproterenol, norepinephrine or SKF 38393 significantly increased the intracellular level of cAMP over the control values (4.6, 3.47 and 1.98 fold, respectively). For further comparison, the concentration of SKF 38393 was then increased to 100 μM resulting in a substantially higher the intracellular level of cAMP than that at the lower concentration (3.81 fold over control values). In a separate experiment, 100 μM of SCH 23390, a selective D_1-dopamine antagonist, was added to a mixture of cultured bovine pinealocytes and 100 μM of SKF 38393. The result was that the stimulatory effect of the D_1-dopamine agonist on the intracellular level of cAMP was completely blocked by the D_1-dopamine antagonist. In yet another experiment quinpirole, a D_2-dopamine agonist, when added to cultured bovine pinealocytes significantly ($p < 0.01$) reduced the intracellular level of cAMP in a concentration-dependent fashion. On the other

hand, forskolin and spiperone, D_2-dopamine antagonists, each significantly induced the intracellular level of cAMP in the pineacolytes but this stimulatory effect was negated in the presence of quinpirole. This result indicated that D_1-dopamine receptor, enhanced the intracellular level of cAMP, whereas D_2-dopamine receptor, inhibited the intracellular level of cAMP, This supports the previous studies indicating that a selective D_1-agonist enhanced *N*-acetyltransferase (NAT) activity and increased melatonin level, whereas, a selective D_2-agonist inhibited NAT activity and decreased melatonin level (Santanavanich et al., 2003).

Dopamine may have an important role in regulating melatonin synthesis in pineal glands. The supports for this mechanism came from the findings that norepinephrine content in the Syrian hamster pineal gland did not exhibit daily variations, although marked nocturnal increases in the levels of DA and DOPAC were evident. The result showed that the correlations between the daily fluctuations in the contents of pineal indoleamines and methoxyindoles were influenced by the photoperiod and that dopamine may play a role in the regulation of pineal function (Miguez et al., 1995). Furthermore, the increase in circulating melatonin levels from the daytime to nighttime was associated with decreases in the contents of DOPAC in both the hypothalamus and pituitary and in the DOPAC/DA ratio in the pituitary. The data suggested that the inhibition of the hypothalamic-pituitary dopaminergic metabolism may be a specific mechanism of melatonin action in the trout brain that might operate following changes in the secretion of the hormone from the pineal gland (Hernandez-Raura et al., 2000). The role of retinal dopamine (DA) receptors in the light induced suppression of melatonin biosynthesis in the chicken pineal gland. The activity of serotonin *N*-acetyltransferase and melatonin content significantly decreased at night in the pineal gland. This suppressive action of light was blocked by intraocular administration of SCH 23390 (a selective antagonist of D_1-DA receptors), but was not affected by sulpiride (a selective antagonist of D_2-DA receptors). These findings indicated that light activation of retinal dopaminergic neurotransmission, with concomitant stimulation of D_1-DA receptors positively coupled to the cAMP generating system, played an important role in a cascade of events regulating pineal activity (Zawilska et al., 2004).

The influence of dopaminergic stimuli on CREB phosphorylation in the bovine pinealocyte

In order to investigate the D_1-dopamine-dependent phosphorylation of the transcription factors, various combinations of the β-adrenergic agonists, norepinephrine and isoproterenol, the D_1-dopamine receptor agonist, SKF 38393, and the D_1-dopamine receptor antagonist, SCH 23390, were added to samples of the cultured bovine pinealocytes. Cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) and phosphorylated CREB (pCREB) were then determined from each treated sample. Immunoblots showed that a protein of 43 kDa corresponding to CREB accounts for the pCREB and confirmed that SKF 38393, a selective D_1-agonist, is effective in inducing CREB phosphorylation (pCREB), as does norepinephrine and isoproterenol. The amount of total (phosphorylated and unphosphorylated) CREB (T-CREB) was not changed upon stimulation of the cells with either norepinephrine or SKF 38393. The stimulatory effect, as a result of treatment with SKF 38393, was blocked by SCH 23390, a D_1-dopamine antagonist. In the present study, in isolated bovine pinealocytes, immunoblots showed that the dopamine as same as norepinephrine-induced immunoreaction was due to phosphorylation of the transcription factor CREB. This study also indicated that quinpirole, a selective D_2-agonist, significantly inhibited the basal level and forskolin stimulated CREB phosphorylation (pCREB), whereas the D_2-dopamine antagonist, spiperone, significantly induced CREB phosphorylation (pCREB) and this stimulatory effect was abolished by the addition of quinpirole.

However, two groups of mammals can be distinguished on the basis of the molecular mechanisms leading to stimulation of AA-NAT activity. In a group comprising many rodent species, the nocturnal increase in AA-NAT activity results firstly from the cAMP/PKA-dependent stimulation of *Aa-nat* gene expression (100–150-fold) with the subsequent synthesis of new molecules of AA-NAT. Additionally, cAMP/PKA phosphorylates AA-NAT, which allows its interaction with a chaperone protein 14-3-3 and inhibits proteasomal proteolysis of the AA-NAT molecules. During the day, AA-NAT activity is relatively low because of the low basal expression of the

Aa-nat gene and low levels of the proteolysis inhibitor (Simonneaux and Ribelayga, 2003).

In bovine pinealocytes, Aanat mRNA was constitutively expressed and AANAT protein was continually synthesized. Elevated cAMP levels resulted in marked differences in the mechanism involved in regulating AANAT activity are seen when rodents are compared to ungulates. The most obvious was the difference in the night/day levels of AANAT mRNA: in the rat, the night/day ratio is >100 (Roseboom et al. 1996), whereas it was ~1.5 in ungulates (Coon et al. 1995). During the day, in the absence of cAMP, AA-NAT protein is constantly translated but instantly degraded by proteosomal proteolysis; in contrast, during the night, β1-AR activation increases the levels of cAMP and PKA activity which, in turn, protects the protein from degradation and thereby enhances AA-NAT activity (Schomerus et al., 2000). Similar mechanisms may also operate in primates because the AANAT mRNA night/day ratio in the Rhesus monkey is small (Klein et al. 1997) and the dynamics of melatonin secretion are similar to those in ungulates (Arendt 1995).

