1992

Amino Acids and Pineal Gland Function in the Rat

Laura Kus
Loyola University Chicago

Amino Acids and Pineal Gland Function in the Rat

by

Laura Kus

A dissertation submitted to the faculty of the Graduate School of Loyola University Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 1992

To the two men who taught me the love of science
Drs. James E. Woods and Robert C. Thommes

Acknowledgements

I wish to thank my advisor, Dr. John A. McNulty and the members of my dissertation committee, Drs. Robert J. Handa, Edward J. Neafsey, George Battaglia and Nicholas V. Emanuele for their help. I would like to extend special thanks to Dr. Handa for allowing me to use his lab facilities. The support of my fellow students, Mette Schulz, JoAnn O'Keefe, and Diane Workman, during my graduate career has been invaluable. I would like to express to them my most heartfelt thanks.

The author, Laura Kus, was born on July 24, 1964 in Evergreen Park, Illinois to Richard and Barbara Kus. She attended DePaul University in Chicago, and graduated with a Bachelor of Science degree in Biology and a minor in Chemistry in June of 1986. In August of 1986, Laura joined the Department of Cell Biology, Neurobiology and Anatomy in the Graduate School of Loyola University Chicago. She was the recipient of four Loyola University Basic Science Fellowships while in the department, and in 1989 she received a Grants-in-Aid of Research award from the research society Sigma Xi. Laura taught in the Medical School Gross Anatomy and Neuroscience courses. She served as the vice-president of the Graduate Student Council and as the student representative to the Ph.D. Council in 1989. Membership in professional societies includes the Society for Neuroscience and the Association for Women in Science. Laura's community and civic activities have included volunteer work as a court approved Citizen Reviewer for the child advocacy group Illinois Action for Children (1989-1990). She is presently on the board of directors of the Southwest Parish and Neighborhood Federation, and serves on the St. Gall's Area Neighborhood Court Watch Council in Chicago.

Table of Contents

List of Abbreviations

ACPD	1-Amino-cyclopentane-1,3-dicarboxylic acid
HEAT	2-[β-(4-hydroxyphenyl)-ethylaminomethyl]-tetralone
APB	2-amino-4-phosphonobutyric acid
AP5	2-Amino-5-phosphonopentanoate
CPP	3-((+)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid
HAP	3-amino-1-hydroxpyrrolidone
5HIAA	5-Hydroxyindole acetic acid
HPETE	5-hydroxyperoxy-6, 8, 11, 14-eicosatetraenoic acid
5HT	5-Hydroxytryptamine (serotonin)
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxalepropionic
ATP	Adenosine triphosphate
Ala	Alanine
AAD	Amino acid decarboxylase
ANOVA	Analysis of variance
Arg	Argenine
Asp	Aspartate
CSF	Cerebral spinal fluid
cDNA	Complimentary deoxyribonucleic acid
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate

DAA	D-α-aminoadipate
DNA	Deoxyribonucleic acid
DAG	Diacylglycerol
K_d	Dissociation constant
GABA	γ-Aminobutyric acid
Glu	Glutamate
GAD	Glutamate decarboxylase
GDEE	Glutamic acid diethyl ester
Gln	Glutamine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HIOMT	Hydroxyindole-O-methyltransferase
IP3	Inositol triphosphate
IP	Intraperitoneal
Iso	Isoproterenol
KA	Kainate
PE	L-Phenylephrine
LDH	Lactate dehydrogenase
B_{max}	Maximum number of binding sites
MAO	Monoamine oxidase
NMDA	N-Methyl -D-aspartate
NPY	Neuropeptide Y
NE	Norepinephrine
OAG	Oleoyl acetyl glycerol
PVN	Paraventricular nucleus
PMA	Phorbol myristate acetate

PKC	Protein kinase C
RIA	Radioimmunoassay
SAD	Seasonal affective disorder
Ser	Serine
SNAT	Serotonin N-acetyltransferase
SA	Specific activity
SCG	Superior cervical ganglion
SCGx	Superior cervical ganglionectomy
SCN	Suprachiasmatic nucleus
Tau	Taurine
TBH	Threo-β-hydroxyaspartate
TPH	Tryptophan hydroxylase
TH	Tyrosine hydroxylase
VIP	Vasoactive intestinal peptide

CHAPTER I

INTRODUCTION

Biological rhythms are present throughout nature. Frequencies of these rhythms can range from several cycles per second to only a single cycle per year. Midway between these extremes are circadian (circa = around, dia = day) rhythms. These rhythms cycle over a 23 - 26 hour period and include the rhythms of sleep, locomotor activity, hormone secretion and body temperature. The time frame over which these rhythms repeats is genetically determined. They are maintained in the absence of external cues. Synchronization of these rhythms with the 24 hour light-dark cycle requires the input of an outside "zeitgeber" or "time giver". In mammals, the most powerful zeitgeber appears to be light. Information about changes in the light-dark cycle is conveyed directly from photoreceptors in the retina to an endogenous rhythm generator within the brain, the suprachiasmatic nucleus (SCN). From the SCN, photic information is processed into neuronal and hormonal signals that convey physiological information to the animal about the time of day or season.

The pineal gland, via its secretion of the hormone melatonin, is an important component of the neuroendocrine system regulating the rhythmicity of an organism. The reception of light by the retina inhibits the synthesis of melatonin, via a multisynaptic pathway that includes the SCN. Synthesized from the amino acid tryptophan, melatonin has been suggested to play a role in such diverse phenomena as the regulation of body

temperature (Morton 1987), thyroid gland function (Vriend et al. 1977), and pigmentation (Bagnara and Hadley 1970). One of the most extensively studied functions of the pineal gland is its interaction with the hypothalamic-pituitary-reproductive axis (Reiter 1980). More recently, several studies in humans have suggested possible roles for melatonin in chronobiological and psychobiological functions (Lowry et al. 1985; Vaughan 1984).

Neural regulation of melatonin synthesis has been well characterized. At the onset of darkness, norepinephrine (NE) is released from nerve terminals within the pineal gland and stimulates, via α- and β-adrenergic receptors on pinealocytes, the N-acetylation of serotonin (derived from the amino acid tryptophan). The product of this reaction, N-acetylserotonin, is methylated to form melatonin, which is then released into the general circulation.

The idea that melatonin synthesis can be modulated by neurotransmitters or circulating factors other than NE has recently gained attention. One potential modulator, the amino acid glutamate, is an important excitatory neurotransmitter within the central nervous system (Mayer and Westbrook 1987). The recent report that glutamate is the dominant excitatory neurotransmitter within the neuroendocrine hypothalamus also suggests the possibility that this amino acid may play an important role in other neuroendocrine systems (Van den Pol et al. 1990).

The present study is based on the observation that the pineal gland possesses high levels of free glutamate and a number of "orphan" receptors whose role in melatonin synthesis are unknown. The experiments in this study were designed to test the hypothesis that glutamate plays a neuromodulatory role in pineal gland function in the rat. Based on the fact

that the NE content of the pineal gland exhibits a distinct circadian rhythm (Craft et al. 1984), the first series of experiments examined amino acid content of the pineal gland over the 24 hour light-dark cycle. The effect of sympathetic denervation on amino acid content was also examined, as several pineal gland rhythms, e.g. serotonin and melatonin content, are known to be synchronized by the rhythmic release of NE from neurons of the SCG. While glutamate has been previously demonstrated to inhibit the NE-stimulated activation of the rate limiting enzyme in the synthesis of melatonin in the rat *in vitro* (Govitrapong and Ebadi 1988), a source of "transmitter" glutamate for the gland has not been demonstrated. To determine if glutamate was concentrated in nerve terminals, semiquantitative immunocytochemistry was employed.

The second series of experiments established the presence of [^{3}H]glutamate binding sites in the pineal gland of the rat. Not previously demonstrated in the pineal gland of this species, [^{3}H]glutamate binding site number, affinity, and subtype were assessed with the use of *in vitro* receptor autoradiography. The hypothesis that maintenance of glutamate binding site number and affinity is dependent on an intact sympathetic innervation was tested by examining [^{3}H]glutamate binding after superior cervical ganglionectomy (SCGx). [^{3}H]Glutamate binding was also examined in pineal glands obtained from animals sacrificed during the middle of the light and dark periods.

A third series of experiments tested the hypothesis that glutamate modulates pineal gland indole metabolism. The effect of glutamate and its analogues on the adrenergic-stimulated secretion of melatonin *in vitro* was examined.

CHAPTER II

REVIEW OF THE LITERATURE

The pineal organ is present in almost all vertebrate species. As demonstrated by the fossil record, the pineal organ is an ancient structure, believed to have been present in certain tetrapods of the Devonian and Silurian periods (Oksche 1965). During evolution, there are few organs in the body that have undergone as much change morphologically and functionally as the pineal organ. The pineal organ of lower vertebrates, e.g. lamprey, fish, and amphibians, consists of cone-like photoreceptors that synapse onto second order neurons, whose axons project to various brain areas (Collin and Oksche 1981). Pineal photoreceptors, like retinal photoreceptors, possess an outer and inner segment, cell soma, and synaptic terminal. Pineal photoreceptors possess many of the same cellular markers found in retinal photoreceptors. Molecules found in both retinal and pineal photoreceptors include opsin, a-transducin, S-antigen, and various photolabile pigments (Collin et al. 1986; Oksche and Hartwig 1979). Cellular organelles specific to the retinal photoreceptor such as the 9 X 2 + 0 cilium located in the outer segment and synaptic ribbons located in the synaptic pedicle are also present in pineal photoreceptors (McNulty 1984). In the retina, communication between photoreceptors and secondary neurons is mediated by excitatory amino acids released from the photoreceptor synaptic pedicle (Copenhagen and Jahr 1989). These amino acids bind to glutamate receptors of the 2-

amino-4-phosphonobutyric acid (APB) subtype located on secondary neurons (bipolar and horizontal cells) (Muller et al. 1988; Slaughter and Miller 1981). The presence of autoreceptors for excitatory amino acid on photoreceptors is also suggested by physiological studies that demonstrate the ability of glutamate to depolarize photoreceptors (Sarantis et al. 1988; Tachibana and Kaneko 1988).

In reptiles and birds, the typical photoreceptor becomes modified (Collin and Oksche 1981). The most obvious changes are seen in the outer segment where there is a decrease in the number of disks present and in the synaptic pedicle where there is a disconnection from, and disappearance of, second order neurons. While it is not clear whether all modified photoreceptors can detect light directly, information about the light/dark cycle can be relayed to the photoreceptor indirectly via pathways originating in the retina (Kappers 1965).

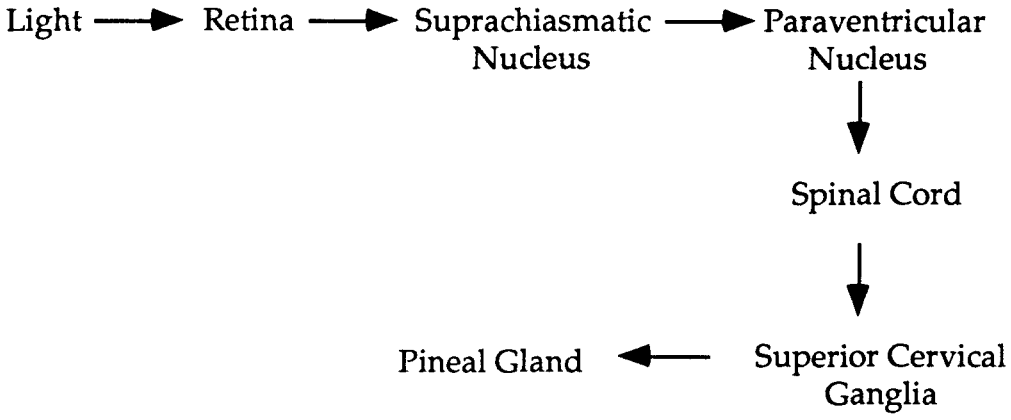
In mammals and snakes, the modified photoreceptor has been almost completely replaced by the secretory pinealocyte (Collin et al. 1986). The pineal organ in these species consists primarily of two cell types, glial cells and chief cells (pinealocytes). Chief cells, while retaining many of the cellular markers found in photoreceptors, e.g. opsin (Korf et al. 1985), S-antigen (Korf et al. 1985), and a-transducin (Van Veen et al. 1986), are mainly involved in indole metabolism (Reiter 1991). Cilia of the 9 X 2 + 0 variety (Karasek 1983) and synaptic ribbons (McNulty et al. 1987) are also present in the secretory pinealocyte. The pineal organ of mammals no longer possesses any direct photoreceptive capacity but receives information about changes in the light/dark cycle via a multisynaptic pathway originating in the retina (Moore 1978).

Peripheral Innervation of the Mammalian Pineal Gland

Information about changes in the light/dark cycle reaches the mammalian pineal gland via a neural pathway originating in the retina (Moore et al. 1964) (Fig. 1). Ganglion cells within the retina send unmyelinated fibers to the suprachiasmatic nuclei (SCN) of the hypothalamus via the optic nerve (Moore 1973). Most of the fibers in this tract, referred to as the retinohypothalamic tract, cross before terminating in the contralateral SCN (Moore and Lenn 1972). While it is known that lesioning the SCN results in the loss of the cyclic production of the pineal hormone melatonin (Moore and Klein 1974), the pathways that connect the SCN to the pineal gland have not been fully defined. One possible route may involve SCN projections to the paraventricular nucleus (PVN) of the hypothalamus (Silverman et al. 1981). Lesions of the PVN have been shown to disrupt the melatonin generating capacity of the pineal gland (Klein et al. 1983). While a direct projection from the PVN to the deep pineal (= pineal tissue present within the dorsal posterior diencephalon) has been reported (Reuss and Moller 1986), neurons in the PVN also have direct projections to the preganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord (Swanson and Kuypers 1980). Axons from these preganglionic neurons, which are located primarily in spinal cord levels T1 - T3 (Gilbey et al. 1982), leave the spinal cord via the ventral roots and travel to the superior cervical ganglion (SCG) where they synapse with postganglionic sympathetic neurons (Rando et al. 1981). Projections from the SCG enter into the brain by traveling with branches of the internal carotid artery (Hedger and Webber 1976). Fibers destined for the pineal gland pass through the tentorium cerebelli and reform as one or two nerves referred to as the nervi

conarii (Kappers 1964). These nerves enter the pineal gland at its apex and terminate in the pericapillary space (Kappers 1960) where they release the neurotransmitter NE.

Fig. 1



Central Innervation of the Pineal Gland in the Rat

While the sympathetic innervation of the rat pineal gland has been extensively studied, knowledge of pinealopetal projections is limited. The observation that all or most nerve terminals in the gland disappeared after removal of the SCG led earlier investigators to believe that the gland was innervated exclusively by the sympathetic nervous system (Kappers 1960). More recent anatomical and electrophysiological studies, however, have suggested that various brain regions project directly to the pineal gland via the pineal stalk. Injection of the neuronal tracer horseradish peroxidase (HRP) into the superficial portion of the pineal gland produced labeled fibers and neurons within the habenular complex (Dafny 1983), while lesioning of the medial habenular nuclei resulted in the appearance of degenerating fibers

and terminals within the pineal gland (Ronnekleiv and Moller 1979). When HRP was used as an anterograde tracer and injected into the PVN, labeled fibers were found in the deep pineal and the pineal stalk (Reuss and Moller 1986). Injection of the more sensitive anterograde tracer *Phaseolus leucoagglutinin* into the intergeniculate leaflett of the lateral geniculate nucleus (Mikkelsen and Moller 1990) or the anterior and tuberal regions of the hypothalamus (Fink-Jensen and Moller 1990) also produced labeled nerve fibers that terminated in the deep pineal and pineal stalk.

Further evidence for the existence of central pineal connections has been obtained from electrophysiological studies. In a study by Martin and Meissl (1990), extracellular potentials evoked by brief photic stimulation of the eyes were recorded from single cells in the pineal gland before and after removal of the SCG. Removal of the sympathetic innervation did not impede evoked extracellular potentials, suggesting that light information can reach the pineal gland via central connections. Evoked potentials were also recorded in the pineal gland after electrical stimulation of the habenula (Ronnekleiv et al. 1980).

Although there appears to be considerable evidence in support of a central innervation of the pineal gland, not all studies agree. Patrickson and Smith (1987) noted, for example, that after careful injection of HRP into the pineal gland labeled neurons could be found only within the SCG. It was noted by these authors that "the encapsulation and lack of rigid attachment of the pineal make it very difficult to penetrate" (Patrickson and Smith 1987). They found that even when the pineal gland was surgically exposed and carefully injected, tracer sometimes leaked from the site of entry. In cases where tracer was found to leak, neurons in various brain areas were found to

contain the HRP label (Patrickson and Smith 1987). A similar pattern of labeling was observed by Stanley et al. (1987) after pineal glands were injected with the neuronal tracers HRP, wheat germ agglutinin-HRP conjugate, fast blue, and rhodamine microspheres. Additionally, the observation that some sympathetic fibers from the SCG project into the medial habenular nuclei after passing through the pineal gland (Patrickson and Smith 1987) suggests that the degenerating nerve terminals found in the pineal gland after lesioning the habenular nuclei (Ronnekleiv and Moller 1979) may not be pinealopetal projections but simply retrogradely degenerating sympathetic fibers. Likewise, the evoked potentials noted in the pineal gland after stimulation of the habenula (Ronnekleiv et al. 1980) could be the result of antidromic conduction along sympathetic fibers.

Biochemistry of Melatonin Synthesis

The pineal hormone melatonin is synthesized from the amino acid tryptophan via a multienzymatic pathway. Tryptophan, found in low concentrations within the pineal gland, is actively taken up from the circulation and converted to 5-hydroxytryptophan by the mitochondrial enzyme tryptophan hydroxylase (TPH) (Fig. 2) (Klein et al. 1981; Sugden 1979). The activity of TPH is reported in rats to increase at night with peak activity occuring 7 hours after lights out (Sitaram and Lees 1978). 5-Hydroxytryptophan is subsequently oxidized to 5-hydroxytryptamine (5HT or serotonin) by the cytoplasmic enzyme L-amino acid decarboxylase (L-AAD) (Fig. 2). While the substrate specificity of this enzyme is low, the activity of L-AAD is reported to be higher in the pineal than in any other tissue examined

(Klein et al. 1981). Changes in L-AAD enzyme activity over the light-dark cycle have not been reported.

Relative to other brain areas the pineal gland possesses high concentrations of 5HT (King et al. 1984; Quay 1963). The levels of this indole vary over the light-dark cycle with concentrations being highest during the light period and lowest at night (Quay 1963; Snyder et al. 1967). If the light-dark cycle is reversed and the animal is allowed to acclimate, the production of 5HT will likewise be reversed (Snyder et al. 1967). Because these rhythms are endogenously generated, animals kept in constant darkness will have a persistent 5HT rhythm that will cycle approximately once every 24 hours. Animals kept in constant light, on the other hand, display no rhythm in 5HT production, demonstrating the inhibitory nature of light on this rhythm generator (Snyder et al. 1965).

In addition to its role as a melatonin precursor, 5HT can be processed via several different pathways within the pineal gland (Fig. 3). It can be released unchanged into the general circulation or CSF (Aloyo and Walker 1988). It can be methylated to form 5-methoxytryptamine, a putative pineal hormone (Pevet 1983). It can be converted by the mitochondrial enzyme monoamine oxidase (MAO) into the unstable 5-hydroxyindole acetaldehyde, which is subsequently converted to either 5-hydroxy indole acetic acid (5HIAA) by the enzyme aldehydehydrogenase or to 5-hydroxytrptophol by the enzyme alcohol dehydrogenase (Klein et al. 1981).

N-acetylation of 5HT by serotonin N-acetyltransferase (SNAT), the rate limiting enzyme in the synthesis of melatonin, produces N-acetylserotonin (NAS) (Fig. 2). While there are two known types of N-acetyltransferase enzymes within the pineal, the enzyme responsible for the production of

NAS is highly specific for arylalkylamines (aromatic compounds that possess a side chain with a primary ring) (Voisin et al. 1984). For this reason it is often referred to as arylalkylamine N-acetyltransferase, or more commonly, serotonin N-acetyltransferase (SNAT). A 24 hour rhythm in the activity of SNAT has been demonstrated with a 20-100 fold increase in enzyme activity during the night (Klein and Weller 1970). A full melatonin response can be achieved with only a 10% activation of SNAT (Wheler et al. 1979). The enzymatic conversion of 5HT to NAS is the rate limiting step in the production of melatonin.

The conversion of NAS to N-acetyl 5-methoxytryptamine (melatonin) is catalyzed by the cytosolic enzyme hydroxyindole O-methyltransferase (HIOMT) (Fig. 2). Found in high concentrations in the pineal gland, HIOMT makes up approximately 4% of the total soluble proteins (Jackson and Lovenberg 1971). Although one early study reported no day-night variations in HIOMT activity (Axelrod et al. 1965), this finding has not been substantiated by other studies. Melatonin is released from the pineal gland at night in a pulsatile fashion, with pulses occuring at the rate of 2 - 4 per hour in the rat (Pang and Yip 1988).

Fig. 2

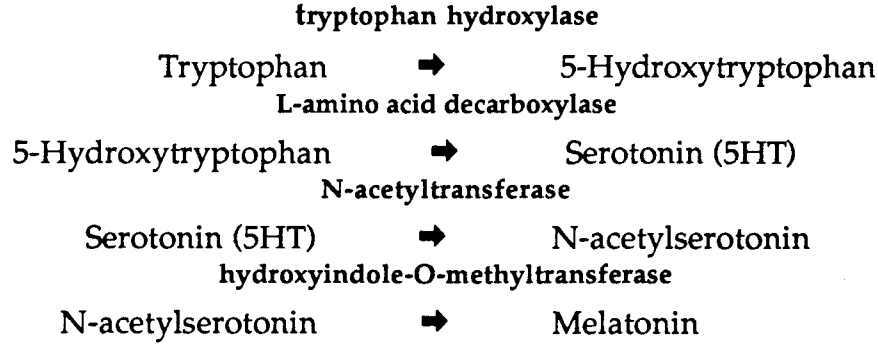
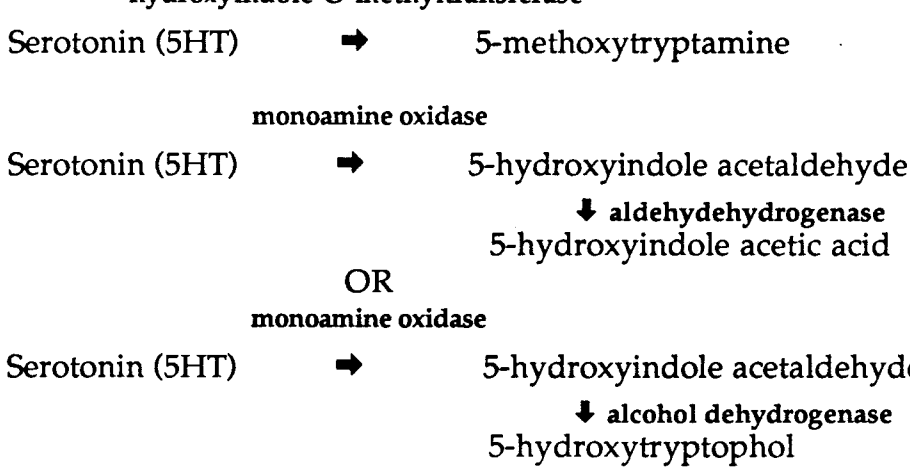


Fig. 3



Adrenergic Stimulation of Melatonin Production

Axelrod et al. (1969) first demonstrated the ability of NE to stimulate melatonin synthesis in the rat pineal gland *in vitro*. It was soon shown that the addition of the β-adrenergic antagonist, propranolol, to NE-stimulated glands prevented the expected increase in melatonin production. The addition of the α-adrenergic antagonist, phenoxybenzamine, did not inhibit melatonin production. It was assumed from these studies that NE exerted its effects exclusively via the β-adrenergic receptor (Wurtman et al. 1971). It was later shown, however, that activation of the α1-receptors by NE plays a role in potentiating the β-adrenergic induced response (Sugden et al. 1984).

β-Adrenergic Receptor Activation in the Rat Pineal Gland

β-Adrenergic receptors have been characterized on rat pinealocyte membranes using a variety of ligands including [^{3}H]alprenolol (Kebabian et al. 1975) and [^{3}H]dihydroalprenolol (Cantor et al. 1981). More recent studies,

which have used the β-adrenergic ligand, [^{125}I]iodocyanopindolol, have identified a receptor with an apparent affinity (K_d) = 170 ± 30 pM and a B_{max} = 548 ± 13 fmol/mg protein (Craft et al. 1985). The binding of NE to β-adrenergic receptors initiates the interaction of the receptor with a stimulatory guanine nucleotide protein (Gs) composed of three peptide chains, α, β, γ. The α subunit of the Gs molecule binds GTP and uncouples from the Gβ and Gγ chains to activate adenylate cyclase, a membrane bound enzyme responsible for the conversion of ATP to cyclic AMP (cAMP). Activation of the β-adrenergic receptor alone leads to approximately a 10 fold increase in the cAMP content in the gland (Vanecek et al. 1985).

While peak levels of cAMP are reached within 10 minutes after adrenergic stimulation and decline thereafter, levels remain elevated over baseline throughout the dark period (Klein et al. 1978). Similarly, the activity of phosphodiesterase, the enzyme responsible for degrading cAMP, is found to be high throughout the dark phase (Minneman and Iverson 1976). In the rat, SNAT activation with subsequent melatonin production occurs approximately 1 - 2 hours after β-adrenergic receptor stimulation (Klein et al. 1983; Simonneaux et al. 1989). This increase in SNAT activity can be blocked by the administration of actinomycin D (an agent that binds DNA) as well as cycloheximide (a peptidyltransferase inhibitor), suggesting that both transcription and translation are necessary for the activation of this enzyme (Romero et al. 1975). Activation of the pinealocyte β-adrenergic receptor also stimulates the production of cGMP 2 - 4 fold (Vanecek et al. 1985). The role of cGMP in pineal gland indole metabolism is unknown (Sugden 1989).

α-Adrenergic Receptor Activation in the Rat Pineal Gland

The ability of α-adrenergic agonists to mimic the NE-induced stimulation of phospholipid metabolism was the first suggestion that α-adrenergic receptors were present on the rat pineal gland. (Hauser et al. 1974). The receptor was later defined pharmacologically using the ligand [^{3}H]dihydroergocryptine (Vacas et al. 1980) and shown to activate phosphatidylinositol metabolism via a postsynaptic mechanism (Smith et al. 1979). α1-Adrenergic receptors have been subsequently characterized with the use of the specific ligand [^{125}I]-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]-tetralone ([^{125}I]HEAT) and found to possess a K_d = 48 ± pM and B_{max} = 341.6 ± 126.4 fmol/mg protein (Sugden and Klein 1985). An approximate 1:2 ratio of α to β receptors has been reported in the rat pineal gland (Dickinson et al. 1986).

Noradrenergic stimulation of the pineal gland leads to the activation of the membrane bound enzyme phospholipase C (Ho et al. 1988). Based on the observation that the NE-stimulated production of inositol monophosphates is inhibited by the addition of α1-adrenergic antagonists *in vitro*, it is postulated that the activity of this enzyme is controlled by the α1-adrenergic receptor. In many cell types, phospholipase C cleaves inositol-containing membrane phospholipids to produce the second messengers inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). IP3 production causes the release of Ca^{2+} from internal cellular stores while DAG stimulates the translocation of protein kinase C (PKC) from the cytosol to the cell membrane where it is activated. In the pineal gland little, if any, IP3 is formed in response to NE stimulation (Sugden et al. 1988; Sugden et al. 1987). Conversely, the activity of PKC in pinealocytes is high (Sugden 1991). While DAG is known to

activate PKC in many cell types, there is some question as to whether DAG activates PKC in the pinealocyte. It has been shown, for example, that maximal PKC activity can be detected in pinealocytes three minutes after stimulation without any prior or concurrent increase in DAG. The isoforms of PKC present in the pineal gland are of the α (80%) and β (20%) variety (Yoshida et al. 1988).

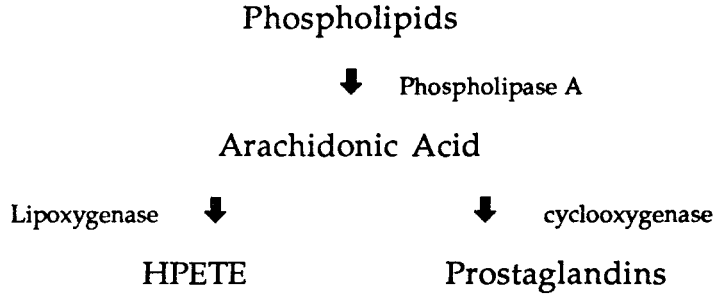
In addition to the stimulation of phospholipase C, activation of the α1-adrenergic receptor leads to an influx of Ca^{2+} into the pinealocyte (Sugden et al. 1987). Intracellular levels of Ca^{2+} increase from a basal level of 100nM to approximately 350 nM in one minute and remain high for about 30 minutes after stimulation (Sugden, et al. 1987). The inability of nifedipine (a Ca^{2+} channel blocker) to inhibit this increase suggests that Ca^{2+} is not entering the cells via the well know voltage-sensitive Ca^{2+} channel present on pinealocytes (Sakai and Marks 1972). While an increase in intracellular Ca^{2+} appears to be necessary (and perhaps solely responsible) for the translocation and activation of PKC (Ho et al. 1988) , the addition of Ca^{2+} ionophores by themselves are not sufficient to activate phospholipase C.

PKC has been reported to have a negative feedback effect on the activation of the α1-adrenergic receptor. A one minute pretreatment of pinealocytes with phorbol myristate acetate (PMA) (an activator of PKC) before the administration of NE reduced the α1-adrenergic induced increase in intracellular Ca^{2+} by 50 % (Sugden et al. 1988). Likewise, pretreatment with PMA inhibited the ability of NE to stimulate the production of inositol monophosphate (Sugden et al. 1988). These authors have suggested that activation of the α1-adrenergic receptor leads to an incease in intracellular Ca^{2+} which causes the translocation of PKC which inhibits the further

increase in intracellular Ca^{2+}. It is believed that PKC phosphorylates and desensitizes tha α1-adrenergic receptor to further stimulation (Leep-Lundberg et al. 1986).

Additionally, a role for arachidonic acid metabolites in pineal gland function has been suggested by the observation that lipoxygenase inhibitors decrease NE-stimulated melatonin production while the products of this enzyme, 12- and 15-hydroxyperoxy-6,8,11,14-eicosatetraenoic acid (HPETE), increase melatonin production (See Fig. 4) (Sakai et al. 1988). Based on the

Fig. 4



observation that activation of the pinealocyte α1-adrenergic receptor, as well as administration of Ca^{2+} ionophores and phorbol esters to pinealocytes stimulates the release of arachidonic acid, it has been suggested that the α1-adrenergic receptor mediates arachidonic acid metabolism. It has also been shown that activation of the α1-adrenergic receptor or direct activation of PKC by phorbol esters causes an increase in intracellular pH (Ho et al. 1989).

α-Adrenergic Potentiation of β-Adrenergic Stimulated cAMP and cGMP Production

Stimulation of the α1-adrenergic receptor by itself does not increase cyclic nucleotide production (Vanecek et al. 1985). Activation of this receptor does, however, potentiate the β-adrenergic induced production of cAMP and cGMP in pinealocytes 60 to 100 fold (Sugden 1990; Vanecek et al. 1985). Based on the observation that synthetic activators of PKC, e.g. phorbol 12-myristate, 13-acetate (PMA) and oleoyl acetyl glycerol (OAG), mimic the ability of α1-adrenergic agonists to potentiate the β-adrenergic induced response, PKC has been suggested to mediate this process *in vivo*. Likewise, if intracellular calcium is increased in pinealocytes by the use of Ca^{2+} ionophores or depolarizing concentrations of K^+ (treatments known to activate PKC), β-adrenergic induced cAMP and cGMP production is enhanced. The fact that α1-adrenergic receptor activation can potentiate cAMP and cGMP production stimulated by either cholera toxin or forskolin suggests that PKC exerts its effects either via the Gs protein and/or adenylate cyclase and probably not via the β-adrenoceptor. Activation of the α1-adrenergic receptor does not appear to potentiate the β-adrenergic induced increase in cAMP and cGMP by inhibiting the enzyme phosphodiesterase because α-adrenergic potentiation of the β-adrenergic induced response is still noted when high concentrations of phosphodiesterase inhibitors, e.g. IBMX, are added to pinealocyte cultures (Sugden and Klein 1988).

The α1-adrenergic potentiation of β-adrenergic induced cGMP production appears to differ somewhat from that of cAMP. While an increase in intracellular calcium potentiates β-adrenergic induced cGMP formation, activation of PKC alone had no effect (Sugden et al. 1985). Activators of PKC

can, however, potentiate the β-adrenergic induced cGMP production in a dose dependent manner if intracellular Ca^{2+} is simultaneously increased. The exact role of Ca^{2+} in cGMP potentiation is not clear.

Differential Regulation of cAMP by PKC Isozymes

There are several isoforms of the enzyme PKC. The α, βI, βII, and γ forms are usually present in large quantities within cells while the δ, ε, and ζ forms are found in smaller amounts (Nishizuka 1988). Functional differences between these isozymes have only recently been discovered when it was determined that two of the PKC subspecies differentially affect cAMP production (Gusovsky and Gutkind 1991). In cells that only possess the α PKC isozyme, e.g. NIH 3T3 cells, activation of PKC leads to an inhibition in forskolin-stimulated cAMP production. Activation of PKC in cells that possess both the α and γ isozymes, e.g. PC12 cells, results in a potentiation of cAMP generation (Gusovsky and Gutkind 1991). While only the α and β isoforms of PKC have been identified in the pineal gland (Yoshida et al. 1988), the exact isozyme involved in potentiating the β-adrenergic induced cAMP and cGMP response has not been determined. In addition to the ability of PKC to potentiate cAMP generating systems, it remains a possibility that cAMP levels may be inhibited by receptors that activate the α PKC isozyme. In the pineal gland, 80% of the PKC present is of the α variety (Yoshida et al. 1988).

Functional Implications of the α-Adrenergic Potentiation of the β-Adrenergic Induced Response

The exaggerated cAMP response initially caused by activation of both α- and β- adrenergic receptors by NE, or by PE-potentiation of cholera toxin stimulated cAMP production is of limited duration (Sugden et al. 1988; Sugden and Klein 1988). In both systems, cAMP levels declined to non-potentiated (equivelent to β-adrenergic induction alone) levels by 60 minutes. Activation of the α1-adrenergic receptor quickly leads to its desensitization (Sugden et al. 1988). The physiological role of this large initial increase in cAMP may determine the extent of SNAT activity while the lower steady state levels of cAMP that follow function to maintain SNAT activity. It is known, for example, that the addition of β-adrenergic antagonists to pinealocytes *in vitro* several hours after SNAT induction leads to a rapid decline in SNAT activity (Deguchi and Axelrod 1972).

The potentiation in cAMP and cGMP levels seen after both α- and β-adrenergic receptor activation are paralleled by a two fold potentiation in SNAT activity (Klein et al. 1983). Whether an amplification in melatonin production also occurs remains controversial. In hamster pineal glands cultured *in vitro,* addition of PE potentiated the isoproterenol (Iso) -induced increase in melatonin production in the gland, while no potentiation in the amount of melatonin secretion into the media was noted (Santana et al. 1989). In an *in vivo* experiment in which hamsters were injected with prazosin, an α-adrenergic antagonist, during the middle of the dark phase, an inhibition of pineal and serum melatonin was noted one hour after injection, while later time points (2 and 3 hours post-injection) were not affected (Stankov et al. 1990). In ovine pinealocytes cultured *in vitro,* PE potentiated

the Iso-induced production of melatonin within cells without any potentiation in the cAMP response (Howell and Morgan 1991).

It appears that little PKC is necessary in pinealocytes to potentiate the β-adrenergic response. After downregulating PKC by 91 - 97%, β-adrenergic induced cAMP was inhibited only 45-55%, while SNAT activity was not inhibited at all (Sugden 1991). This suggests that not all of the cAMP produced by the activation of both α- and β-adrenergic receptors is necessary for the full induction of SNAT. The author of this study (Sugden 1991) concluded by stating "...the physiological role of the α1-adrenergic potentiation mechanism in regulating SNAT induction, and hence melatonin synthesis, *in vivo* is uncertain".

Other Receptor/Binding Sites Present in the Rat Pineal Gland

In addition to the well characterized adrenergic receptors, the pineal gland possesses many other receptor types whose role in indole metabolism is not known (Table 1). Many of these compounds appear to play a neuromodulatory role, i.e. they have no effect on their own but modulate adrenergic-induced SNAT activity and subsequent melatonin production.