Studies performed so far in different mammalian models show that the nocturnal increase in melatonin synthesis is primarily triggered by an increase in AA-NAT activity resulting from accumulation of the AA-NAT protein itself. Nevertheless, fundamental differences in the mechanisms involved in the accumulation of stable and active AA-NAT molecules exist. Two groups of mammals can be distinguished: first, the rodent species (“rat type”), in which an increase in the expression of the *Aa-nat* gene and synthesis of new AA-NAT molecules are a requirement, and secondly the nonrodent species (“sheep-type”), in which *Aa-nat* mRNA is constitutively present at a high level and AANAT protein accumulation results basically from stabilization of the constantly translated protein. These different mechanisms are responsible for the different patterns of melatonin synthesis and secretion observed between the two groups (Klein et al., 1997; Stehle et al., 2001) with a long delay (several hours) from dark onset to melatonin onset in rodents and a very short delay (a few minutes) from dark onset to melatonin onset in nonrodents. Analyses of these findings, however, show that, although NE is probably an important neurotransmitter regulating daily

melatonin synthesis, most of these species are not fully responsive to NE, suggesting the involvement of other transmitters to obtain a full melatonin response.

In this study showed that dopamine receptor can enhance the level intracellular of cAMP and induce phosphorylation of CREB. According to immunoblots of norepinephrine-dependent activation of transcription factors transcription factor CREB in the bovine pineal gland, Schomerus et al. (2003) have shown that NE-induced immunoreaction was due to phosphorylation of the transcription factor CREB and another protein, presumably the activating transcription factor 1 (ATF-1). Isoproterenol (ISO) or forskolin mimicked the response to NE indicating that NE acted through the beta-adrenergic/cAMP pathway (Schomerus et al., 2003). PKA type II catalyzed the phosphorylation of the transcription factor cAMP-response-element-binding protein (CREB) which was essential for the transcriptional induction of the arylalkylamine- N-acetyltransferase (AANAT), the rate limiting enzyme of melatonin biosynthesis. Furthermore, PKA may control protein levels and enzyme activity via two PKA-dependent phosphorylation sites in the AANAT molecule (Koch and Korf, 2002).

The present data indicated that dopamine receptors in the pineal gland might have an independent function in regulating melatonin synthesis. Intracellular levels of cAMP are regulated primarily by adenylyl cyclase. Increased cAMP levels directly affect the function of the tetrameric protein kinase A (PKA) complex. Binding of cAMP to two PKA regulatory subunits releases the catalytic subunits, enabling them to phosphorylate target proteins. In the nucleus, the phosphorylation state of transcription factors and related proteins appears to directly modulate their function and thus the expression of cAMP-inducible genes. These results suggest that, both DA- and NE-induced CREB phosphorylation in the bovine pinealocytes. However, the genes targeted by pCREB may vary from one mammalian species to the other. Data from the present study suggested that each system, D_1-dopamine or norepinephrine receptors acted independently. This data also indicated that melatonin production was controlled via transcriptional mechanisms.

The gene expression of dopamine transporter in bovine pineal glands

The dopamine transporter has been identified and characterized in the bovine pineal gland (Govitrapong et al., 2003). Interestingly, the K_d and B_{max} values obtained from bovine pineal were closer to those values obtained from bovine striatum and frontal cortex. Furthermore, the B_{max} value of dopamine transporter sites in the bovine pineal gland was higher than those reported in rat caudate nucleus (Janowsky et al., 1987), rat nucleus accumbens (Maloteaux et al., 1988), human putamen (Allard et al., 1994), and various human brain regions (Hitri et al., 1991). These results indicated that a high affinity dopamine transporter exists in the bovine pineal, which may exhibit circadian periodicity.

In this study, the expressions of dopamine transporter mRNA was not demonstrated in bovine pineal tissue. There are the whole systems of dopamine such as receptor, neurotransmitter but DAT mRNA is not found in the pineal gland. Dopamine nerve fiber may originate outside the pineal gland (Figure 6.3) from the central nervous system (CNS), superior cervical ganglion (SCG), or peripheral endocrine organs.

According to Jin et al. (1988) demonstrated dopamine fibers do innervate the golden hamster pineal gland and in the rat and gerbil pineal glands. Double immunostaining for tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) in the pineal gland revealed many TH-positive but DBH-negative, neuron-like cells in golden hamsters, but not in rats and gerbils. Bilateral superior cervical ganglionectomy diminished strikingly the number of TH- and DBH-positive nerve fibers, but did not affect the number of TH-positive cells (Jin et al., 1988). According to, the presence of tyrosine hydroxylase (TH) in the rat pineal gland was studied using a combination of immunochemical and biochemical methods. In superior cervical ganglionectomized (SCGx) animals and in isolated pineals incubated for 72 hr, both TH immunoreactive (TH-IR) fibers and TH biochemical activity were still present but reduced. The results suggested a central pinealopetal catecholaminergic pathway which could use dopamine as a neurotransmitter (Hernandez et al., 1994).

Clinical relevance of dopamine receptors and melatonin

Dopamine (DA) is the predominant catecholamine neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. This catecholamine also plays multiple roles in the periphery as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility. The dopaminergic systems have been the focus of much research, mainly because several pathological conditions such as Parkinson's disease, schizophrenia, Tourette's syndrome, and hyperprolactinemia have been linked to a dysregulation of dopaminergic transmission. Dopamine receptor antagonists have been developed to block hallucinations and delusions that occur in schizophrenic patients, whereas DA receptor agonists are effective in alleviating the hypokinesia of Parkinson's disease. However, blockade of DA receptors can induce extrapyramidal effects similar to those resulting from DA depletion, and high doses of DA agonists can cause psychoses. The therapies of disorders resulting from DA imbalances are thus associated with severe side effects (Missale et al., 1998).

From this study, D_1- and D_2-dopamine receptors have influence in the mealatonin synthesis in pineal gland. D_1-dopamine receptor induces melatonin production whereas D_2-dopamine receptor decreases the melatonin level. Melatonin has been suggested to act i) as a regulator of aging and senescence (Reiter et al., 2002) and (ii) as an intracellular scavenger of hydroxyl and peroxyl free radicals both in vitro and in vivo (Reiter, 1997). Thus, melatonin has a protective role for neurons and other cells. The interaction between melatonin and the dopaminergic system in the pineal gland may be the potential clinical use in dopamine-related disorders in the central nervous system.