Table 1. - Receptor/Binding Sites in the Pineal Gland

Monoamines

Receptor/ binding site [*]ligand	Affinity (K_d) nM	Receptor number (B_{max})	Effect of SCGx	Function	Ref.
β-adrenergic [^{125}I]iodo-cyanopindolol	0.17	548 fmol /mg protein	increase B_{max}	Stim. cAMP & cGMP; increase SNAT activity & melatonin prod.	1
α_1-adrenergic [^{125}I]HEAT	0.05	342 fmol /mg protein	increase B_{max}	Stim. PI hydrolysis & Ca^{2+} influx; increase PKC activity	2
α_{2D}-adrenergic [^{3}H]rauwolscine	1.40	71 fmol /mg protein	Not determined	Inhibits SNAT activity	3
D1-Dopaminergic [^{3}H]SCH 23390	0.56	974 fmol /mg protein	Not determined	Unknown	4
D2-Dopaminergic [^{3}H]spiroperidol	0.18 & 2.10	37 & 630 fmol/ mg protein	Not determined	attenuates NE-stimulated SNAT activity	5
$5HT_2$ [^{3}H]Spiperone	1.26	193 fmol/mg protein	Not determined	Unknown	6

1) Craft et al. 1985 (rat)

2) Sugden and Klein 1984 (rat)

3) Simmonneaux et al. 1991 (bovine); Alphs et al. 1984 (rat/function)

4) Simmonneaux et al. 1990 (bovine)

5) Govitrapong et al. 1984 (bovine); Govitrapong et al. 1989 (rat/function)

6) Govitrapong et al. 1991 (bovine)

Table 1 - Continued

Amino acids

Receptor/ binding site [*]ligand	Affinity (K_d) nM	Receptor number (B_{max})	Effect of SCGx	Function	Ref.
Glutamate [^{3}H]Glutamate	534	4840 fmol /mg protein	Not determined	Inhibits NE-stim. SNAT activity	7
GABA [^{3}H]GABA	16.5 & 567	121 & 2470 fmol/mg protein	Not determined	Inhibits melatonin prod.	8

Benzodiazepines

Peripheral-type Benzodiazepine [^{3}H]Ro 5-4864	2.46	24000 fmol/mg protein	Decrease B_{max}	Enhances Iso-stim. mel. production	9
Peripheral-type Benzodiazepine [^{3}H]PK 11195	4.40	2040 fmol/mg protein	Not determined	Unknown	10
Central-type Benzodiazepine [^{3}H]flunit-razepam	2.80	124 fmol/mg protein	Not determined	Enhances Iso-stim. mel. production	11

7) Govitrapong et al. 1986 (bovine); Govitrapong et al. 1988 (rat/function)

8) Ebadi and Chan 1980 (bovine); Rosenstein et al. 1989 (rat/function)

9) Weissman et al. 1984 (rat); Winters et al. 1991 (rat/function)

10) Suranyi-Cadotte et al. 1987 (human)

11) Suranyi-Cadotte et al. 1987 (human); Winters et al. 1991 (rat/function)

Table 1 - Continued

Peptides

Receptor/ binding site [*]ligand	Affinity (K_d) nM	Receptor number (B_{max})	Effect of SCGx	Function	Ref.
VIP [^{125}I]VIP	Not determined[1]	Not determined[1]	Not determined	Stim. SNAT act. /melatonin production	12
Arginine-vasopressin $_{4-9}$ [^{35}S]VP $_{4-9}$	Not determined[1]	Not determined[1]	Not determined	Enhances NE-stim SNAT activity	13
Neuropeptide Y [^{125}I]NPY	1.00	40 fmol/mg protein	Not determined	See note[2]	14

[1]Note: Autoradiographic visualization of [^{125}I]VIP and [^{35}S]VP$_{4-9}$ binding in whole brain demonstrated the presence of a high concentration of VIP and VP4-9 binding sites in the pineal gland *relative* to other brain areas. K_d and B_{max} values were determined for whole sections and did not differentiate between brain and pineal gland.

[2]Note: The role of NPY in pineal gland function is controversial. Olcese (1991) reported that NPY is a potent inhibitor of NE-stimulated melatonin secretion *in vitro* (IC_{50} 0.4 nM) while Vacas et al. (1987) reported that NPY (10nM) enhances NE-stimulated melatonin production *in vitro.*

12) Besson et al. 1986 (rat); Kaneko et al. 1980 (rat/function); Simonneaux et al. 1990 (rat/function)

13) De Kloet et al. 1985 (rat); Stehle et al. 1991 (rat)

14) Olcese 1991 (rat); Vacas et al. 1987 (rat/function)

Table 1- Continued

Other

Receptor/ binding site [*]ligand	Affinity (K_d) nM	Receptor number (B_{max})	Effect of SCGx	Function	Ref.
Estrogen nuclear complex [^{3}H]estradiol	0.42	1.6 fmol /mg tissue	Decrease B_{max}	Unknown	15
Sigma opiate [^{3}H]1,3-di-ortho-totyl-guanidine	Not deter-mined[1]	Not deter-mined[1]	Not determined	Unknown	16
Muscarinic-Cholinergic [^{3}H]QNB	0.01	32 fmol /mg protein	No change	Stim. PI hydrolysis	17
A2-Adenosine [^{3}H]NECA	51	1060 fmol/mg protein	Not determined	Enhances NE-stimulated mel. prod.	18

[1]NOTE: Autoradiographic visualization of [^{3}H]DTG binding in whole brain demonstrated the presence of a high concentration of σ binding sites in the pineal gland *relative* to other brain areas. K_d and B_{max} were not determined.

15) Cardinali 1977 (rat)

16) Jansen et al. 1990 (rat)

17) Taylor et al. 1980 (ovine and rat); Laitinen et al. 1989 (rat-function)

18) Sarda et al. 1989 (rat); Vasas et al. 1989 (rat-function)

Melatonin - Its Role *In Vivo*

Over the course of a year, animals in their natural habitat are subjected to changes in day length. In seasonally reproductive animals, e.g sheep and hamster, the length of the photoperiod that an animal is exposed to per day determines reproductive success. This is most clearly seen in the hamster where a minimum of 12.5 hours of light is required to maintain normal testicular size (Elliott 1976). If the animal is exposed to anything less than 12.5 hours of light, the testes will begin to regress and spermatogenesis is inhibited (Elliott 1976). This inhibitory effect of shortened photoperiod can be reversed by removal of the pineal gland (Reiter 1972). The fact that testicular atrophy can be induced in pinealectomized hamsters by a daily regimen of melatonin administration (3 injections of 25 ug at three hour intervals), suggests that melatonin is responsible for this inhibition *in vivo* (Tamarkin et al. 1977). It should be noted, however, that melatonin's role in reproductive function is not always inhibitory. In sheep, exposure to short days stimulates reproductive activity (Wayne et al. 1990). Mating in this species occurs in the fall resulting in lambs that are born in the spring. Administration of melatonin to female sheep in the summer has been reported to advance the onset of cyclical ovarian activity (Nowak et al. 1990).

The site of action of melatonin within the central nervous system is not known. Receptors for melatonin have only recently been characterized and appear to be present in the pars tuberalis and SCN regions of the brain (Reppert et al. 1988; Williams and Morgan 1988). It has been postulated that melatonin acts via the SCN to alter the release of gonadotropin releasing hormone (GnRH) and thereby alters reproductive function.

The human pineal gland, like the pineal gland of all other animals, has elevated levels of melatonin secretion at night (Lynch et al. 1978). The function of this hormone in humans is not clear however. Administration of exogenous melatonin (1mg/kg) to human subjects during the day has been reported to induce subjective sleepiness (Lieberman et al. 1984). A role for melatonin in seasonal affective disorder (SAD), a form of clinical depression noted during the winter months when daylength is shorter, has been postulated (Thompson et al. 1990). Symptoms of this disorder include a greater need for sleep, weight gain, and a craving for carbohydrates. Therapy for this disorder includes exposing the patient to high intensity light (Oren et al. 1991). Melatonin levels abruptly drop in humans subjected to bright light during the night when melatonin levels are normally high (Thompson et al. 1990).

Excitatory Amino Acids / Pharmacology

Within the human brain, there are an estimated 10^{12} - 10^{14} neuronal synapses responsible for the transduction of chemical information between cells (Purves and Lichtman 1985). Inhibition at these synapses are mediated by a number of well established neurotransmitters including dopamine, GABA, norepinephrine, and serotonin. By contrast, most excitatory synaptic transmission appears to be mediated by a single compound or family of compounds chemically related to the amino acid L-glutamate. The excitatory properties of glutamate were first demonstrated in 1954 when it was reported that the injection of L-glutamate or L-aspartate directly into the brain or carotid artery of laboratory animals resulted in seizures (Hayashi 1954). The earlier observation that tissue glutamate levels were highest in the brain had

already suggested an important role for glutamate in neuronal function (Waelsch 1951). A known intermediate in the tricarboxylic acid cycle, glutamate was believed to be linked to the high metabolic demands of the tissue. Later studies, noting the chemical similarities between this amino acid and the known neuroinhibitor GABA, suggested that glutamate had, in addition to its metabolic role, a neuroactive role. Early reports describing an inhibitory role for glutamate noted its ability to initiate spreading depression within the brain (Purpura et al. 1959; Van Harreveld 1959). However, these studies were not substantiated and evidence accumulated for glutamate's role as an excitatory agent. It had been demonstrated, for example, with the use of intracellular recording techniques that both L-glutamate and L-aspartate exerted a depolarizing effect on spinal cord neurons (Curtis et al. 1959). Unlike other known neurotransmitters, however, glutamate was found to stimulate neurons in nearly all areas of the central nervous system tested, including brain stem neurons (Curtis and Koizumi 1961), cerebral cortical neurons (Phillis and Krnjevie 1961) and thalamic neurons (Curtis and Davis 1962). To earlier investigators, this non-specific nature of L-glutamate and the fact that high concentrations (10^{-3} to 10^{-4} M) were needed to evoke a response made its role as a neurotransmitter seem unlikely. In addition, no specific enzymatic system capable of terminating glutamate's stimulatory action appeared to be present, suggesting that if a separate excitatory system did exist it was not glutamate, but a related compound that acted as the endogenous transmitter. Extensive structure-activity studies were undertaken in an attempt to determine the functional groups responsible for glutamate's stimulatory effect. These studies resulted in the discovery of several new "excitants" including N-methyl-D-aspartate (NMDA), L-cysteic acid, D-

homocysteic acid, and D,L-aminoadipic (Watkins 1962). For many years the nature of glutamate's excitatory action within the CNS remained undefined. In addition to the obvious differences between glutamate and other known neurotransmitters, inhibition of glutamate's stimulatory effect had not been demonstrated with the use of specific receptor antagonists. Early reports of regional differences (spinal cord and cerebral cortex) in the rank order potencies of various glutamate analogues were the first suggestion of a more specific nature of action for this amino acid (Curtis and Watkins 1960; Krnjevic and Phillis 1963), but the data were not conclusive. One of the first definitive reports of the specific nature of glutamate's stimulatory action came several years later when it was demonstrated in the cat spinal cord that ventral horn neurons were significantly more responsive to L-aspartate than L-glutamate, while dorsal horn neurons preferentially responded to L-glutamate (Duggan 1974). In another study that same year, NMDA and kainic acid were proposed to act at different receptor sites; NMDA appearing to act at an "aspartate preferring" site and kainic acid acting at a "glutamate preferring" site (McCulloch et al. 1974). Relatively selective antagonism of L-glutamate and L-aspartate responses had already been obtained with the compounds L-glutamic acid diethyl ester (GDEE) and 3-amino-1-hydrxpyrrolidone (HAP), respectively (Haldemann and McLennan 1972) further supporting the idea of receptor subtypes. In addition, membrane binding studies utilizing glutamate demonstrated for the first time that glutamate binding was saturable, of reasonably high affinity, and inhibited by other neuroexcitatory amino acids (Michaelis et al. 1974; Roberts 1974)

NMDA Receptor

It was becoming increasingly evident that glutamate's actions in brain were mediated by at least two distinct receptor subtypes. The first unequivocal report of receptor differentiation was the demonstration by Evans et al. that NMDA responses could be selectively inhibited by the divalent cation Mg^{2+}. Responses to quisqualate and kainate were unaffected by concentrations of Mg^{2+} as high as 20mM (Evans et al. 1977). It was also noted at this time that D-alpha-aminoadipate (DAA), a compound previously shown to have weak excitatory action in its racemic form, selectively inhibited responses to NMDA (Biscoe et al. 1977). It was later discovered that substitution of the carboxy group of DAA with a phosphonate group produced an antagonist, D(-)-2-amino-5-phosphonopentanoate (AP5), that was much more selective and potent than DAA (Davies et al. 1981). This antagonist and several other related antagonists were soon superceeded in potency by heterocyclic derivatives of AP5 which included the compound 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), an antagonist approximately 5 times more potent than AP5 (Harris et al. 1986) More recently another heterocyclic derivative of AP5, CGS-19755 was found to be an even more potent antagonist at the NMDA site (Lehmann et al. 1988).

NMDA Receptor - Ionophore Complex

In addition to the transmitter recognition site which binds compounds such as NMDA, DAA, AP5, CPP, and CGS-19755, several other sites are present within the receptor -ionophore complex. These include (a) a Mg^{2+} binding site (Mayer et al. 1984; Nowak et al. 1984); (b) an allosteric regulatory site that binds glycine (Johnson and Ascher 1987; Kleckner and Dingledine

1988); (c) a phencyclidine binding site (Anis et al. 1983; Coan and Collingridge 1987; Wong et al. 1986); (d) a Zn^{2+} binding site (Peters et al. 1987; Westbrook and Mayer 1987); and (e) a polyamine binding site (Ransom and Stec 1988).

Non-NMDA Receptors: AMPA and Kainate

Often referred to as the non-NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors have not been clearly distinguished from one another on the basis of selective antagonist binding. It was the observation of differences in rank order potencies when receptors were labeled with either [^{3}H] kainate (domoate> kainate> quisqualate>> L-glutamate) (Monaghan and Cotman 1982) or [^{3}H] AMPA (quisqualate> AMPA L-glutamate> kainate) (Honore et al. 1982) that first supported the idea of separate receptors. The most convincing evidence for separate kainate and AMPA receptors came from studies in which the anatomical localization of [^{3}H] kainate and [^{3}H] AMPA binding within the brain was examined. It was demonstrated that kainate binding is concentrated in the hippocampal CA3 stratum lucidum, deep cerebral cortical layers, striatum and granule cell layer of the cerebellum (Monaghan and Cotman 1982; Unnerstall and Wamsley 1983) whereas [^{3}H] AMPA binding sites are more numerous in the CA1 stratum radiatum, outer cortical layers, lateral septum, and molecular layer of the cerebellum (Monaghan et al. 1984; Olsen et al. 1987). The recent development of the antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) has provided specific inhibition of a kainate/quisqualate response but could not distinguish between the two receptor subtypes (Honore et al. 1988). Another recently developed antagonist, 2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionic

acid (AMOA), has been reported to selectively inhibit kainate responses without significantly decreasing AMPA-induced responses (Frandsen et al. 1990).

APB Receptors

In the early 1980's the antagonistic properties of APB had been reported at several glutamate using synapses (Collins 1982; Davies and Watkins 1982; Lanthorn et al. 1984). APB did not, however, appear to antagonize the effects of NMDA, quisqualate, kainate, and L-glutamate if applied simultaneously, suggesting that APB was acting at a fourth receptor subtype (Evans et al. 1982). Recent evidence suggests a presynaptic site of action for APB (Forsythe and Clements 1988).

Glutamate Metabotropic/ Trans-ACPD Receptor

Glutamate has been shown to stimulate the production of inositol phosphate in various neuronal preparations (Nicoletti et al. 1986; Sladeczek et al. 1985). When the pharmacological properties of the receptor mediating this effect were examined, it was noted that L-glutamate, quisqualate, ibotenate, and 1-amino-cyclopentyl-1,3-dicarboxylate (ACPD) stimulated inositol phosphate production while NMDA, AMPA and kainate did not (Monaghan et al. 1989; Nicoletti et al. 1986). This stimulation of inositol phosphate production could not, however, be blocked by known quisqualate/kainate or NMDA antgonists, indicating that this receptor differed from the known glutamate receptors (Schoepp and Johnson 1988). ACPD, the most selective agonist at this receptor, appears to be linked to a pertussis toxin-sensitive G protein that mediates the production of inositol phosphates. This receptor

appears to be important during normal neuronal development and synaptic stabilization as demonstrated by the fact that receptor efficacy decreased after synaptogenesis and reappeared following deafferentation (Schoepp et al. 1990).

Molecular Biology of Glutamate Receptors - Are There More than Five Receptor Subtypes?

Glutamate-evoked responses at neuronal synapses are mediated by the opening of ion channels (ionotropic receptors) and/or the stimulation of second messenger systems (metabotropic/ACPD receptors). Glutamate receptor subtypes linked to ion channels include the AMPA, kainate, NMDA and APB receptors. The AMPA and kainate receptors have been demonstrated to gate ion channels that permit the influx of monovalent cations such as Na^+ and K^+, while the NMDA receptor complex is believed to modulate a voltage-sensitive Ca^{2+} channel (Watkins et al. 1990). The recent cloning of the genes for several of the glutamate receptor subtypes suggests a more complicated arrangement. It is now known that there are at least 4 subtypes of AMPA receptor, refered to as GluR-A GluR-B, GluR-C, GluR-D (or GluR-1, -2, -3, -4) (Hollmann et al. 1989; Keinanen et al. 1990). In addition, the cDNA nucleotide sequence of each receptor subtype possesses 115 base pairs that are found to exist in 1 of 2 sequence versions. These two versions are refered to as "flip" and "flop" (Sommer et al. 1990). This increases the number of molecular species to 8. Each receptor subtype demonstrates a unique mRNA expression pattern in the brain (Keinanen et al. 1990).

While these receptors display typical AMPA pharmacology (Keinanen et al. 1990), the expression of two of the subtypes, GluR-A or GluR-C, produces ion channels that are highly permeable to Ca^{2+} (not an AMPA-like

characteristic) (Hollmann et al. 1991). If GluR-B is expressed along with GluR-A or GluR-C an ion channel that is impermeable to Ca^{2+} is formed (Hollmann, et al. 1991). This led Richard Miller to state that "...if the potential number of kainate /AMPA receptors available from combinations of multiple gene products and splice varients are considered, the resulting diversity is truly mind boggling... although it could be said that all these receptors will be activated by excitatory amino acids, wide variations in their pharmacology and biophysical properties should be expected" (Miller 1991).

While each receptor subtypes forms a functional ion channel when expressed alone, the composition of native receptors is not known. Three subtypes of kainate receptor have also been cloned, GluR-E (GluR-5) (Bettler et al. 1990), GluR-F (GluR-6) (Egebjerg et al. 1991) and KA1 (Werner et al. 1991), Like the AMPA receptor, each receptor subtype possesses a unique anatomical distribution. KA1, while binding [^{3}H]kainate, does not appear to be functional in electrophysiological studies. The metabotropic receptor has also been cloned (Masu et al. 1991), and may exist in multiple forms (Henley and Johnston 1991). The glutamate binding site of the NMDA receptor complex has been the most recent glutamate receptor cloned (Kumar et al. 1991).

Glutamate Uptake Sites

After it is released from nerve terminals glutamate is removed from the extracellular space by uptake into neurons and glia. Distinguished by their ion dependencies, two distinct systems for the uptake of glutamate exist. The first site to be described, a Na^+-dependent uptake site, displayed high

affinity for the glutamate analogues D-aspartate, L-aspartate, threo-β-hydroxyaspartate (TBH), and L-cysteine sulfinic acid . Early binding studies reported K_d values ranging from 2400 - 4000 nM and B_{max} values ranging from 75000 - 336000 fmol/mg protein (Foster and Fagg 1984). A more recent autoradiographic study utilizing D-[^{3}H]aspartate has characterized a Na^+-dependent binding site with a K_d = 577 nM and a B_{max} = 3710 fmol/mg protein (Anderson et al. 1990). Anatomical heterogeneity in L- and D-TBH inhibition of D-[^{3}H]aspartate binding has suggested that two distinct population of Na^+-dependent binding sites may exist (Anderson and Vickroy 1990). The process of glutamate uptake into the cell is electrogenic. When a single glutamate anion is transported into a cell, it is accompanied by the simultaneous uptake of three Na^+ ions while one K^+ ion is transported out of the cell (Barbour et al. 1988; Brew and Attwell 1987). Because one positive charge enters the cell for every glutamate taken up by the carrier, an inward current is generated. The second class of uptake sites, the Cl^--dependent sites, are not as numerous as the Na^+-dependent sites. Saturation analysis of [^{3}H]glutamate binding in the presence of Cl^- yield a binding site with a K_d = 315 nM and B_{max} = 656 fmol/mg protein (Anderson et al. 1990). Binding at this site is most potently inhibited by L-glutamate, L-α-amino adipic acid, quisqualate, and L-serine-*o* -sulfate.

In addition to the Na^+ and Cl^--dependent glutamate uptake sites present on the plasma membrane, uptake sites for glutamate are present on synaptic vesicles. Uptake at these sites is Na^+-independent but potentiated by the presence of Cl^-. These binding sites have an affinity for glutamate in the millimolar range (K_m = 2mM) and do not transport aspartate or other glutamate analogues (Carlson et al. 1989).

Glutamate Induced Neurotoxicity

Excitotoxicity, now a well known property of glutamate, was first reported by Lucas and Newhouse (1957), when it was demonstrated that large amounts of glutamate injected subcutaneously in mice caused extensive retinal damage. The process of L-glutamate induced cell death is believed to be mediated by several different mechanisms initiated by the binding of glutamate to the NMDA, kainate, and possibly AMPA receptors. One mechanism for this unique type of neurotoxicity, which damages only neuronal cell bodies and not axons of passage, is believed to be an excess influx of Ca^{2+} into cells that results in the stimulation of various Ca^{2+} dependent proteases (Choi 1987). Others have suggested that a passive influx of Cl^- ions may be responsible for the associated cell swelling and death (Olney et al. 1986; Rothman 1985). Still more recently Coyle and collegues have suggested that glutamate induced neurotoxicity may be mediated by oxidative stress (Murphy et al. 1989). Excitatory amino acids have been suggested to play a role in such neurodegenerative diseases as Huntington's chorea (McGeer and McGeer 1976), epilepsy (Sloviter 1983), lathyrism (Spencer et al. 1986), and Alzheimer's disease (Greenamyre and Young 1989). Attenuation of glutamate induced neuronal damage has been demonstrated with the use of both competitive and non-competitive glutamate antagonists (Boast et al. 1988; Woodruff et al. 1987).

Excitatory Amino Acid Modulation of Noradrenergic/ Pineal Function

A novel function for excitatory amino acids was first suggested by Baudry and collegues who noted that glutamate and its analogues inhibited carbachol and histamine induced [^{3}H]inositol phosphate accumulation in the

hippocampus (Baudry et al. 1986). A similiar observation was made in prefrontal cortex (Noble et al. 1989). NE-induced inositol phosphate production did not appear to be inhibited by glutamate. It was noted by others, however, that if the assay was modified and glutamate was added prior to the incorporation of [^{3}H]inositol into the larger phospholipid molecule, glutamate could the NE-stimulated synthesis of [^{3}H]IP$_3$ (Jope and Li 1989). How glutamate inhibits this norepinephrine induced synthesis is not known, but a reduction in the number of α1-adrenergic receptors, known to mediate the NE-induced IP$_3$ production, does not appear responsible for this decrease (Nicoletti et al. 1986). In agreement with the observation that glutamate decreases NE-induced phosphoinositide hydrolysis, it has been demonstrated *in vitro* that SNAT activity can be decreased by the addition of glutamate to NE-stimulated pineal glands (Govitrapong and Ebadi 1988). The recent observation that photoreceptors (from which mammalian pinealocytes evolved) release glutamate upon stimulation suggests a possible feedback mechanism that can be applied to the pineal gland (Copenhagen and Jahr 1989).

This dissertation, which hypothesizes a neuromodulatory role for glutamate in pineal gland function, examines several aspects of glutamate - pineal gland interaction. In the first series of experiments, the amino acid content of the pineal gland over the 24 hour light-dark cycle is examined. The effect of sympathetic denervation on amino acid content within the gland was assessed to determine if maintenance of amino acid content was dependent on an intact innervation. Anatomical localization of glutamate within the pineal gland was determined with the use of semiquantitative immunocytochemistry.

In the second series of experiments the presence of a binding site for [^{3}H]glutamate in pineal gland was ascertained with the use of *in vitro* receptor autoradiography. To determine if binding sites were located on either sympathetic terminals or other pineal gland cell types, [^{3}H]glutamate binding was also examined in sympathetically denervated pineal glands. Day -night differences in [^{3}H]glutamate binding site number was determined by examining [^{3}H]glutamate binding in pineal glands obtained from animals sacrificed during the middle of the light and dark periods.

The third series of experiments examines the effect of glutamate and various glutamate agonists on the adrenergic-stimulated secretion of the pineal gland hormone melatonin *in vitro*.

CHAPTER III

PINEAL GLAND AMINO ACIDS IN THE RAT I. IMMUNOCYTOCHEMISTRY AND CIRCADIAN BIOCHEMICAL ANALYSIS AFTER SUPERIOR CERVICAL GANGLIONECTOMY

Abstract

Semiquantitative immunocytochemistry was employed to determine the anatomical distribution of glutamate- and glutamine-like immunoreactivity within the pineal gland of the rat. The greatest amount of glutamate- and glutamine-like immunoreactivity was found over pinealocytes and glia respectively. At the subcellular level, glutamate-like immunoreactivity was highest over pinealocyte mitochondria and lowest over lipid inclusions. Pineal levels of glutamate and glutamine as measured by high performance liquid chromatography (HPLC), did not vary over a light-dark cycle. Superior cervical sympathetic denervation, which abolishes pineal gland melatonin synthesis, resulted in a nearly 50% reduction in pineal glutamate levels, but had no effect on levels of glutamine and taurine. Other amino acids (alanine, arginine, aspartate, serine) were reduced between 16-36% following sympathectomy.

Introduction

The mammalian pineal gland, a transducer of photoperiodic information, synthesizes and secretes the hormone melatonin in highest quantities at night (Klein and Weller 1970). The rate-limiting enzyme in the synthesis of melatonin, SNAT, is activated by the nocturnal release of the neurotransmitter NE from sympathetic nerve terminals located within the pineal gland. Activation of this enzyme leads to the conversion of the indoleamine serotonin (5HT) to N-acetylserotonin. A second enzyme, hydoxyindole-O-methyltransferase subsequently methylates NAS to form the methoxyindole, melatonin (Klein 1985). The resulting circadian rhythms in these indole derivatives are driven by clock mechanisms in the SCN and are dependent on an intact sympathetic innervation (Moore 1978).

Recently, numerous other factors have been implicated in the control of melatonin synthesis and secretion. In addition to monoamines (Ebadi and Govitrapong 1986), neuromodulatory peptides such as vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), vasopressin (VP) and oxytocin (OT) have varying effects on SNAT activity and melatonin secretion (Olcese 1991; Simonneaux et al. 1990; Simonneaux et al. 1990; Stehle et al. 1991). Levels of OT and VP, as well as other neuropeptides such as somatostatin and an immunoreactive arginine vassopressin-like peptide have been reported to vary with time and/or physiological status of the pineal gland implying a functional relationship to melatonin synthesis (Liu and Burbach 1987; Liu and Burbach 1988; Moujir et al. 1990; Webb et al. 1984). These observations clearly indicate that the mechanisms regulating synthesis and secretion of melatonin in the pineal gland are complex and multifaceted.

To date, little information is available on a possible role for neuroactive amino acids in modulating melatonin synthesis/secretion in the pineal gland. Two such amino acids, glutamate and taurine, are present in high concentrations within the pineal (Govitrapong and Ebadi 1988; McNulty et al. 1990; Nir et al. 1973), and glutamate binding sites have been characterized in the gland (Govitrapong et al. 1986; Kus et al. 1990). Administration of glutamate to glands *in vitro* inhibited adrenergic receptor activation of SNAT and melatonin secretion (Govitrapong and Ebadi 1988; Kus et al. 1991). Taurine has also been shown to affect SNAT activation by adrenergic mechanisms, but in a stimulatory fashion (Wheler et al. 1979). An hypothesized functional relationship of these neuroactive amino acids with adrenergic-mediated indole metabolism was further suggested by developmental changes in day-night levels of amino acids coincident with the ontogenetic onset of circadian rhythms in indole constituents leading to the production of melatonin (McNulty et al. 1990).

The purpose of this investigation was to provide semiquantitative immunocytochemical data on the distribution of glutamate and glutamine within the different cellular compartments of the rat pineal gland. The subcellular localization of glutamate within pinealocyte organelles was also analyzed. A second aim of the study was to determine if steady state levels of free amino acids exhibit circadian rhythms. In view of the above cited studies suggesting an interaction of glutamate with adrenergic receptor mechanisms, we also investigated the effects of sympathetic denervation on amino acid levels over the 24 hour light-dark cycle.

Materials and Methods

Animals

A total of 112 adult male Sprague-Dawley rats (Sasco-King, Oregon, WI) were used in this study. Animals were housed in a fully accredited animal care facility and were used according to the recommendations in the *Guide for the Care and Use of Laboratory Animals* and *The Guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center.* All animals were entrained for 4 weeks to programmed light:dark cycles in the animal room (1000 lux) or in environmental chambers illuminated by Vita lights (2200 lux) connected to automatic 24 hour timers. Temperature in the environmental chambers was regulated by fans. All animals had access to food and water *ad libitum.* For the experiments measuring effects of denervation of pineal free amino acids, animals (n=6 per time point per group) were sacrificed by rapid decapitation at 6 time points over the light:dark cycle (mid-light, mid-dark, and 1 hour either side of lights on and lights off). A dim red light (Kodak filter 1A) was used for dark-time sampling. Animals used for immunocytochemical analysis were sacrificed by cardiac perfusion after sodium pentobarbitol anesthesia.

Superior Cervical Ganglionectomy Procedures

For bilateral removal of the superior cervical ganglia, animals (n=36) were anesthetized with sodium pentobarbitol (65 mg/kg), placed in an aseptic surgical field, and a ventral midline incision made on the neck. The carotid sheath and contents were exposed and the sympathetic chains followed rostrally to the superior cervical ganglia (SCG). The ganglia were removed

bilaterally with fine forceps and micro-scissors and the incision closed with sterile silk suture. Sham operations (n=36) involved all of the above except removal of the ganglion. An additional unoperated control group (n=36) was included in the study. A 4 week recovery period from the surgery was allowed and the animals checked for ptosis to confirm superior cervical ganglionectomy (SCGX). Individual glands were also assayed for norepinephrine by HPLC (see below) to further confirm degeneration of noradrenergic terminals.

High Performance Liquid Chromatography (HPLC)

General methods for HPLC have been described elsewhere (McNulty et al. 1987; McNulty et al. 1990). Briefly, pineal glands were quickly dissected, frozen on dry ice, and stored at -70°C until assayed. Glands were homogenized in cold 0.05 M monobasic phosphate buffer (pH 7.0). Homogenates were centrifuged at 15,000X g for 2 minutes and aliquots of supernatant were injected directly into the chromatographic system consisting of a Rainin Rabbit pump, a Rheodyne 7125 inject port with a 100 ml sample loop, and a C18 reverse phase column (10 cm X 4.6 cm, 3 mm particle size, Rainin Instruments, Woburn, MA). Levels of free amino acids were determined using pre-column derivatization with o-phthaldialdehyde (OPA; Sigma) as described previously (McNulty et al. 1990). Fluorescence emitted by the derivatized compounds was measured with a LDC Fluoromonitor III (Milton Roy, Riviera Beach, FL) equipped with standard excitation (340-380 nm) and emission (418-700 nm) filters. The detector was connected to a HP 3390A integrator (Hewlett Packard, Avondale, PA) used to analyze the peaks. The mobil phase consisted of 0.1 M monobasic sodium

phosphate mixed with HPLC-grade methanol (32 %) and delivered at a rate of 1.0 ml/min. A separate chromatograph was used to measure pineal levels of NE as described previously (McNulty et al. 1990). Briefly, NE was separated using a C18 reverse phase column and electrochemically detected with a glassy carbon electrode (0.7 V vs Ag/AgCl reference electrode) connected to a BAS LC-4B electrochemical transducer. The mobil phase comprised 0.1 M sodium phosphate with 3 mM octanesulfonic acid (Kodak) and 4% methanol delivered at 1.0 ml/min with a 110A Altex metering pump. Identification of eluted compounds were based on retention times, and the concentration of samples were determined by comparison with synthetic standards made daily from stock solutions. Aliquots of supernatant were reserved for the measurement of soluble proteins according to the method of Lowry et al. (1951).

Quantitative Electron Microscopic Immunocytochemistry.

Electron microscopic immunocytochemistry of amino acids followed previously published procedures (Ji et al. 1991; Ottersen 1988; Somogyi et al. 1986; Storm-Mathisen and Ottersen 1990). Animals (n=4) were anesthetized with pentobarbital (65 mg/kg) during the middle of the photoperiod and perfused through the left cardiac ventricle with the fixative solution comprising 2.5 % glutaraldehyde and 1 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The fixative perfusion was preceded by a brief flush of a 2% solution of dextran (MW 70,000) in the same buffer. After dissecting the glands, they were postfixed in the glutaraldehyde/paraformaldehyde fixative for 3 hours before post-fixation for 1 h with 1% osmium tetroxide in phosphate buffer. Glands were

subsequently dehydrated in ethanol and propylene oxide, then infiltrated and embedded in epoxy resin (Durcupan). Ultra-thin sections (70-80 nm) were cut with a diamond knife and mounted on *Ni* mesh grids for post-embedding immunocytochemical procedures. Sections treated with periodic acid and sodium periodate were exposed to the primary antisera, diluted 1:500. The sera had been raised against either glutamate (code 03; (Ji et al. 1991)), or glutamine (code 34; (Laake et al. 1986)) coupled to carrier protein by glutaraldehyde following the procedure originally described by Storm-Mathisen et al. (Storm-Mathisen et al. 1983). The secondary antibodies were coupled to 15 nm gold particles. The specificity of labelling was monitored in each experiment by analysis of test sections (Ottersen 1987) that had accompanied the tissue sections throughout the immunocytochemical procedures. These test sections carried model conjugates prepared by reacting different amino acids with brain protein in the presence of glutaraldelhyde. Each antiserum reacted selectively with the amino acid used for immunization as demonstrated in a series of previous reports (Ji et al. 1991; Ottersen 1988). As a further specificity control the antisera were preadsorbed with their respective antigens. This was done by adding 400 uM glutaraldehyde complexes of either glutamate or glutamine to the appropriate antiserum on the day before the immunocytochemical incubation (Ji et al. 1991; Ottersen 1988). This procedure reduced the gold particle density over the tissue to a level similar to that over tissue-free resin. Labelling was also abolished when a pre-immune serum was used in lieu of the primary antiserum.

Micrographs of the sections were made with an Hitachi H-600 electron microscope at primary magnifications of 8,000X and 20,000X. Counts of the

area densities of gold particles over specific cell compartments were obtained by computerized image analysis using a Mac IIcx running *NIH Image* software. The freehand-selection tool was used to obtain profile areas of specific cellular compartments and the number of gold particles counted within that profile area after thresholding the gray-scale to include only the electron dense gold particles. From 10-50 profiles of each cell type were analyzed at 8,000X with 5-10 areas measured in each profile. Additional measurements of area density of gold particles in mitochondria, cytoplasm (n=22) were obtained from micrographs taken at 20,000X. Area densities for pinealocyte cytoplasm and mitochondria obtained from low and high magnification micrographs did not differ significantly.

Statistics

Means and standard errors of amino acid levels were calculated and grouped according to treatment and time of sacrifice. The F-statistic for the effect of time was calculated using a one-way ANOVA followed by Bonferroni simultaneous confidence intervals. The F-statistic for the effect of time and treatment was calculated using a two-way ANOVA. For immunocytochemical data the F-statistic between groups was calculated using a one-way analysis of variance. A $p < 0.05$ was considered significant.

Results

Pineal Gland Free Amino Acids and Protein

Circadian analysis

Estimates of the steady state levels of free amino acids in the pineal gland of rats entrained to a 10:14 hour light-dark cycle revealed that glutamate and taurine occurred in the highest concentrations (Table 1). Only one of the amino acids assayed, alanine, exhibited significant differences over the light-dark cycle (Fig. 1 and Table 2). Steady state levels of other amino acids assayed (glutamate, taurine, glutamine, aspartate, arginine, and serine) did not vary significantly over the 24 hour light-dark cycle (Figs. 2, 3 and Table 2). There were no differences in protein content of the glands related to time of sacrifice over the light-dark cycle (data not shown).

Effect of SCGx

Variables from sham operated animals were not significantly different from unoperated controls at any time point based on two-way ANOVA. Data points from both the control and sham operated animals did not differ significantly and the data were therefore combined (Table 1). Bilateral SCGx significantly decreased pineal levels of glutamate, aspartate, alanine, and arginine over the light-dark cycle (Figs. 1, 2 and Table 3). Sympathetic denervation of the gland had the greatest effect on glutamate, decreasing levels by 48% for samples pooled over the light:dark cycle. By comparison, levels of aspartate, alanine, and arginine were decreased to a lesser extent (16-36%) following SCGx, while the levels of serine were only marginally affected ($p=0.10$) and glutamine, taurine, and protein were unaffected by denervation

(Fig. 3 and Table 3). Sympathectomy also abolished the circadian changes in pineal alanine content (Fig. 1). NE levels were not detectable in any of the sympathectomized glands (data not shown).