Accumulating evidence indicates decreased melatonin levels in patients with schizophrenia. Insomnia, mainly difficulty in falling asleep at night, is commonly reported in this population. Association of insomnia with low or abnormal melatonin

rhythms has been repeatedly documented. Melatonin is an endogenous sleep promoter in humans. Melatonin improves sleep efficiency in patients with schizophrenia whose sleep quality is low. In addition, melatonin treatment exaggerates the first-night effect in patients with chronic schizophrenia, thereby suggesting an improved ability of these patients to mobilize alertness in unfamiliar surroundings (Shamir et al., 2000). In addition, anti-psychotics remain the mainstay of drug intervention in the management of schizophrenia. However, long-term treatment with antipsychotics is associated with a variety of movement disorders, the most disabling of which is tardive dyskinesia (TD), which occurs in up to 50% of patients hospitalized with chronic schizophrenia. The pathophysiology of TD is still unclear and no definite treatment exists. Both dopamine receptor supersensitivity and oxidative stress-induced neurotoxicity in the nigrostriatal system are apparently implicated. The pineal hormone melatonin is a potent antioxidant and attenuates dopaminergic activity in the striatum and dopamine release from the hypothalamus. Thus, it may have a beneficial effect for both the treatment and prevention of TD. Melatonin appears to be effective in the treatment of tardive dyskinesia. a severe movement disorder associated with long-term blockade of the postsynaptic dopamine D_2 receptor by antipsychotic drugs in schizophrenic patients (Shamir et al., 2001).

Recent findings indicate that melatonin may modulate dopaminergic pathways involved in movement disorders in humans (Zisapel, 2001). In Parkinson patients melatonin may, on the one hand, exacerbate symptoms (because of its putative interference with dopamine release) and, on the other, protect against neurodegeneration (by virtue of its antioxidant properties and its effects on mitochondrial activity). Furthermore, the low doses of 6-hydroxydopamine (6-OHDA) induce apoptosis of naive (undifferentiated) and neuronal (differentiated) PC12 cells, and this system has been proposed as an adequate experimental model for the study of Parkinson's disease. The mechanism by which this neurotoxin damages cells is via the production of free radicals. Melatonin can prevent the apoptosis caused by 6-OHDA in naive and neuronal PC12 cells (Mayo et al., 1998). Exploration of the mechanisms used by melatonin to reduce programmed cell death revealed that this chemical mediator prevents the 6-OHDA induced reduction of mRNAs for several antioxidant

enzymes. This endogenous agent has no known side effects and readily crosses the blood-brain-barrier. This study supports that melatonin have a high clinical potential in the treatment of Parkinson's disease and possibly other neurodegenerative diseases, although more research on the mechanisms is yet to be done. The interaction of melatonin with the dopaminergic system may play a significant role in the nonphotic and photic entrainment of the biological clock as well as in the fine-tuning of motor coordination in the striatum. These interactions and the antioxidant nature of melatonin may be beneficial in the treatment of dopamine-related disorders.

Melatonin may potentially play a role in the processes of aging, the prolongation of life span and health in the aged; particularly, melatonin may exert a beneficial action on neurodegenerative conditions in humans with debilitating diseases (Reiter et al., 2002). Aging in animals results in a decline in several cellular functions, such as mitochondrial respiration (Wei et al., 1998), lysosomal enzyme activities (Nakamura et al., 1989), and rates of mitochondrial and nuclear DNA repair (Stevnsner et al., 2002). A decline in cellular functions with aging somewhat correlates with the cognitive impairment observed in normal aging and other age-related conditions such as senile dementia, AD and Parkinson’s disease. Melatonin production in the pineal gland declines progressively with age, and in elderly humans the amount of melatonin produced is much less than that in young individuals (Reiter, 1992). Recent studys suggested that age-related changes in mRNA gene expression in the central nervous system (CNS) of mice can be modulated by dietary melatonin (Bondy et al., 2002; Sharman et al., 2002). The addition of melatonin to the diet resulted in a significant increase in brain melatonin levels with a significant reduction in cortical amyloid beta-peptide (Ab) levels. Thus, melatonin supplementation may retard age related neurodegenerative processes by compensating for the depletion of melatonin in the brain of aging mice.

In conclusion, from my study has elucidated the existence of both D_1-and D_2-dopamine receptor mRNAs in the bovine pineal gland. In addition, we have shown that dopamine D_1-receptors stimulate the activity of NAT and, hence, enhances the formation of melatonin, and that the said action is blocked by SCH-23390 whereas dopamine D_2-receptors inhibit NAT activity and melatonin level, and the inhibitory effect is blocked by D_2-antagonists. Furthermore, our study has elucidated the function of both D_1-and D_2-dopamine transporter and receptors in the bovine pineal gland. We have shown that dopamine D_1-receptors stimulate cAMP accumulation and, hence, enhances the phosphorylation of CREB, and that the said action is blocked by SCH-23390 whereas dopamine D_2-receptors inhibit the level intracellular of cAMP and phosphorylation of CREB, and the inhibitory effect is blocked by D_2-antagonists. D_2-dopamine receptors also inhibit forskolin stimulated the level intracellular of cAMP and phosphorylation of CREB. Taken together, these results directly demonstrate for the first time that in the mammalian pineal gland dopamine stimulates NAT activity and enhances melatonin level, and that stimulation is mediated by D_1 dopaminergic receptors (Figure 6.1). Additionally, the data directly identified that in the mammalian pineal gland dopamine stimulates cAMP and that stimulation is mediated by D_1 dopaminergic receptors. This study then determined the role of dopamine-dependent phosphorylation of the transcription factor cyclic adenosine monophosphate responsive element-binding protein (CREB). This result elucidated that dopamine has an effect as same as norepinephrine in stimulation of CREB phosphorylation and melatonin production via transcriptional mechanisms (Figure 6.2). However the presence of dopamine containing fibers and the localization of specific dopamine receptors in pineal need to be further elucidated (Figure 6.3).

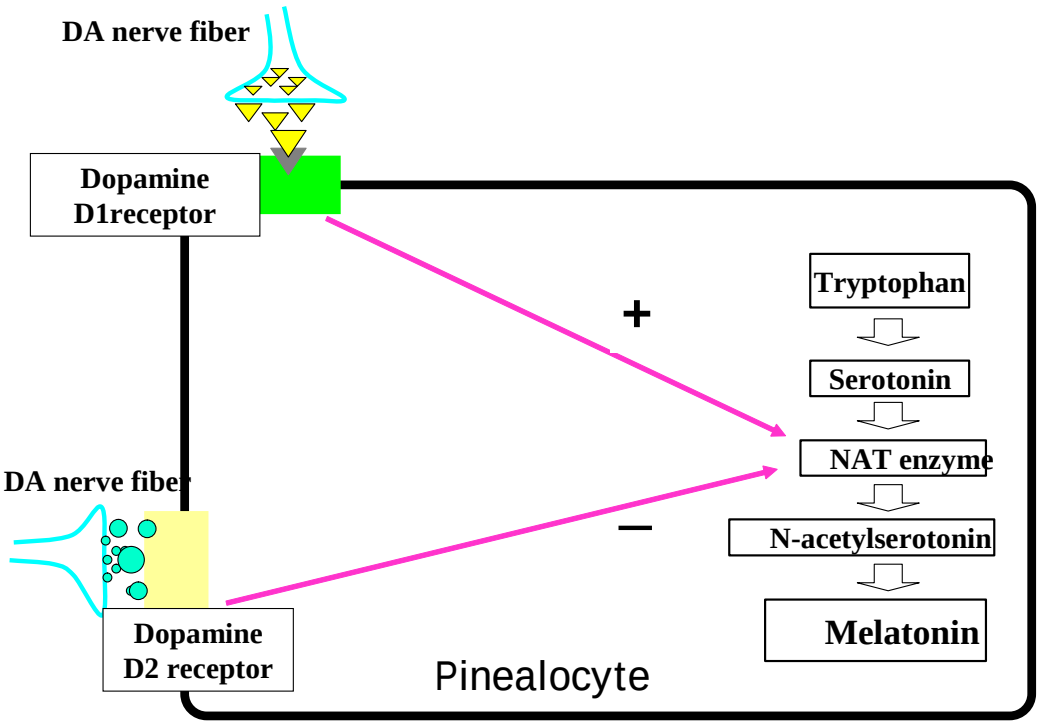


Figure 6.1 Role of D_1- and D_2-dopamine receptor in NAT activity and melatonin synthesis

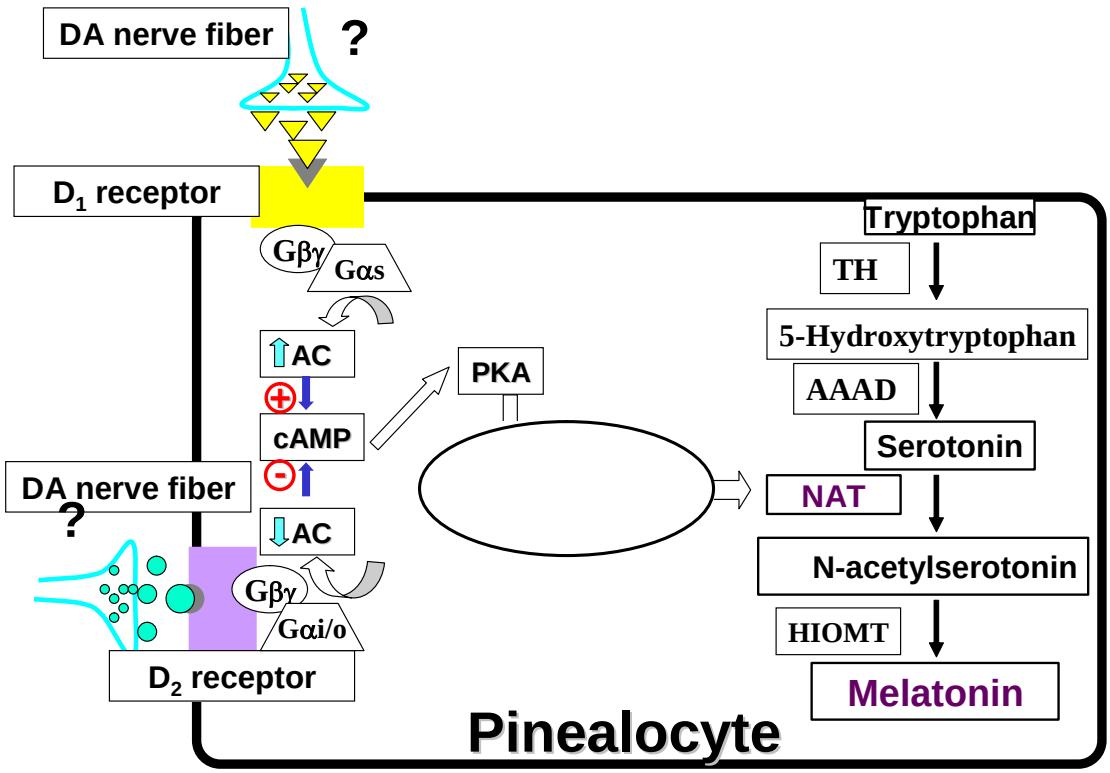


Figure 6.2 Dopamine receptor activation in bovine pinealocyte via a cAMP-dependent transcription pathway

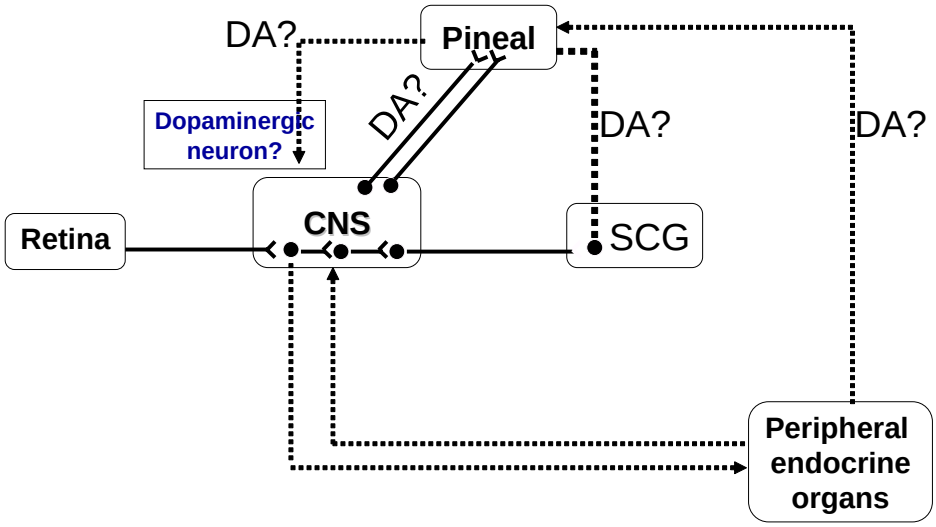


Figure 6.3 Possible pathways of dopaminergic neuron to the pineal gland

CHAPTER 7

CONCLUSION

This study identified the mRNAs for both the D_1- and D_2-dopamine receptors in bovine pineal glands and clarified the mechanism of dopamine in controlling melatonin production in the mammalian pineal gland. Cyclic AMP accumulation and the dopamine-dependent phosphorylation of the transcription factors cyclic adenosine monophosphate responsive element-binding protein (CREB) have been currently determined in cultured bovine pinealocytes. The result indicated that dopamine stimulated NAT activity and enhanced melatonin level and this stimulation acted via a cAMP-dependent transcription mechanism.