Immunocytochemistry

Glutamate

Specific immuno-staining for glutamate was found in each of the cell types present within the pineal gland (Figs. 4, 6). Semi-quantitative analysis of gold particle densities revealed the highest concentration of glutamate immuno-staining over pinealocytes (Figs. 4, 6). The densities of glutamate immunolabelling did not differ significantly between glia, endothelia and sympathetic nerve elements. Analysis of the subcellular cytoplasmic distribution of glutamate-immunolabel within pinealocytes indicated that lipid inclusions were nearly devoid of glutamate immunolabeling and mitochondria had slightly higher label density compared to the surrounding cytoplasm (Fig. 7).

Glutamine

Specific glutamine immuno-staining had the highest area density of label in glia, and was only slightly above background in neural elements (Figs. 5, 6). The overall density of label in each of cell types was lower than that of glutamate.

Discussion

As it is in other brain regions, glutamate is present in high concentration within the rat pineal gland. Immunocytochemically localized within all cell types present in the gland, glutamate is likely to have several functions. In addition to being a reaction product of the citric acid cycle, glutamate is involved in the detoxification of ammonia, and serves as a precursor for the synthesis of glutathione, proteins and GABA (Meister 1979). The presence of these high levels of glutamate in the rat pineal gland may also be related to the gland's phylogenetic transition from a photoreceptive organ. Comparative anatomical and histochemical studies have presented strong evidence that mammalian pinealocytes have evolved from photoreceptors (Collin and Oksche 1981; Oksche 1965). It is not surprising then that glutamate, which is a putative neurotransmitter in retinal photoreceptors (Copenhagen and Jahr 1989; Ulshafer et al. 1990), is found in high concentrations in pinealocytes. It has been recently demonstrated that glutamate, which is released from photoreceptors (Copenhagen and Jahr 1989) and activates second order neurons in the retina, can also influence photoreceptors directly by binding to an autoreceptor and depolarizing the cell (Sarantis et al. 1988; Tachibana and Kaneko 1988).

A role for glutamate in the mammalian pineal gland is suggested by evidence for a specific binding site for this amino acid. Using [^{3}H]glutamate as a ligand, a single, saturable and stereospecific binding site has been characterized in both bovine and rat pineal gland (Govitrapong et al. 1986; Kus et al. 1990). Although the precise function of this binding site has yet to be elucidated, there is evidence that glutamate modulates the adrenergic

activation of melatonin synthesis and secretion in an *in vitro* system (Govitrapong and Ebadi 1988; Kus et al. 1991).

Because the mammalian pineal gland synthesizes and secretes melatonin in highest concentrations at night *in vivo*, the presence of compounds that modulate this process *in vivo* might also be expected to possess a similar 24 hour rhythm in the gland. The question of a circadian rhythm in glutamate concentration in the pineal gland was examined by measuring glutamate concentration in the gland over the 24 hour light-dark cycle. It was observed that glutamate levels do not change over the 24 hour light-dark cycle, a finding that can be interpreted several ways. It is possible that glutamate may be released from sympathetic nerve terminals in the gland in a tonic manner. Changes in the glutamate concentration over the 24 hour light-dark cycle would therefore not be evident. It is also possible that glutamate may be released in very small quantities. Because nerve terminals make up such a small percentage of total pineal gland area, even concentrated amounts of glutamate released by nerve terminals may not be detected within the total pool of glutamate of approximately 2000 ng/gland. NE, for example, only increases from 2.3 ng/gland during the day to 4.7 ng/gland at night (Craft et al. 1984). In addition, glutamate is involved in general metabolism and if released from nerve terminals may not remain as free glutamate within the tissue for a long period of time. It may be immediately shunted by the cells into some other metabolic process such as protein or glutathione synthesis. It is also possible that glutamate is removed from the cell by its uptake carrier because it has been shown that even highly concentrating uptake systems can transport amino acids out of a cell (Van Winkle 1988).

An alternative explanation for the lack of a 24 hour rhythm in the glutamate content of pineal gland is that glutamate is not released from sympathetic nerve terminals, which are responsible for the cyclic release of NE in the gland. This is supported by the fact that immunocytochemical localization of glutamate does not show this amino acid to be highly concentrated within nerve terminals. It is interesting to note, however, that removal of sympathetic nerve terminals by means of sympathectomy, results in a significant decrease in the amount of glutamate present (48%) within the gland. This might be expected if the SCG is the source of "transmitter" glutamate. The effect of sympathectomy, however, was not specific for glutamate as alanine, aspartate, arginine and serine were also decreased, although to a lesser extent (16-36%).

In view of the fact that amino acids are an essential component of intermediary metabolism, an effect of denervation on their content within the pineal gland was not unexpected. Sympathectomy, for example, is known to inactivate the indole biosynthetic pathway. Although it is unlikely that almost half the steady state levels of glutamate is required for cellular mechanisms leading to the synthesis and secretion of melatonin, it is difficult to say with much certainty that the loss of pineal gland glutamate after sympathectomy is due to loss of a transmitter pool located within sympathetic nerve terminals. It is possible that uptake of these amino acids, which in the case of glutamate occurs against a concentration gradient, is somehow inhibited by sympathetic denervation. It is known, for example, that the movement of one glutamate and three Na^+ into the cell, while one K^+ exits the cell requires the maintenance of the Na^+/K^+ concentration gradient (Barbour et al. 1988; Brew and Attwell 1987). The observation that NE

stimulates Na^+/K^+- ATPase activity in rat brain *in vivo* (Swann and Steketee 1989) suggests that inhibition of the Na^+/K^+ pump by denervation could affect amino acid uptake.

The present data suggest that the decline in pineal glutamate was not related to a general effect of denervation on pineal metabolism because several other amino acids (glutamine, taurine, serine) were either unaffected or marginally decreased following sympathectomy. Soluble protein content was also unchanged after denervation, which is in good agreement with the findings of Moore and Rapport (1971) that ganglionectomy had no effect on pineal protein after 30 days.

Glutamine, another important amino acid in intermediary metabolism, is believed to be in dynamic equilibrium with glutamate in nervous tissue where glutamine is partitioned principally within glia. Our immunocytochemical data suggest that a similar glial partitioning of glutamine exists in the pineal gland. Compartmentilzation of glutamate and glutamine is believed to be an important mechanism for separating metabolic regulation of these amino acids in neural functions (Nicklas et al. 1987). The present observation that pineal glutamine levels were not affected by denervation suggests that the effects of sympathectomy are specific and confined primarily to pinealocytes. The subcellular localization of glutamate within pinealocytes is similar to that seen in neurons (Torp et al. 1991).

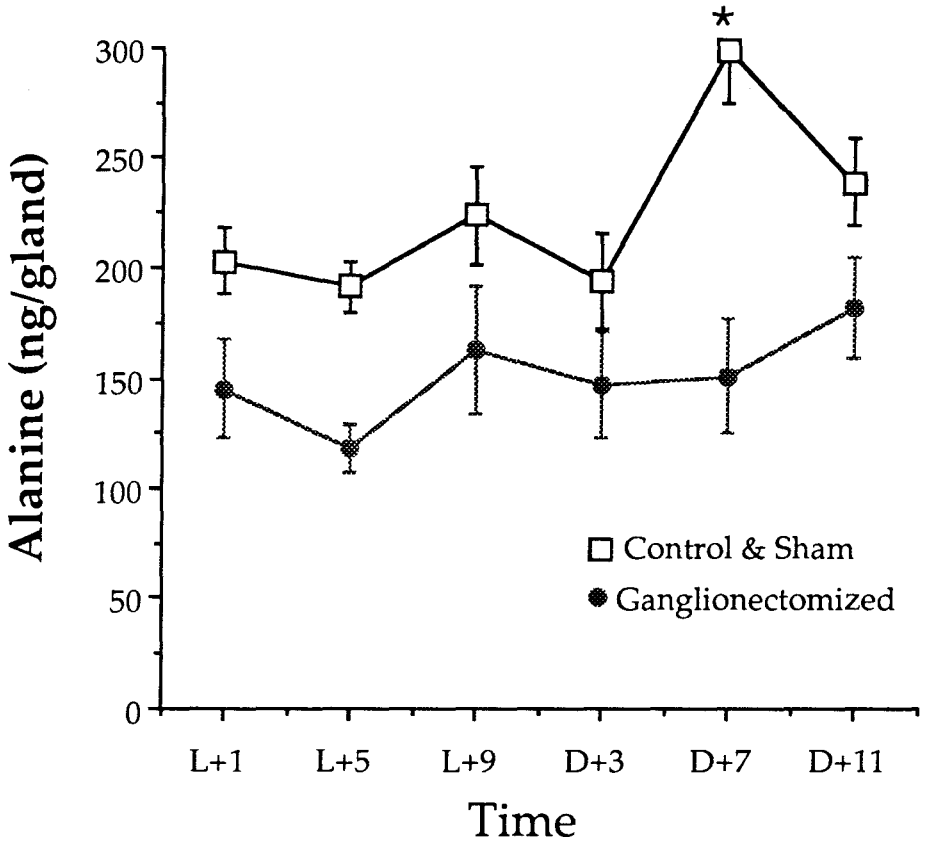


Fig. 1. Alanine content of rat pineal gland over the 24 hour light-dark cycle. Alanine values expressed as mean ± SEM are significant higher at D+7 compared to other time points ($p<0.05$ Bonferroni). Control and sham operated groups did not differ significantly ($p<0.77$ ANOVA) and were combined. n = 12 pineal glands per group per time point. Ganglionectomy significantly decreased alanine level compared to control and sham values ($p<0.01$ ANOVA). n = 6 pineal glands per group per time point.

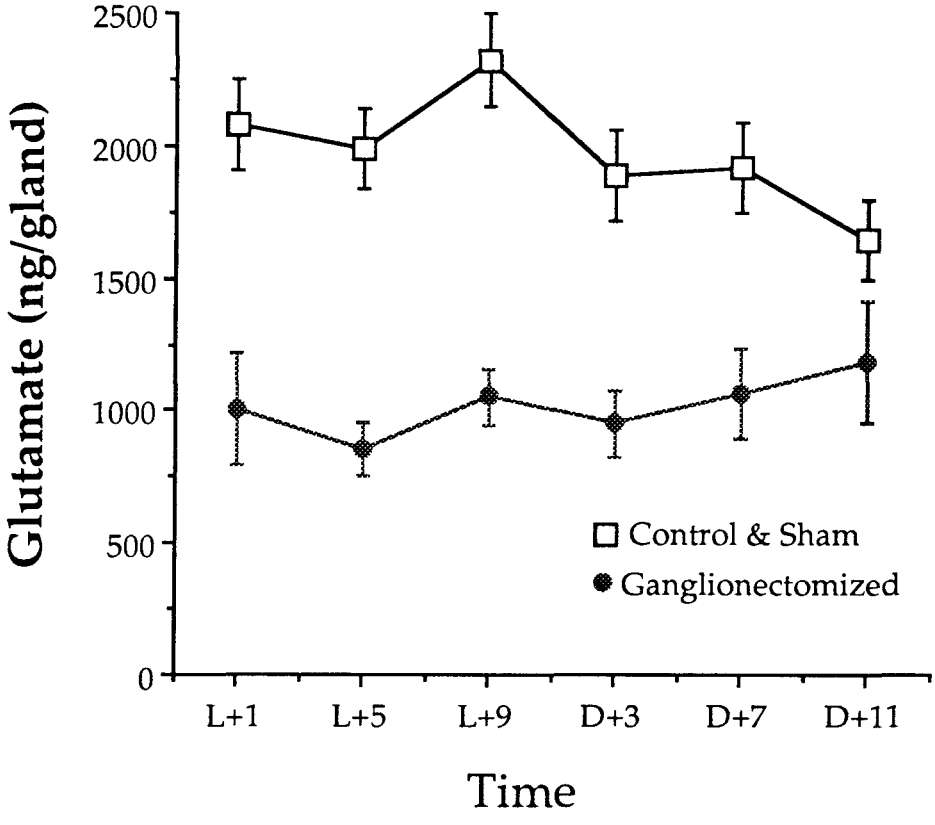


Fig. 2. Glutamate content of rat pineal gland over the 24 hour light-dark cycle. Glutamate levels are expressed as mean $\pm$ SEM. Control and sham operated groups did not differ significantly ($p<0.92$ ANOVA) and were combined. n = 12 pineal glands per group per time point. There was no significant differences in glutamate concentrations over the light-dark cycle ($p<0.09$). Ganglionectomy significantly decreased glutamate levels compared to control and sham operated values ($p<0.01$ ANOVA). n = 6 pineal glands per group per time point.

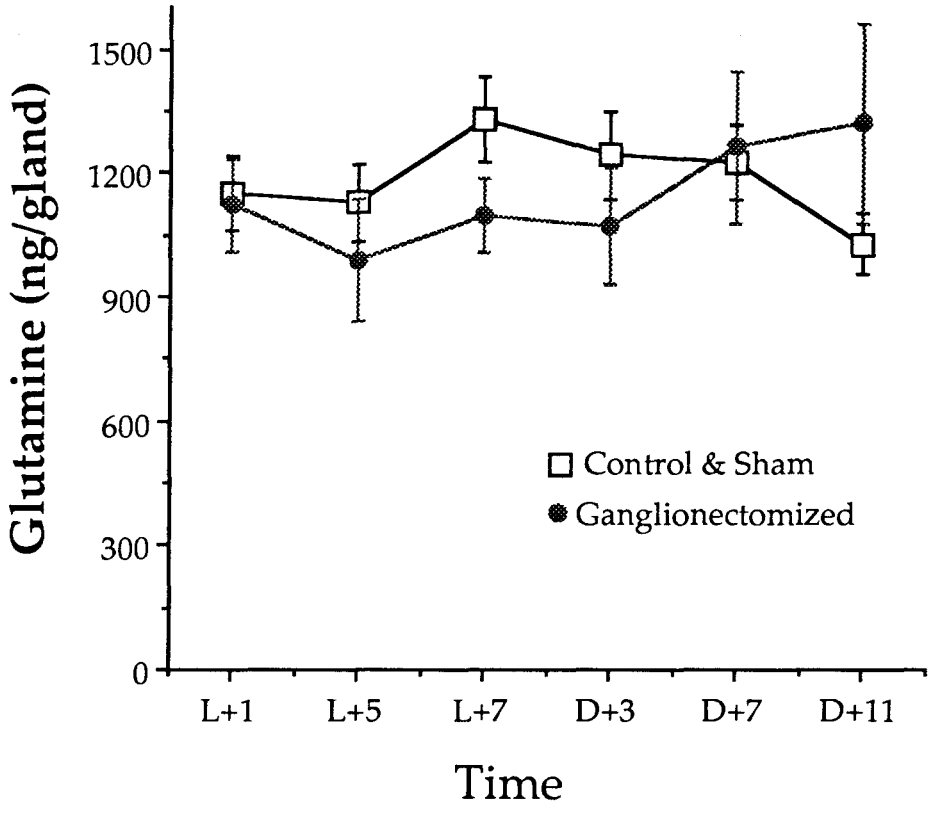


Fig. 3. Glutamine content of rat pineal gland over the 24 hour light-dark cycle. Glutamine levels are expressed as mean $\pm$ SEM. Control and sham operated groups did not differ significantly from one another and were combined. n = 12 pineal glands per group per time. Ganglionectomy did not effect glutamine content of the pineal gland. n = 6 pineal glands per group per time point.

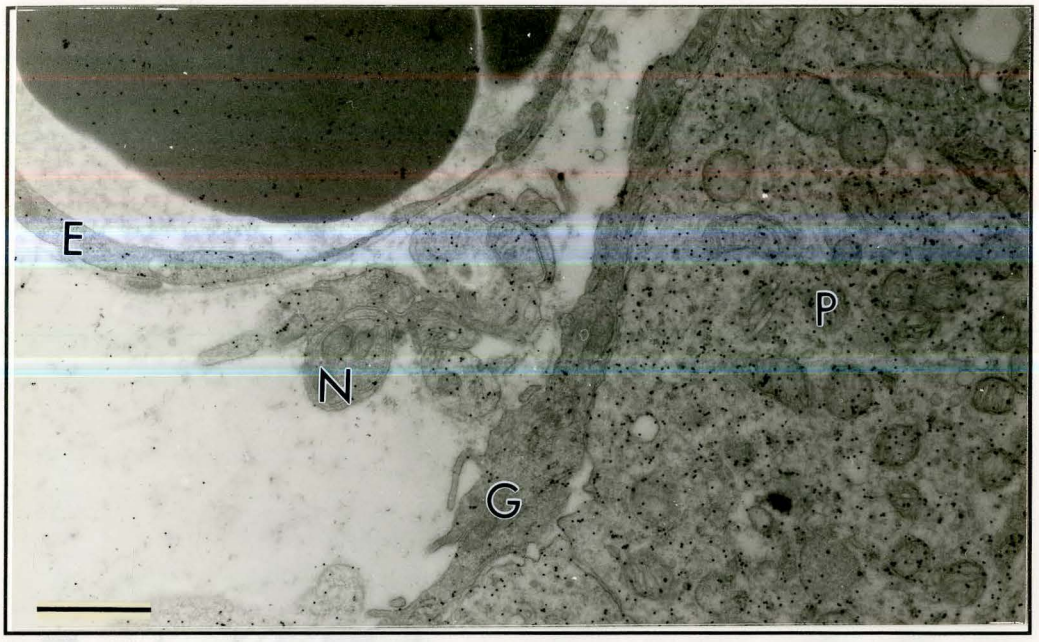


Fig. 4. Electron micrograph of pineal gland immuno-reacted for glutamate illustrating parts of pinealocytes (P), a glial process (G), noradrenergic neural elements (N) and endothelium (E). The density of gold particles tends to be greatest over the pinealocytes. Bar indicates 1.0 μM