The RT-PCR technique was performed to study the gene expression of D_1 and D_2-dopamine receptors in the bovine pineal gland. The results were:

- The mRNA of D_1 and D_2-dopamine receptor was found in the bovine pineal gland. This data indicated that both D_1 and D_2-dopamine receptors existed in bovine pineal gland.

The NAT activity and melatonin determination were performed to study the function of dopamine in controlling melatonin production in the mammalian pineal gland. The results were:

- Various concentrations (1-100 μM) of all D_1-agonists (SKF 38393, CY 208-203, Dihidrexidine) stimulated NAT activity and melatonin production in a concentration-dependent manner on bovine pinealocytes.
- D_1-dopamine receptor directly activated NAT activity and melatonin level in the bovine pineal gland. In order to compare the stimulatory effect of D_1-dopamine agonist with norepinephrine, the same

concentration of β-adrenergic agonists, norepinephrine and isoproterenol and D_1-dopamine receptor agonist, SKF 38393 were added into the cultured bovine pinealocytes. Ten micromolar of isoproterenol, norepinephrine and SKF 38393 significantly ($p < 0.05$) increased (1.96, 1.71, 1.7 folds, respectively) in NAT activity and increased (2.02, 1.59, 1.62 folds, respectively) in melatonin synthesis. When SKF 38393 and norepinephrine were added simultaneously to cultured bovine pinealocytes, the stimulatory effect on NAT (1.72 fold increase) did not differ from each individual effect whereas the stimulatory effect on melatonin synthesis (2.31 fold increase) differs from each individual effect.

- Ten micromolar of SKF 38393 (D_1-agonist) had a significant effect on melatonin synthesis. It caused significant ($p < 0.05$) increase (2.4 folds) in the NAT activity and caused significantly ($p < 0.01$) increased (1.8 folds) melatonin production in the cultured bovine pinealocytes and this stimulatory effect was blocked by SCH 23390, a selective D_1-dopamine antagonist.

- D_2-dopamine receptor directly inhibited NAT activity and melatonin level. Quinpirole, a D_2-dopamine agonist significantly reduced the NAT activity and melatonin production in a concentration-dependent fashion in the cultured bovine pinealocytes. D_2-dopamine antagonists (spiperone, haloperidol and domperidone) significantly increased the basal activity of NAT and melatonin production. The inhibitory effect of quinpirole was prevented by D_2-dopamine antagonists, spiperone, haloperidol and domperidone.

The cAMP accumulation and the phosphorylated cyclic adenosine monophosphate-responsive element-binding protein (pCREB) were performed to clarify the mechanism of dopamine in controlling melatonin production in the mammalian pineal gland. The results were:

- D_1-dopamine receptor stimulated adenylate cyclase that increased cyclic AMP formation. Treatment with 10 μM of isoproterenol, norepinephrine or SKF 38393 significantly increased cAMP accumulation over the control values (4.6, 3.47 and 1.98 fold, respectively). For further comparison, the concentration of SKF 38393 was then increased to 100 μM resulting in a substantially higher cAMP accumulation than that at the lower concentration (3.81 fold over control values). The stimulatory effect of the D_1-dopamine agonist on cAMP accumulation was completely blocked by the D_1-dopamine antagonist.
- D_2-dopamine receptor inhibited adenylate cyclase that decreased cyclic AMP formation. Quinpirole, a D_2-dopamine agonist, when added to cultured bovine pinealocytes significantly ($p < 0.01$) reduced the level of cAMP accumulation in a concentration-dependent fashion. On the other hand, forskolin and spiperone, D_2-dopamine antagonists, each significantly induced cAMP accumulation in the pineacolytes but this stimulatory effect was negated in the presence of quinpirole.
- D_1-dopamine receptor activated pCREB. Immunoblots showed that a protein of 43 kDa corresponding to CREB accounts for the pCREB and confirmed that SKF-38393, a selective D_1 agonist is effective in inducing CREB phosphorylation (pCREB) like norepinephrine. The amount of total (phosphorylated and unphosphorylated) CREB was not changed upon stimulation of the cells with NE or SKF 38393. This stimulatory effect was blocked by SCH 23390, a D_1-dopamine antagonist.

- D_2-dopamine receptor decreased pCREB. Quinpirole, a selective D_2 agonist significantly inhibited the basal level and forskolin stimulated CREB phosphorylation (pCREB) whereas D_2-dopamine antagonists (spiperone) significantly induced the phosphorylation of CREB and this stimulatory effect was abolished by quinpirole.

To detect the dopamine neuron in the bovine pineal gland, the RT-PCR technique was performed to study the gene expression of the dopamine transporter. The results were:

- The mRNA of the dopamine transporter was not found in the bovine pineal gland. This data indicated that dopaminergic nerve fibers may originate outside the pineal gland. They may be from the central nervous system (CNS), the superior cervical ganglion (SCG), or the peripheral endocrine organs.

These results suggest that dopamine may be another real neurotransmitter and have an important role in regulating melatonin synthesis in the pineal gland and elucidate that each system, D_1-dopamine or norepinephrine receptor contributes about the same and dopamine has a synergistic effect like that of norepinephrine in induce CREB phosphorylation and melatonin production via transcriptional mechanisms.

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APPENDIX

Melatonin determination

Melatonin level in cultured media was determined by radioimmunoassay. Cultured media were diluted with assay buffer.

Buffer: Tricine is made up at 0.1M (pH 5.5) with 0.9% NaCl and 0.1% gelatin. It is necessary to heat to 50°C for 30 minutes to dissolve gelatin

17.9g tricine	)	
9.0g NaCl	)	to 1 litre with DGDW
1.0g gelatin	)	
Buffer is made up fresh weekly		

Antiserum: samples were incubated with specific antisera against melatonin (G/S/704-6483) the contents of a vial sufficient for 1,000 assay tubes should be reconstituted with 0.5 ml DGDW to provide an intermediate dilution of 1:10. This is aliquotted into 50µl portions and stored at –20°C. The working solution is made as required by diluting one 50 µl aliquot to 20 ml with assay buffer. They provide sufficient reagent for 100 tubes with an initial dilution of 1:4,000.

Dextran coated charcoal: suspend activated charcoal at 2% w/v in assay buffer. Stir for 5 minutes. Centrifuge at 4°C for 5 minutes at 1000 rpm. Discard the supernatant and any fines around the sides of the vessel. Resuspend charcoal in the original volume of assay buffer and add 0.02% w/v dextran. Stir for at least 1 h at 4°C and make up fresh weekly.

Radiolabel: tritiated melatonin is obtained from Amersham International and stored at –20°C. An intermediate dilution is prepared by diluting 20 µl of this stock to 2 ml with absolute ethanol. This solution is also stored at -20°C. The working solution is prepared by further diluting this intermediate stock with assay buffer such that 100 µl contains approximately 4,000 cpm. The working solution is freshly prepared daily.

Standards: a stock melatonin standard (1mg/ml) is prepared by dissolving 10 mg melatonin in 0.5 ml absolute ethanol and adjusting the volume to 10 ml with DDW. This solution is stable for at least 1yr. The working standard is freshly prepared from this ethanolic stock for each assay as follows:

100 μl (1mg/ml) to 100 ml in DDW = 1 μg/ml

500 μl (1 μg/ml) to 50 ml in DDW = 10 ng/ml

125 μl (10 ng/ml) to 2.5 ml in melatonin free plasma = 0.5ng/ml

Further dilutions with melatonin free plasma provide standards (2.5, 5.0, 12.5, 25, 50, 100 and 250 pg) for the standard curve.

MT standard 0.5 ng/ml(μl)	**MT free plasma (μl)**	**MT concentration pg/0.5ml**
0	500	0
5	495	2.5
10	490	5.0
25	475	12.5
50	450	25.0
100	400	50.0
200	300	100.0
500	0	250.0

The standards are treated in exactly the same way as the plasma samples in the assay.

Method

The volumes required in the assay are as follows:

Samples / standards	500 μl
Antiserum	200 μl
Radiolabel	100 μl
Dextran coated charcoal	500 μl
Total volume	1300 μl

The volumes can be added with ordinary microlitre dispensers or preferably with repeating dispenses. It is necessary to restrict assay sizes to 150 tubes or less in order to eliminate assay drift.

Assay Protocol

1. Add 500 µl of each sample or standard to assay tubes
2. Add 200 µl antiserum to all tubes except total count and non-specific binding tubes. Vortex and incubate at room temperature for 30 minutes
3. Add 100 µl [^{3}H]-melatonin to all tubes, mix again, and incubate for 18 h at 4^{o}C.
4. Separate the antibody bound melatonin from the free fraction by incubation for 15 minutes at 4^{o} C, in ice or on a cold tray, with 500 µl dextran coated charcoal (stirred during addition at 4^{o}C). The dextran coated charcoal is stirred continuously on ice for 30 minutes before use. Charcoal addition and vortexing should be done quickly to reduce intra-assay variation. Do not add dextran coated charcoal to the total tubes.
5. Spin at 1500 g for 15 minutes at 4 ^{o} C.
6. Remove 700 µl aliquots of the supernatant into vials containing 4.0 ml scintillation fluid.

Shake vials at room temperature for 1 h. This is a two-phase counting system, the shaking is necessary to extract all the [^{3}H]-melatonin into the organic phase. It provides greater efficiency of counting than a detergent based system and is less sensitive to quenching by coloured constituents of the plasma. Radioactivity was counted in all tubes by a liquid scintillation spectrometer. Determine the melatonin concentration in the samples from the standard curve. Activity values were normalized against the amount of total protein determined using the method of Bradford (1976).

Cyclic AMP measurement (cAMP [^{125}I] RIA Kit)

Preparation of reagents [store at 2-8^{o}C, 2 months]

1. cAMP sodium acetate buffer

   One vial of concentrated buffer is supplied. Dilute to 500 mL with distilled water. The final solution will contain sodium acetate buffer, pH 6.2, and a stabilizer. The diluted buffer is stable for at least two months when stored at 2 - 8°C. Refer to vial label for exact expiration date of the concentrated reagent.

2. cAMP standard

One vial of lyophilized standard is supplied. Reconstitute the contents with exactly 2.0 mL of distilled water. The reconstituted solution will contain: cAMP at a concentration of 5000 pmol/mL, sodium acetate buffer, 0.1% sodium azide and an inert ingredient. The cAMP Standard has been calibrated spectrophotometrically using the molar absorption coefficient. The reconstituted standard is stable for at least two months when stored at 2 - 8°C. Refer to vial label for expiration date of lyophilized reagent.

3. cAMP antiserum complex

One vial of lyophilized, prereacted, first and second antibody is supplied. Reconstitute with 21 mL of distilled water. The resulting solution will contain the prereacted antibody complex, and an inert ingredient, in sodium phosphate buffer, pH 6.0. This solution may appear cloudy and will settle upon standing. Before use and during prolonged use, mix thoroughly.

4. cAMP [^{125}I] tracer (succinyl cAMP tyrosine methyl ester [^{125}I]

Two vials of concentrated tracer are supplied. Each vial contains less than 74 KBq (2 μCi) on calibration date in one mL of a 1:1 n-propanol water solution. Use one vial at a time as directed. Add 5.0 mL of distilled water to each vial as required. The concentrate and diluted tracer are stable for at least two months when stored at 2 - 8°C.

5. cAMP carrier serum

Two vials of lyophilized carrier serum are supplied. Use one vial at a time. Reconstitute the contents of each vial with exactly 6.0 mL of distilled water. (One vial of reconstituted carrier serum is sufficient for 100 tubes.) The resulting solution will contain carrier serum, 0.1% sodium azide, a stabilizer, and an inert ingredient in sodium acetate buffer, pH 6.2. Refer to vial label for expiration date of lyophilized reagent. Store at 2 - 8°C.

6. cAMP acetic anhydride

One vial containing one mL is supplied. CAUTION: FLAMMABLE, CORROSIVE, LACHRYMATOR. Store tightly closed in the refrigerator. Allow vial to equilibrate to room temperature before use. Protect from

moisture. This material is stable for at least two months under these conditions. Refer to vial label for expiration date.

7. cAMP Triethylamine

One vial containing one mL is supplied. CAUTION: FLAMMABLE, VAPOR HARMFUL. Store tightly closed in the refrigerator. Allow vial to equilibrate to room temperature before use. Refer to vial label for expiration date.

8. cAMP Precipitator

One bottle containing 100 mL is supplied ready to use and contains a precipitation enhancer and sodium azide in sodium acetate buffer. Refer to bottle label for expiration date. Store at 2 - 8°C. Shake well before using.

Preparation of cell pellets

Cell pellets were homogenized in 150 µl assay buffer (50 mM Tris and 4 mM EDTA) to prevent degradation of cAMP and heating 5 min in a boiling water bath to coagulate protein. To remove precipitates, the samples were centrifuged for 10 min (10,000 g) and collected supernatant. The protein pellets were assayed by the Bradford method. Cyclic AMP was then acetylated and its total amount determined by use of [^{125}I]cAMP (a commercial RIA) from PerkinElmer NEK033 according to manufacturer's instructions. For measurements of intracellular cAMP accumulation, cells were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and then stimulated with the indicated drugs in the presence of IBMX.