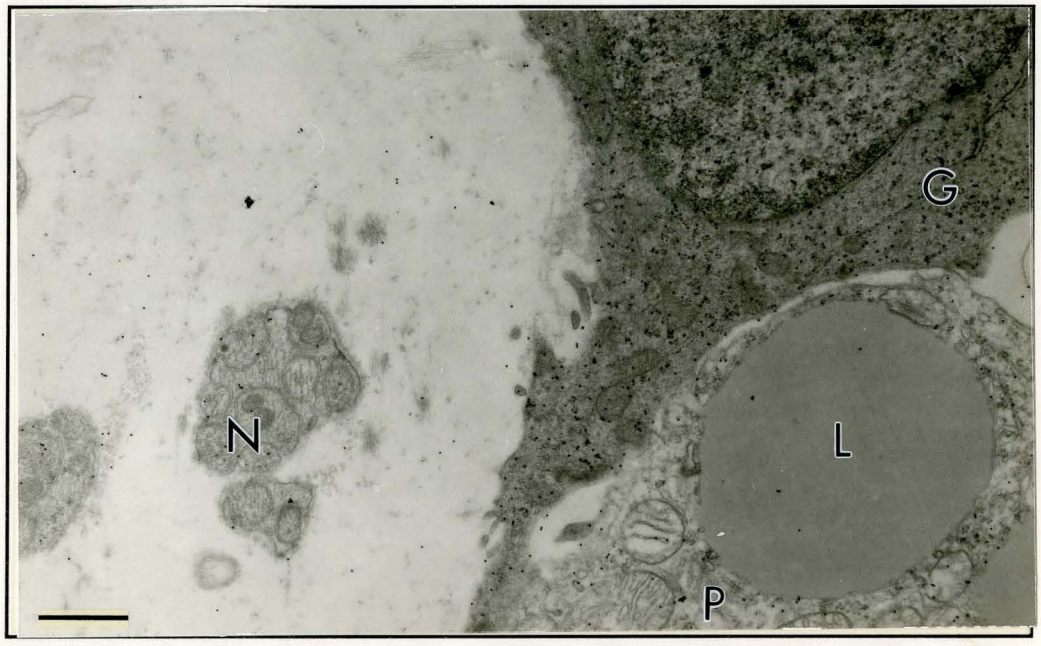


Fig. 5. Electron micrograph of pineal gland immuno-reacted for glutamine. Glia (G) are intensely labelled, and there is sparse labelling over the neural element (N) in the perivascular space. P= pinealocyte L = lipid droplet. Bar indicates 1.0 μM

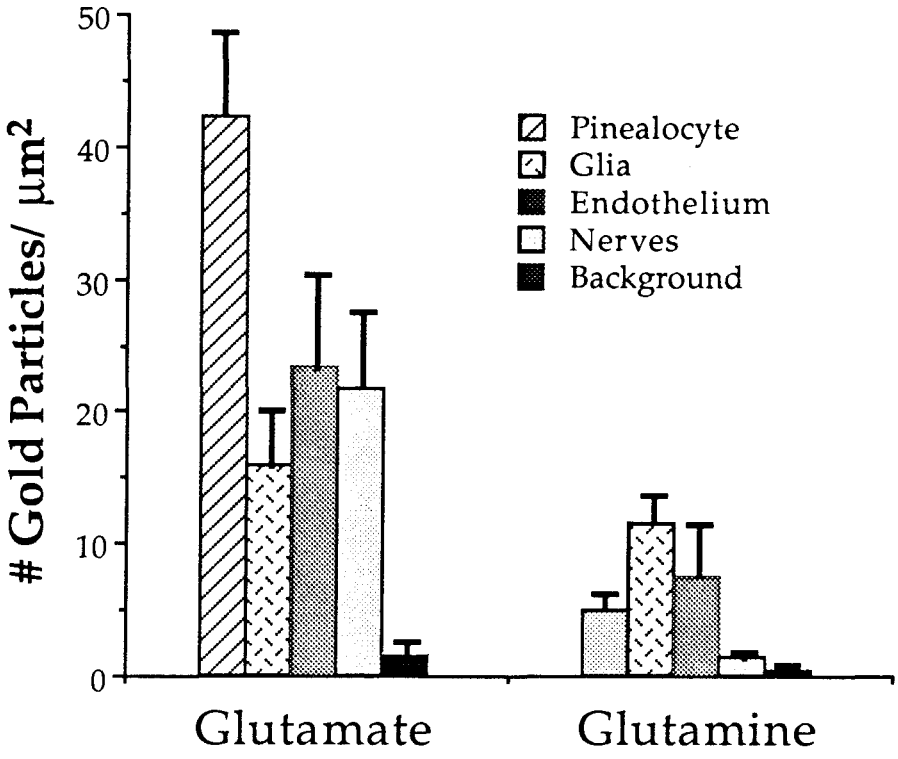


Fig. 6. Means ± SEM of density of gold particles over cell compartments in the pineal gland after immuno -staining for glutamate and glutamine. Gold particle density was measured over cytoplasm (minus mitochondria and lipid) in pinealocytes and glia. Glutamate-like immunoreactivity was highest over pinealocytes ($p < 0.01$ ANOVA). Glutamine-like immunoreactivity was highest over glia ($p < 0.04$ ANOVA). Background represents particle density over the extracellular compartment. n = 4 pineal glands.

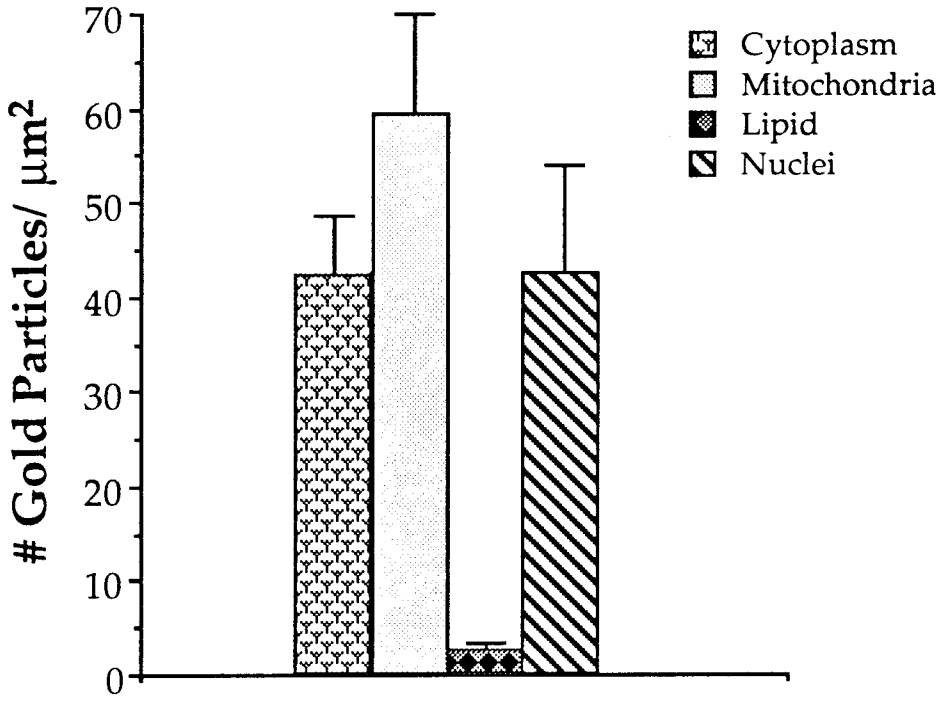


Fig. 7. Means ± SEM of density of gold particles over subcellular compartments of pinealocytes immuno -reacted for glutamate. Cytoplasm includes cytosol and remaining organelles (e.g. endoplasmic reticulum) not specifically sampled. n = 4 pineal glands.

Table 1. This table compares amino acid content of pineal glands obtained from control (C) and sham operated (S) animals. Means ± SEM of amino acids (ng/gland) in the rat pineal gland over the 24 hour light-dark cycle are presented. Time is designated as hours after lights on (L) and hours after lights off (D). n = 6 pineal glands for each group at each time point. P values are based on F statistic for effect of treatment from a two-way ANOVA. A $p < 0.05$ was considered significant. Control and sham operated values do not differ significantly in any of the amino acids. Control and sham values were therefore grouped in later statistical analysis (See Tables 2 and 3).

		L+1	L+5	L+9	D+3	D+7	D+11	Treat p>F
Arg	C	359±60	532 ±126	601±124	623±89	549±99	489±61	
	S	548±55	498±116	711±95	551±80	677±86	480±88	0.09
Asp	C	434±94	388±47	499±95	401±60	396±88	293±56	
	S	565±70	479±110	473±85	446±132	397±102	215±35	0.53
Ala	C	178±22	200±14	241±40	189±33	284±33	268±26	
	S	228±21	182±21	206±25	199±35	313±40	204±28	0.77
Glu	C	1713±243	2105±191	2305±303	2008±211	1886±314	1790±156	
	S	2448±150	1873±255	2342±243	1765±296	1949±187	1472+283	0.92
Gln	C	1010±149	1226±134	1323±198	1350±150	1268±148	1095±29	
	S	1289±90	1034±141	1335±104	1140±158	1189±130	955±166	0.47
Ser	C	471±109	412±41	651±185	371±22	474±66	534±76	
	S	411±56	398±44	484±127	408±63	397±121	320±46	0.09
Tau	C	1587±211	2014±111	2156±379	1966±216	1356±103	1974±171	
	S	2279±217	1886±243	2670±408	1800±341	2005±320	1846±293	0.08

Table 2. This table examines amino acid content of pineal glands over time. Control and sham operated groups are combined as C/S. Values are presented as means ± SEM of amino acids (ng/gland). Time is designated as hours after lights on (L) and hours after lights off (D). P values are based on F statistic for effect of time from one-way ANOVA. A $p < 0.05$ was considered significant. Significant differences over time are seen only in alanine in the C/S group. No differences over time in amino acid content was seen in any ganglionectomized (G) group. n = 12 (C/S) or 6 (G) pineal glands for each group at each time point.

		L+1	L+5	L+9	D+3	D+7	D+11	Time p>F
Arg	C/S	454±48	515±78	656±73	587±56	613±63	485±47	0.13
	G	317±46	339±58	317±34	326±24	377±69	429±98	0.67
Asp	C/S	499±57	433±56	486±58	423±67	396±61	257±34	0.05
	G	251±51	284±50	247±66	333±64	290±49	283±21	0.82
Ala	C/S	203±16	191±12	224±22	194±22	299±24	239±20	0.01
	G	145±22	118±10	163±29	147±24	151±26	182±23	0.54
Glu	C/S	2080±174	1989±149	2324±177	1886±170	1917±167	1646±147	0.09
	G	1005±214	851±98	1052±107	949±122	1068±171	1185±229	0.78
Gln	C/S	1150±91	1130±93	1329±101	1245±105	1228±90	1031±72	0.26
	G	1123±113	988±148	1098±92	1072±142	1264±182	1322±242	0.66
Ser	C/S	441±57	405±27	567±105	389±31	435±64	427±55	0.34
	G	356±35	326±69	449±109	355±36	317±25	441±42	0.56
Tau	C/S	1933±176	1950±123	2413±266	1883±185	1680±184	1916±146	0.11
	G	1910±322	1693±181	1815±144	1705±178	1776±246	2083±410	0.88

Table 3. This table compares amino acid content of pineal glands obtained from control and sham operated (combined as C/S) with ganglionectomized (G) animals. Means ± SEM of amino acids (ng/gland) in the rat pineal gland over the 24 hour light-dark cycle are presented. Time is designated as hours after lights on (L) and hours after lights off (D). n = 6 pineal glands for each group at each time point. P values are based on F statistic for effect of treatment from two-way ANOVA. A $p < 0.05$ was considered significant. Significant differences between control and sham (C/S) and ganglionectomized (G) groups were seen in arginine, aspartate, alanine and glutamate but not in glutamine and taurine.

		L+1	L+5	L+9	D+3	D+7	D+11	Treat p>F
Arg	C/S	454±48	515±78	656±73	587±56	613±63	485±47	
	G	317±46	339±58	317±34	326±24	377±69	429±98	0.01
Asp	C/S	499±57	433±56	486±58	423±67	396±61	257±34	
	G	251±51	284±50	247±66	333±64	290±49	283±21	0.01
Ala	C/S	203±16	191±12	224±22	194±22	299±24	239±20	
	G	145±22	118±10	163±29	147±24	151±26	182±23	0.01
Glu	C/S	2080±174	1989±149	2324±177	1886±170	1917±167	1646±147	
	G	1005±214	851±98	1052±107	949±122	1068±171	1185±229	0.01
Gln	C/S	1150±91	1130±93	1329±101	1245±105	1228±90	1031±72	
	G	1123±113	988±148	1098±92	1072±142	1264±182	1322±242	0.66
Ser	C/S	441±57	405±27	567±105	389±31	435±64	427±55	
	G	356±35	326±69	449±109	355±36	317±25	441±42	0.10
Tau	C/S	1933±176	1950±123	2413±266	1883±185	1680±184	1916±146	
	G	1910±322	1693±181	1815±144	1705±178	1776±246	2083±410	0.34

CHAPTER IV

PINEAL GLAND AMINO ACIDS IN THE RAT II. CHARACTERIZATION OF A [^{3}H]GLUTAMATE BINDING SITE

Abstract

Glutamate, an excitatory neurotransmitter/ neuromodulator involved in cell to cell communication within the central nervous system is now believed to play a role in neuroendocrine function. In this study we describe a single, saturable and stereospecific binding site for [3H]glutamate (K_d = 612 $\pm$ 23 nM, B_{max} = 3170 $\pm$ 330 fmol/mg protein). Binding of [3H]glutamate was temperature, time, and pH-dependent. After removal of the sympathetic innervation to the pineal gland, [^{3}H]glutamate binding displayed a higher appparent affinity (K_d = 412 $\pm$ 28 nM) ($p<0.05$ T-test) without a change in binding site number (B_{max} = 3600 $\pm$ 240 fmol/mg protein). No difference in [^{3}H]glutamate binding site number was observed in pineal glands obtained from animals sacrificed during the middle of either the light or dark periods. These data suggest a possible modulatory role for glutamate in pineal gland function.

Introduction

The excitatory amino acid (EAA) glutamate plays an important role in both normal and pathological brain function. Acting via its AMPA, kainate, and NMDA receptors, glutamate depolarizes postsynaptic neurons by opening ion channels and allowing the influx of Na^+ and Ca^{2+} (Asher and Nowak 1987; Hollmann et al. 1991). Glutamate also stimulates second messenger production by interacting with a fourth receptor subtype, the metabotropic receptor (Sladeczek et al. 1988). Activation of these receptors within the central nervous system is believed to mediate such diverse phenomena as learning (Lynch et al. 1990), visual plasticity (Dudek and Bear 1989), neuronal differentiation (Mattson et al. 1988) and possibly cell death (Choi and Rothman 1990). The recent observation that a majority of excitatory neurotransmission within the neuroendocrine mediobasal hypothalamus is mediated by glutamate (Van den Pol et al. 1990) also suggests that this amino acid may play an important role in the regulation of neuroendocrine systems.

The pineal gland is an important component of the neuroendocrine system regulating circadian rhythmicity. The release of NE from post-ganglionic sympathetic fibers originating in the superior cervical ganglion (SCG) stimulates the production of the pineal hormone melatonin via its binding to adrenergic receptors located on pinealocytes (Sugden 1989). The pineal gland also possesses a number of other receptor types, including those for benzadiazepine, dopamine, GABA and glutamate (Ebadi 1987). While the function of these latter receptors is unclear, a possible role for glutamate in pineal gland function of the rat has been suggested by several observations. First, glutamate is found in relatively high concentrations in the rat pineal

gland (McNulty et al. 1989). Second, glutamate has been demonstrated to inhibit NE stimulated serotonin SNAT activity in the rat pineal *in vitro* (Govitrapong and Ebadi 1988). Due to the small amount of tissue present in the rat pineal, however, identification of a glutamate binding site in this species has not been made. Because the rat is an important animal model in pineal gland research, the goal of this study was to identify and characterize a glutamate binding site in the rat pineal gland with the use of *in vitro* quantitative receptor autoradiography.

In addition, we have also examined several possible ways in which pineal glutamate binding sites can be regulated. With regard to the observation that steady state levels of glutamate can be markedly reduced by SCGx (McNulty et al. 1989), it was important to ascertain the effects of denervation on this binding site. Examination of [^{3}H]glutamate binding after SCGx was also expected to yield additional information about the location of [^{3}H]glutamate binding, i.e binding to pre- or post-synaptic sites. Finally, because NE content, β-adrenergic receptor density, and melatonin levels exhibit significant differences over the light-dark cycle (Gonzalez-Brito and Reiter 1987), pineals were examined for day vs night differences in glutamate binding site number.

Materials and Methods

Chemicals

[^{3}H]Glutamate (SA = 43-56 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Quisqualate, CPP ((+/-) -3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid), and CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione)

were obtained from Research Biochemicals Inc. (Natick, MA). All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

A total of 64 adult male Sprague-Dawley rats (Sasco-King, Oregon, WI) were used in this study. Animals were housed in a fully accredited animal care facility and were used according to the recommendations in the *Guide for the Care and Use of Laboratory Animals* and *The Guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center*. All animals were entrained to a 12:12 hour light-dark cycle and had access to food and water *ad libitum*. Animals were sacrificed by rapid decapitation over the light:dark cycle at times specified in each experiment. A dim red light (Kodak filter 1A) was used for dark-time sampling.

Superior Cervical Ganglionectomy Procedures

For bilateral removal of the superior cervical ganglia (SCG), animals were anesthetized with pentobarbitol (65 mg/kg ip), placed in an aseptic surgical field, and a ventral midline incision made on the neck. The carotid sheath and contents were exposed and the sympathetic chains were followed rostrally to the superior cervical ganglia. The ganglia were removed with fine forceps and micro-scissors and the incision closed with sterile silk suture. A 4 week recovery period was allowed.

Autoradiography

Following sacrifice, pineal glands were rapidly removed and frozen on dry ice. Tissue was subsequently placed in embedding matrix for support and

sectioned with a Leitz (Model II) cryostat at a thickness of 10-14 μm. Sections (3-4) were placed on the ends of individual gel coated slides and frozen at -70°C overnight. A single pineal yielded 50-60 sections, enough for a complete saturation or competition curve. The tissue was preincubated in 0.05M Tris acetate buffer, pH 7.2 at 4°C for 20 min. Sections were dried with a stream of cool air and incubated at 4°C for 60 min in Tris buffer with increasing concentrations of L-[^{3}H]glutamate (further referred to as [^{3}H]glutamate) (25 nM-800 nM). Non-specific binding was determined by addition of 1000 fold excess non-radioactive glutamate and was subtracted from total binding to determine specific binding. Competition assays to determine receptor subtypes utilized a single concentration of [^{3}H]glutamate (200 nM) and increasing concentrations of various non-radioactive compounds (25 nM-200 mM). To terminate the binding reaction, sections were rinsed in ice cold Tris acetate buffer for 2 minutes with 1 buffer change. Sections were rapidly dried under cool air and placed in X-ray cassettes with appropriate radioactive standards (Microscales, Amersham). Tissue was apposed to tritium sensitive Hyperfilm (Amersham) for 3-6 weeks at 4°C. The film was developed in fresh Kodak D19 for 5 minutes, fixed, rinsed and dried. Autoradiograms were analyzed by computer assisted microdensitometry (RAS 5000 system, Amersham). Bound radioactivity was determined by a computer generated polynomial regression analysis which compares film densities produced by pineal sections with those produced by tritium standards. Saturation data were analysed according to the method of Scatchard (Scatchard 1949). K_i values were calculated from IC_{50} values using the equation of Cheng and Prusoff: $Ki = IC_{50}/(1 + [^3H\text{-}Glu]/ K_d)$ (1973).