Acetylated (Plasma) Radioimmunoassay Protocol

1. Dilute the reconstituted cAMP stock standard reagent (5,000 pmol/mL) fifty-fold in a 13 x 100 mm test tube by adding 100 µL of the 5,000 pmol/mL solution to 4.9 mL of Assay Buffer. The resulting solution will be 100 pmol/mL. Prepare a 40 pmol/mL solution by adding 1.0 mL of the 100 pmol/mL solution to 1.5 mL of Assay Buffer. Place 200 µL of the 40 pmol/mL solution in a marked glass tube.

2. Prepare the plasma samples for acetylation as follows: Pipet 100 µL of the plasma sample into 400 µL of Assay Buffer (1:5 dilution). Place 100 µL of the 1:5 dilution in a marked glass test tube.

3. Prepare 150 µL of acetylation reagent by mixing together in a glass test tube 100 µL of Triethylamine and 50 µL of Acetic Anhydride. Vortex before using. Make up a fresh solution each time.

4. Prepare 10 mL of Modified Assay Buffer by adding 50 µL of the acetylation reagent (Step 3) to 10 mL of Assay Buffer. Mix well and let incubate at room temperature for at least three minutes. Modified Assay Buffer is used for the preparation of the cAMP standards and in the "blank" and "zero standard" tubes.

5. Acetylate the 40 pmol/mL standard prepared in Step 1 (i.e., the 200 µL aliquot) by adding to it 10 µL of the freshly prepared acetylation reagent. Allow the reaction to proceed for at least three minutes at room temperature and then add 1.8 mL of Assay Buffer. Label this 4 pmol/mL standard as tube A. The amount of acetylation reagent used is sufficient to acetylate 25,000 pmols of cAMP.

6. Acetylate the previously prepared 100 µL aliquot of diluted sample (Step 2) by adding 5 µL of acetylation reagent to each tube. Immediately vortex and let the sample incubate for at least three minutes at room temperature. Then add 900 µL of Assay Buffer to each tube. This makes a 1:50 dilution of the sample.

7. Dilute the 4 pmol/mL standard with Modified Assay Buffer (see Step 4) as shown below. The standards should be prepared fresh each day. Do not store for reuse.

8. Prepare Working Tracer Solution by adding one volume of diluted cAMP [^{125}I]-Tracer to one volume of the reconstituted cAMP Carrier Serum. Make enough of the Working Tracer Solution to run the desired number of tubes (e.g., 1.0 mL of cAMP Carrier Serum and 1.0 mL of cAMP [^{125}I]-Tracer will provide 2.0 mL of solution which is theoretically sufficient for a maximum of 20 tubes). Any remaining volume is to be discarded appropriately. Do not store and reuse this solution.

Pipet mL cAMP Std.	From Tube	Add mL Modified Assay Buffer	Into Labeled Tube	Concentration (pmol/mL)
-	-	-	A	4.0
1.0	A	1.0	B	2.0
1.0	B	1.0	C	1.0
1.0	C	1.0	D	0.5
1.0	D	1.0	E	0.25
1.0	E	1.5	F	0.10
1.0	F	1.0	G	0.05

9. Number a series of 20 tubes to be used for the standard curve, plus two additional tubes for each sample. The assay may be set up at room temperature.
10. Tubes 1 and 2 measure the total counts added and receive only the Working Tracer Solution (Table 4.3).
11. Add 200 µL of Modified Assay Buffer to Tubes 3 and 4 (blank tubes).
12. Add 100 µL of Modified Assay Buffer to Tubes 5 and 6 (zero standard tubes).
13. Add 100 µL of each standard solution or sample to the appropriate tubes.
14. Add 100 µL of Working Tracer Solution to all tubes.
15. Thoroughly mix Antiserum Complex and add 100 µL of to all tubes, except total count tubes and blank tubes.
16. Mix all tubes, except 1 and 2, by using a vortex mixer.
17. Cover and incubate overnight (16 - 18 hours) at 2 - 8°C.
18. Set Tubes 1 and 2 aside, and add 500 µL of 2 - 8°C cAMP Precipitator to all the other tubes. Mix well with a vortex mixer and centrifuge at 2 - 8°C for 15 minutes at approximately 1200 x g.
19. Decant by gently inverting all tubes once, preferably at the same time, discarding the supernatant into a radioactive waste container. Keeping the tubes inverted, place them on absorbent paper for blotting. To facilitate removal of remaining droplets, gently tap the rims of the tubes on the paper. Allow tubes to drain for 20 - 30 seconds.
20. Count all tubes, including Tubes 1 and 2, in a gamma counter. At the usual counting efficiency of 50 - 70%, a counting time of one minute should be sufficient. Include an instrument blank.

Table 1 Protocol for Acetylated Standard Curve

	Tube	Assay Buffer	Stds.	Sample	Working Tracer	Antiserum Complex	Precipi-tator
Total Cts.	1, 2	-	-	-	100	-	-
Blank	3, 4	200	-	-	100	-	500
"0" Std.	5, 6	100	-	-	100	100	500
0.05 Std.	7, 8	-	100		100	100	500
0.10 Std.	9, 10	-	100	--	100	100	500
0.25 Std.	11, 12	-	100	-	100	100	500
0.5 Std.	13, 14	-	100	-	100	100	500
1.0 Std.	15, 16	-	100	-	100	100	500
2.0 Std.	17, 18	-	100	-	100	100	500
4.0 Std.	19, 20	-	100	-	100	100	500
Sample	21, 22	-	-	100	100	100	500

Westernblot analysis

1. Preparing of the Sodium Dodecyl Sulfate-Polyacrylamine Gel Electrophoresis (SDS-PAGE)

The glass plates were cleaned with water and detergent then thoroughly rinsed with deionized water to removed detergent residues. The plates were wiped with 70% ethanol. The glass plate sandwiches were collected and were locked to the casting stand.

2. Pouring the separation gel

The separating gel solution was prepared by mix the solution in a Falcon 50 ml tube, gently swirl to mix. Using a Pasteur pipette, transfer the separating gel solution to the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is about 11 cm from the bottom of the gel plate. Using another Pasteur pipette, slowly cover the top of the gel with a layer of distilled water by gently squirting the distilled water against the edge of one of the spacers. The gel was allowed to polymerize 30 min at room temperature.

3. Pouring the stacking gel

The layer of dH_20 was poured off completely. The stacking gel solution was prepared by mix the following in a Falcon 50 ml tube, gently swirl to mix. Using a Pasteur pipette, slowly allow the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is about 3 cm. A Teflon comb was inserted into the layer of stacking gel solution ensuring that there was no air bubble trapped in the tooth edges of the comb. Additional stacking gel was added to completely fill the spaces in the comb and the stacking gel solution was allowed to polymerize 45 min to 1 hr at room temperature. The comb was removed and the wells of the gel were washed with dH_20 before the gel was used.