Statistics

Statistical analysis of the data was performed with the Student's t-test.

Results

Binding Assays

[^{3}H]Glutamate binding in intact animals

Saturation analysis of [^{3}H]glutamate binding revealed a single binding site with a Hill coefficient = 0.932 $\pm$ 0.028 (Fig. 2), an apparent dissociation constant (K_d) of 612 $\pm$ 23 nM and a B_{max} of 3170 $\pm$ 330 fmol/mg protein (Fig. 1). Binding was time, temperature, and pH dependent with optimal conditions at 60 minutes (Fig. 3), 4^{o}C (Fig. 4), and pH 7.2 (Fig. 5). Various glutamate agonists and antagonists demonstrated a range of competition for bound [^{3}H]glutamate. L-Glutamate was the best competitor with a K_i value of 536 $\pm$ 37 nM (Fig. 6), while the D-isomer was inactive. Quisqualate displayed moderate inhibition of binding with a K_i value of 34000 $\pm$ 5500 nM (Fig. 7). All other compounds tested were inactive (Table 1).

Mid-light vs mid-dark [^{3}H]glutamate binding

Specific [^{3}H]glutamate binding in the pineal gland of animals entrained to a 12:12 hour light-dark cycle and sacrificed during the middle of the dark period did not differ significantly from binding observed in pineals obtained from animals sacrificed during the middle of the light period (Fig. 8).

[^{3}H]Glutamate binding in SCGx animals

SCGx was confirmed in all animals by the presence of ptosis bilaterally. Saturation analysis of [^{3}H]glutamate binding in this group demonstrated a single binding site with a Hill coefficient of 0.987 $\pm$ 0.021, a K_d of 412 $\pm$ 28 nM and a B_{max} of 3600 $\pm$ 240 fmol/mg protein. Sympathectomized pineal glands, when compared to intact controls, possessed a higher apparent affinity for [^{3}H]glutamate binding ($p<0.05$) without a change in binding site number (Fig. 9).

Discussion

In this paper, we describe a single, saturable, stereospecific, temperature, time, and pH-dependent binding site for glutamate in the pineal gland of the rat which has not been previously characterized. Our values are consistent with values reported by other investigators for glutamate binding in other brain regions (Cincotta et al. 1989; Fagg et al. 1983) as well as in the bovine pineal (Govitrapong et al. 1986). Interestingly, the only compound that demonstrated a relatively high affinity for [^{3}H]glutamate binding was L-glutamate itself. While quisqualate inhibited [^{3}H]glutamate binding, the high K_i value of 34000 $\pm$ 5500 nM (relative to that observed for quisqualate inhibtion in other brain regions), suggests that it is unlikely that this corresponds to the functional quisqualate/AMPA receptor described in other brain regions. Contamination of quisqualate by L-glutamate is believed to be, in part, responsible for this low affinity component of quisqualate binding (Cha et al. 1989).

It should be noted that the binding of [^{3}H]glutamate in tissue can represent binding to several membrane components, including receptors linked to ion channels/second messenger systems, uptake sites, and enzymes. In addition, sequestration of ligand by lipid vesicles within tissue can occur. To minimize the possibility of [^{3}H]glutamate uptake, the present saturation and competition studies were performed in sodium, chloride, and calcium-free buffer. No inhibition of [^{3}H]glutamate binding was observed in the presence of D-aspartate, an amino acid with relatively high affinity for the Na^{2+}-dependent glutamate uptake site. The possibility that [^{3}H]glutamate may have bound to an enzyme similiar to the one responsible for the cleavage of the endogenous peptide N-acetyl-aspartyl-glutamate (Slusher et al. 1990; Stauch et al. 1989) is not likely for several reasons. First, as outlined by Slusher et al. (1990) and Stauch et al. (1989), this enzyme, N-acetylated-alpha-linked acid peptidase (NAALA dipeptidase), has been demonstrated to have a much higher affinity for quisqualate than L-glutamate. We have observed the opposite rank order for these 2 compounds in the pineal gland. Second, NAALA dipeptidase appears to have an absolute requirement for chloride for its activation and we did not include chloride in our incubation buffer. Third, binding of [^{3}H]glutamate in this study was greater at 4^{o}C than at 37^{o}C, not a common feature of enzyme-substrate interactions. While the type of glutamate binding site present in the rat pineal gland is not clear, it does not appear to be one of the major glutamate receptor subtypes (AMPA, kainate, or NMDA) as even high concentrations of competitor did not inhibit [^{3}H]glutamate binding. It is possible that this binding site is similar to the Na^{2+} and Cl^--independent glutamate binding site described in the neurohypothysis (Yoneda and Ogita 1989). Glutamate binding at this site

possesses a K_d = 340 nM and is sensitive to inhibition by quisqualate (IC_{50} = 56μM) but not other glutamate analogues. Dissimilarities, however, include the fact that binding site number increase if the tissue is incubated at 30°C when compared to 2°C. Functional studies to examine the role of glutamate on the adrenergic stimulated production of melatonin may help to elucidate the role of glutamate in pineal gland function.

The observed increase in the affinity of [^{3}H]glutamate binding after SCGx without a corresponding change in receptor number may have been due, in part, to a decrease in the amounts of endogenous ligand present after SCGx. The intact pineal gland of the rat possesses relatively high levels of glutamate that decrease by approximately 50% after SCGx (McNulty et al. 1989). It is possible that tissue preincubation may not have removed all the free glutamate present within the tissue sections, thereby decreasing the apparent affinity of [^{3}H]glutamate binding in intact glands without altering binding site number. A similar problem with contamination by L-glutamate was noted in homogenous preparations of retinal membranes (Yoneda and Ogita 1989).

Based on the observation that glutamate stimulates the release of [^{3}H]NE from synaptosomes prepared from hippocampus, olfactory bulb, and cerebral cortex, it is believe that presynaptic receptor for glutamate are present on noradrenergic terminals (Wang et al. 1992). The observation that receptor number did not change after SCGx suggests that binding sites for [^{3}H]glutamate were located either on pinealocytes and/or astrocytes and not on noradrenergic nerve terminals.

Unlike noradrenergic receptors (Pangerl et al. 1990), a day-night difference in [^{3}H]glutamate binding site number was not observed. In

addition, the observation that glutamate levels in the pineal gland do not vary over the light-dark cycle (McNulty et al. 1989) suggests that the role of these binding sites may be tonic in nature. Previous demonstration of glutamate's ability to inhibit the NE-induced production of inositol phospholipids in various brain regions (Jope and Li 1989) suggests a mechanism by which glutamate may interact with the adrenergic system to inhibit melatonin production.

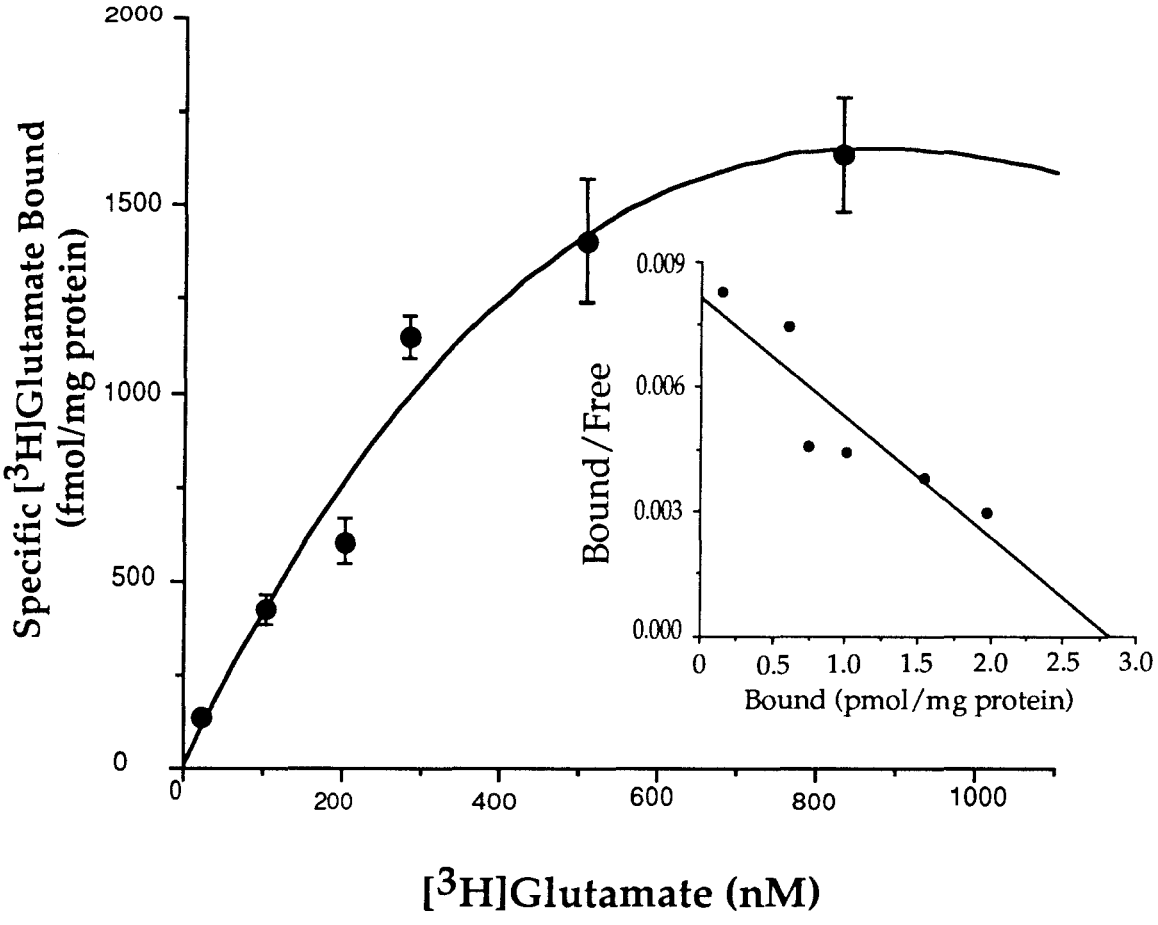


Fig. 1. Saturation and Scatchard analysis of [^{3}H]glutamate binding in rat pineal glands. These studies, performed in Cl^-, Na^+, and Ca^{2+} free buffer, utilized ligand concentrations between 25 nM and 800 nM. Non-specific binding was determined by the addition of 1000 fold excess inert glutamate. Each pineal gland provided a complete saturation curve. Six pineal glands were analysed. Binding at each concentration of [^{3}H]glutamate was performed in triplicate. K_d = 612 + 23 nM, B_{max} = 3170 + 330 fmol/mg protein.

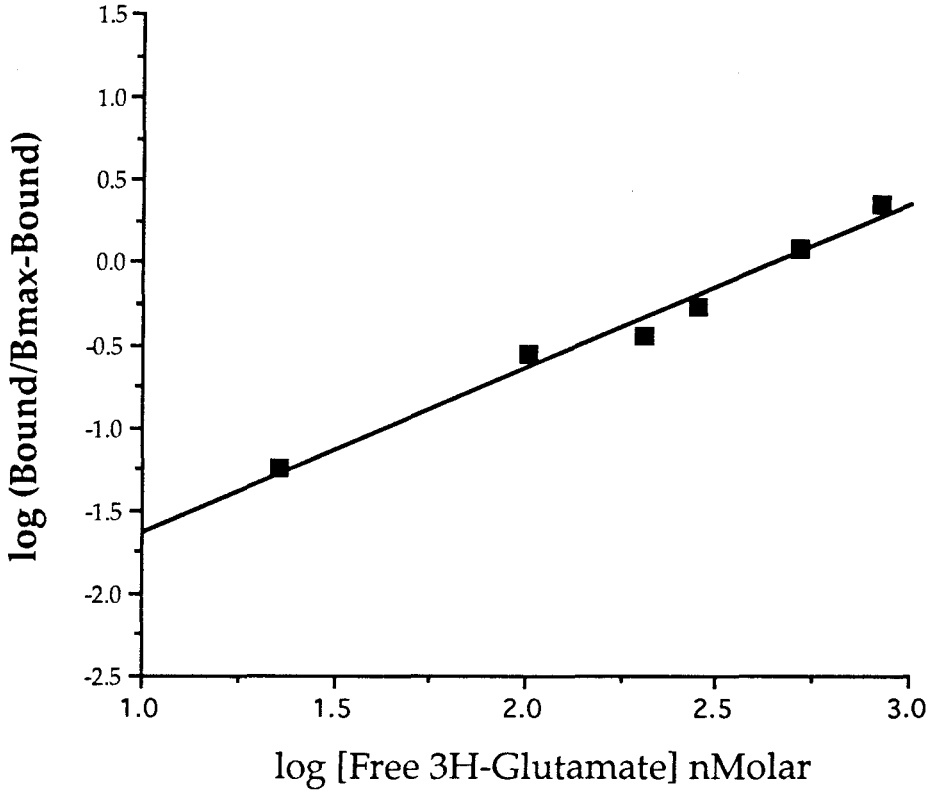


Fig. 2. Representative Hill plot of [^{3}H]glutamate saturation binding data from rat pineal glands. Hill coefficient = slope of line. Equation of representative line is $y = -2.630 + 0.995x$. Hill coefficients obtained from six saturation curves (six pineal glands) yield a mean $\pm$ SEM of 0.932 ± 0.028. These data suggests a single [^{3}H]glutamate binding site.

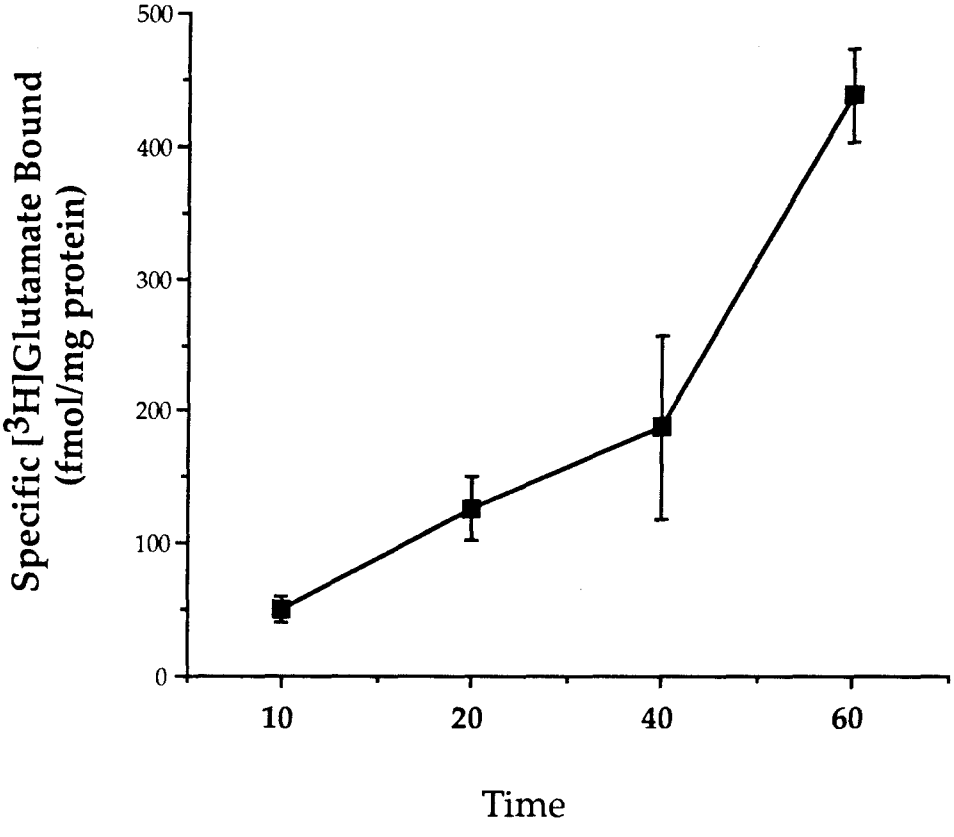


Fig. 3. Specific [^{3}H]glutamate binding (288nM, SA = 43 Ci/ mmol) over time in rat pineal gland. Each bar represents the mean $\pm$ SEM of 3 (40 and 60 min) or 4 (10 and 20 min) pineal glands. Each point was performed in triplicate. Temperature = 4°C and pH = 7.2.

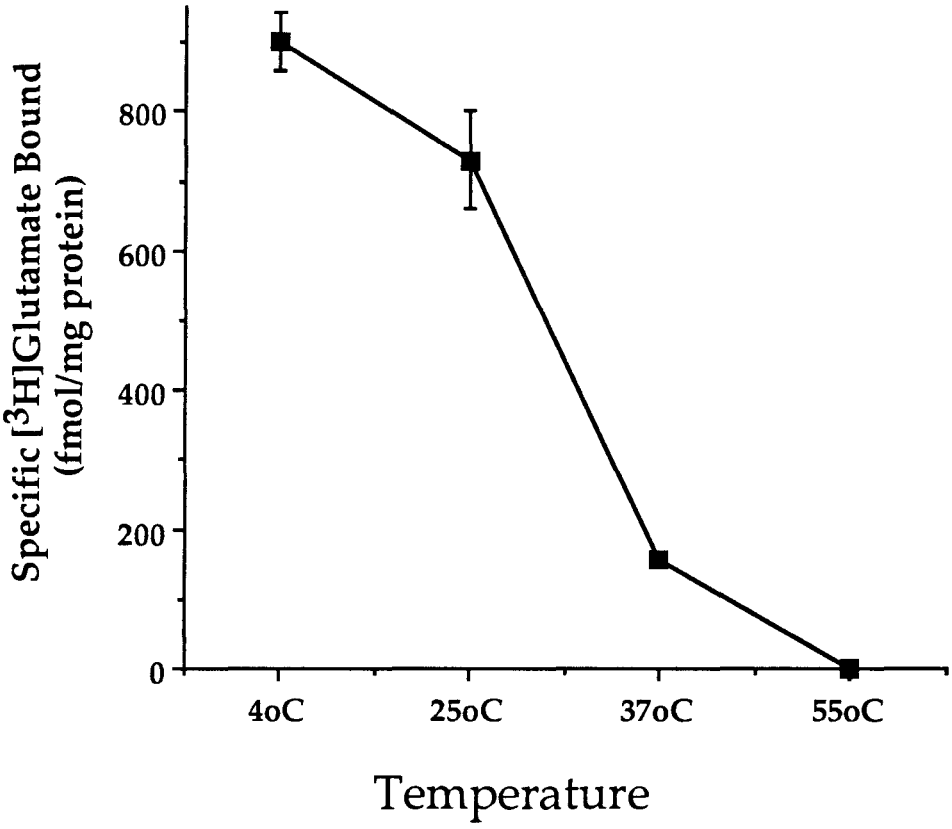


Fig. 4. Temperature-dependency of specific [^{3}H]glutamate binding (309nM, SA = 43 Ci/mmol) in rat pineal gland. Incubation time = 60 min, buffer = pH 7.2. Each bar represents the mean $\pm$ SEM of 2 pineal glands. Each point was performed in triplicate. Binding was not detectable at 55°C.