4. Sample preparation

In each experiment, the protein concentration of all samples was adjusted to the same concentration and was mixed with an equal volume of 2x loading buffer. All samples were boiled for 5 minutes at 100 °C and loaded into the well of the gel.

5. Loading the gel

An aliquot of the protein was diluted to be analyzed at least 1:2 (vol/vol) with 2x SDS/sample buffer and were boiled 5 min at 100°C. Then the Teflon comb was removed carefully without tearing the edges of the polyacrylamide wells. The gel plates were gently placed at both sides of the gel support frame in the chamber. The 1.5 liters of 1x SDS/electrophoresis buffer was poured into the lower buffer chamber. The sandwich was placed to attach to upper buffer chamber into lower buffer chamber. The upper buffer chamber was filled partially with 300 ml of 1x SDS/electrophoresis buffer so that the sample wells of the stacking gel were filled with buffer. Using a 25- or 100-ml syringe with a flat-tipped needle, load the protein sample into one or more wells by carefully applying the sample as a thin layer at the bottom of the well.

6. Running the gel

The power supply was connected to the cell and run at 120 volts of constant current for a slab gel 1.50 mm thick until the tracking dye enters the separating gel.

After the Bromphenol Blue tracking dye has reached the bottom of the separating gel, disconnect the power supply. The total run time for a gel is 1 hr 20 min. At the end of time the upper buffer chamber and the attached sandwich were carefully removed. The sandwich was laid on a sheet of absorbent paper. Carefully slide one of the spacers halfway from the edge of the sandwich along its entire length. Using the exposed spacer as a lever to pry opens the glass plate, exposing the gel. The gel was carefully removed from the lower plate. The gel can be stained with Coomassie Brilliant Blue or transferred to the Nitrocellulose membrane for Western blotting.

7. Transferring proteins from gel to Nitrocellulose membrane

Nitrocellulose membranes were prewetted with electroblotting buffer about 30 min. Place a piece of cut, marked, and wetted Nitrocellulose membrane directly onto the anode (positive electrode) side of the gel and remove all air bubbles. Place another piece of wetted Whatman filter paper on the anode side of the Nitrocellulose membrane and remove all air bubbles. Place another Scotch-Brite pad on top of this filter paper Place this sandwich into a plastic support and place the support containing the sandwich into the electroblotting apparatus in the correct orientation. Fill the tank with electroblotting buffer and connect the leads of the power supply to the corresponding anode and cathode site of the electroblotting apparatus. Electrophoretically transfer the protein from the gel Nitrocellulose membrane at 20 volt and set current at maximum 400 mA, for overnight at 4°C lay down chamber in the refrigerator, stabilizer and power supply stay outside. Transfer time is dependence on the thickness of the gel and the size of the protein being transferred. In general, proteins are transferred within 1-6 hr, but high-molecular-weight molecules may take longer. Overnight transfer is reliable and convenient.

8. Immunoblotting membrane

The nitrocellulose membranes were washed for 5 min with TBS-T. The membranes were incubated in 10 ml of 2% non-fat milk blocking solution for 1 hr, at room temperature and then washed for 5 min with TBS-T. The membranes were incubated with primary antibody P-CREB (rabbit polyclonal IgG) diluted 1:1000 in 2.5 blocking solution with gentle shaker for 2 hr at room temperature. The membranes

were washed 3 times for 5 min with TBS-T. The membranes were incubated with secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000) in 2.5 blocking solution with gentle shaker for 1 hr at room temperature and then washed 3 times for 5 min with TBS-T.

9. ECL plus western blotting detection of proteins

The membranes were incubated with ECL plus western blotting detection reagents for 5 min. The solution A and B were mixed in a ratio of 40:1 (3.5ml solution A + 88 ml solution B). The final volume of detection reagent required is 0.1 ml/cm2. Drain the excess wash buffer from the washed membranes and place protein side up on a sheet of Saran Wrap. Pipette the mixed detection reagent on to membrane. Drain the excess detection reagent by holding the membrane gently in forceps and touching the edge against a tissue. Place the blot protein side down on to a fresh piece of Saran wrap. Wrap up the blot and gently smooth out any air bubbles. Place the wrapped blots, protein side up in a film cassette. Then place a sheet of Hyperfilm ECL on top of the membrane closes the cassette and exposed with film. Processing the film in Developer solution and Fixative solution, then measured the band density by Scion Image (Beta 4.0.2). Developer solution was diluted from stock solution 25 ml in to 100 ml distilled water and diluted fixative solution from stock solution 25 ml in to 100 ml distilled water (Kodak Co, Ltd.).

Technique of Analyzing Electrophoresis Gels

The following is one of possible procedure for using Scion Image to analyze a one-dimensional electrophoretic gel. It also demonstrates some of the less obvious features in Scion Image, and also a few shortcuts. Note that this technique cannot be used to compare bands on different gels unless the gels are calibrated to known standards. Any results obtained using this procedure should not be trusted without testing using standards with known concentrations or by comparing with results obtained using a densitometer.

1. Use the Calibrate command to calibrate the image to standards included in the gel or to a calibrated optical density step tablet. Failure to do this

could result in incorrect and misleading measurements. For calibrated images, results are reported in calibrated units such as integrated OD.

2. Use the rectangular selection tool to outline the first lane. This is the left most lane for vertically oriented lanes and the top lane for horizontal lanes.
3. Select Mark First Lane in the Special menu. A copy of the image will be displayed with the first lane outlined.
4. Move the rectangular selection (by clicking inside it and dragging) and outline (using Mark Next Lane) each of the other lanes in succession.
5. Use Plot Lanes to generate the lane profile plots.
6. Use the line drawing tool to draw base lines and drop lines so that each peak defines a closed area as shown above. Note that you can hold the shift key down to constrain lines to be vertical.
7. Measure the areas of the peaks by clicking inside each one in succession with the wand tool.
8. Scroll Lock-click with the text tool to automatically label peaks, in reverse order, with the area measurements. The area measurements are also recorded in tabular form, and can be displayed to a spreadsheet.

Note that this macro package enables Wand Auto-Measure so that the area is automatically measured when the wand tool is clicked under a peak.

BIOGRAPHY

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INSTITUTIONS ATTENDED:	
1982-1986	B. Sc. (Nursing and Midwifery) Mahidol University, Bangkok, Thailand
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