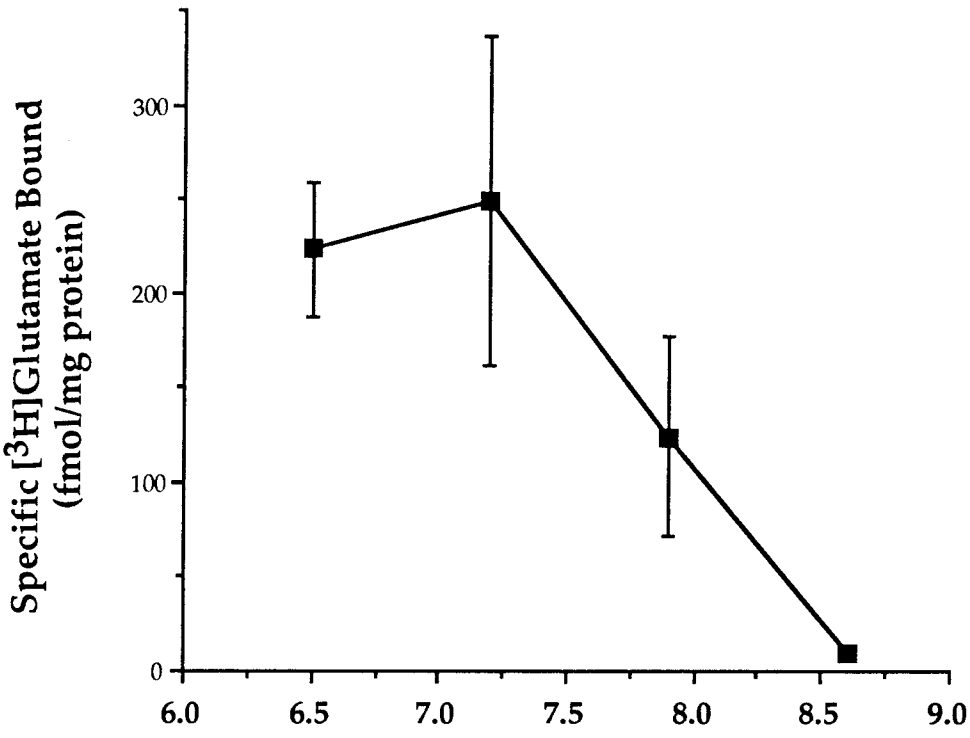


Fig. 5. pH-dependency of [^{3}H]glutamate binding (100nM, SA = 56 Ci/mmol) in rat pineal gland. Each bar represents the mean $\pm$ SEM of 4 pineal glands. Each point was performed in triplicate. Incubation time = 60 min, temperature = 4°C.

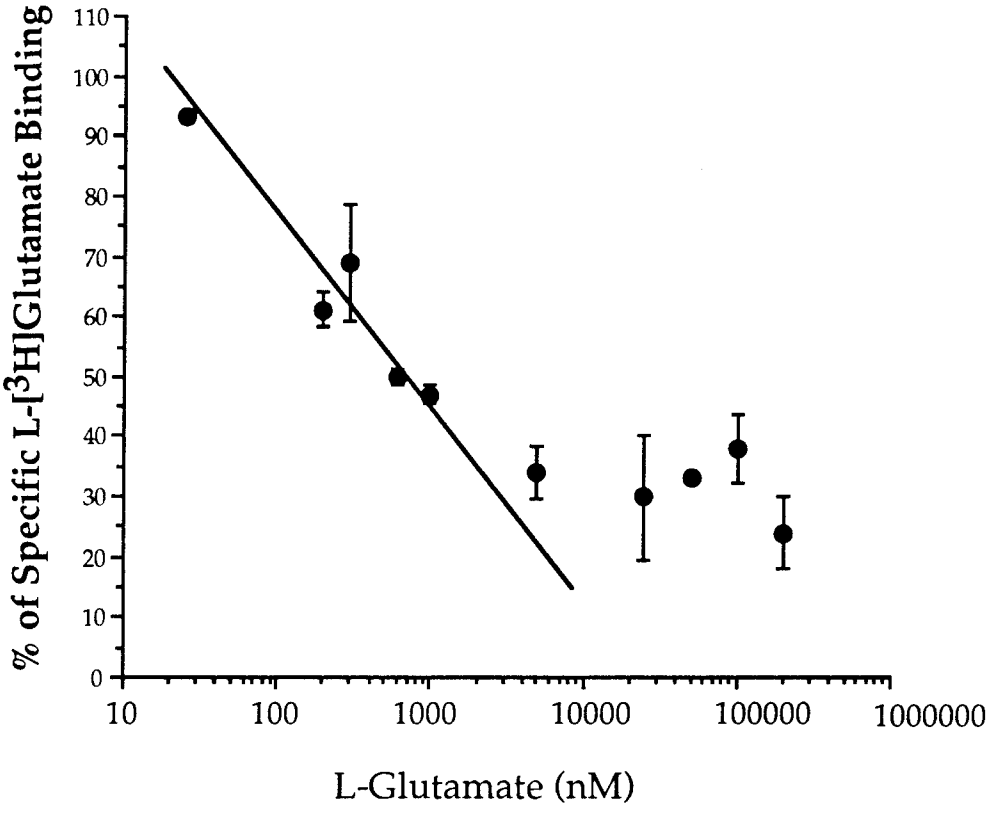


Fig. 6. Inhibition of [^{3}H]glutamate binding (218nM, SA = 46 Ci/mmol) in rat pineal gland by L-glutamate. Mean K_i value $\pm$ SEM (calculated from IC_{50} values) is 535 $\pm$ 37 nM. n = 5 pineal glands.

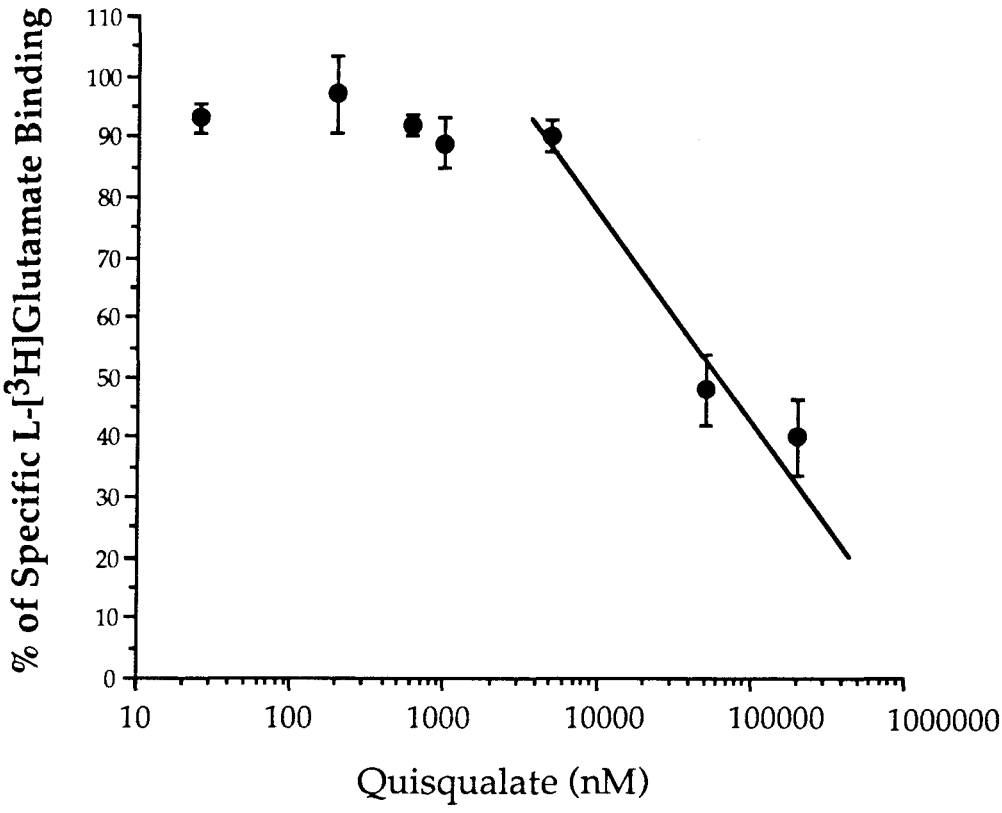


Fig. 7. Inhibition of [^{3}H]glutamate binding (279 nM, SA = 46 Ci/mmol) in rat pineal gland by quisqualate. Mean K_i value $\pm$ SEM (calculated from IC_{50} values) is 34000 $\pm$ 550 nM. n = 3 pineal glands.

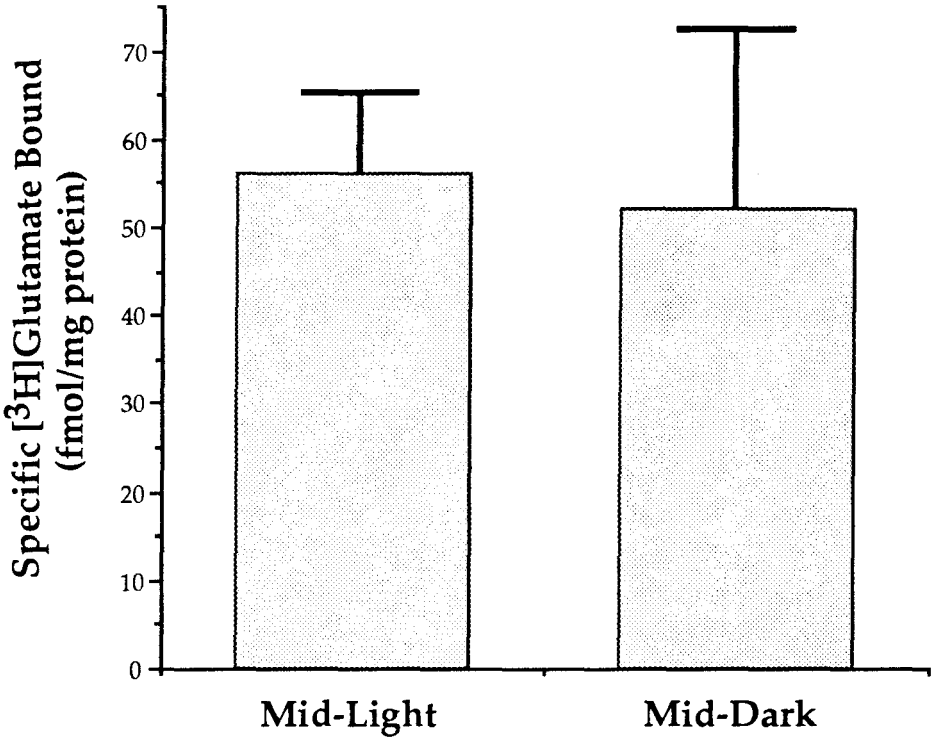


Fig. 8. Specific [^{3}H]glutamate binding (277nM, SA = 21 Ci/mmol) in pineal glands obtained from rats sacrificed during the mid-light or mid-dark period. Each bar represents the mean $\pm$ SEM of 4 pineal glands. No significant differences were detected.

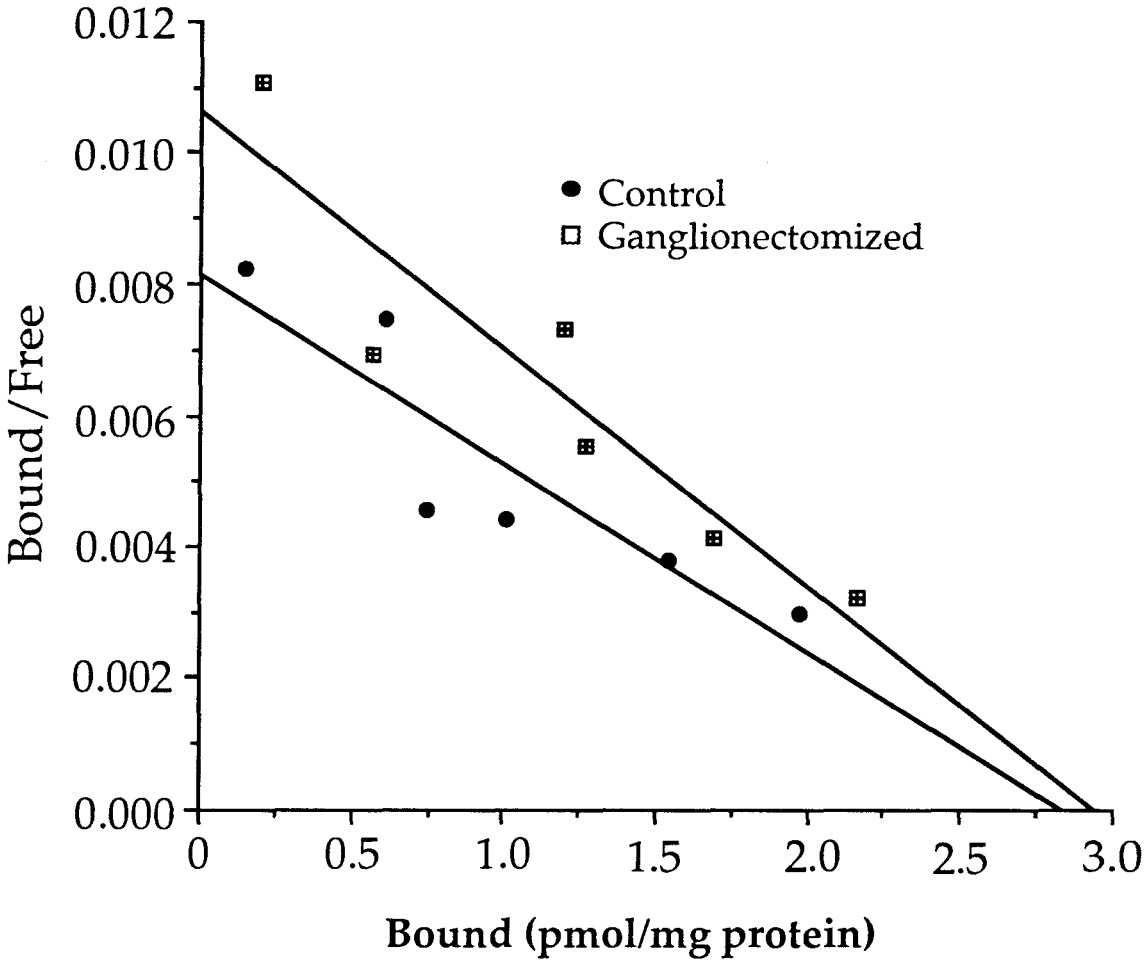


Fig. 9. Representative Scatchard graphs comparing [^{3}H]glutamate binding in pineal glands from intact and ganglionectomized animals (6 animal = 6 Scatchard graphs/group). Denervated glands (squares) possess a significantly higher apparent affinity (K_d= 412 $\pm$ 28 nM) $p<0.002$ for L-[^{3}H]glutamate binding than intact glands (circles) (K_d=612 $\pm$ 23 nM). Receptor number (B_{max}) did not change with ganglionectomy (3600 $\pm$ 240 vs 3170 $\pm$ 330 fmol/mg protein - gangX vs control, respectively).

Table 1. Inhibition of [^{3}H]glutamate binding (100 - 300 nM) in rat pineal gland by various non-radioactive compounds. Data represent the means $\pm$ SEM of 3 - 5 inhibition curves (1 pineal gland/curve). K_i values were calculated from IC_{50} values using the equation of Cheng and Prusoff (1973). Number of pineal glands are shown in parenthesis.

	K_i (nM)
L-Glutamate (5)	535 $\pm$ 37
D-Glutamate (3)	>200000
Quisqualate (3)	34000$\pm$550
CNQX (5)	>200000
N-Methyl-D-Aspartate (4)	>200000
CPP (4)	>200000
Kainate (4)	>200000
D-Aspartate (4)	>200000
APB (3)	>100000
Taurine (3)	>200000
Melatonin (3)	>200000
Norepinephrine (3)	>200

CHAPTER V

GLUTAMATE INHIBITION OF THE ADRENERGIC-STIMULATION SECRETION OF MELATONIN IN THE RAT PINEAL GLAND *IN VITRO*

Abstract

The effect of L-glutamate on the adrenergic stimulated release of melatonin in the rat pineal gland was examined using an *in vitro* perifusion system. L-Glutamate by itself had no effect on melatonin production while L-glutamate administered prior to (-)isoproterenol (β-adrenergic agonist) and L-phenylephrine (α-adrenergic agonist) inhibited melatonin production by 42 %. L-Glutamate did not inhibit melatonin production when glands were stimulated with (-)isoproterenol alone. D-glutamate as well as the L-glutamate agonists, kainate, NMDA, quisqualate and trans-ACPD had no effect on melatonin release. The possibility that L-glutamate may be converted to another neuroactive compound (GABA) prior to the addition of (-)isoproterenol and L-phenylephrine is suggested by the observation that simultaneous administration of L-glutamate with (-)isoproterenol and L-phenylephrine did not inhibit melatonin secretion.

Introduction

In the pineal gland of every mammalian species studied, the autonomic nervous system plays a pivotal role in regulating the production of the pineal hormone melatonin (Kappers 1965). NE released from the postganglionic neurons of the SCG activates the rate limiting enzyme in melatonin synthesis, SNAT, via its binding to α- and β-adrenergic receptors (Klein et al. 1987). A number of other receptor types have been described in the pineal gland including D2-dopaminergic, GABAergic, glutaminergic and cholinergic, the functions of which are presently unknown (Ebadi et al. 1986). The characterization of a binding site for L-[^{3}H]glutamate in the pineal gland of the rat (Kus et al. 1990) together with the observation that high amounts of glutamate are normally present in the gland (Govitrapong and Ebadi 1988; See Chapter IV) prompted the present investigation of the function of this binding site.

Known to act as an excitatory neurotransmitter in most regions of the central nervous system, glutamate has been reported to inhibit SNAT activity in the pineal gland of the rat *in vitro* (Govitrapong and Ebadi 1988). Inhibitory effects of glutamate are, however, rare (Mayer and Westbrook 1987). Accordingly, we examined the role of glutamate in pineal gland function by testing the effects of various glutamate agonists on the adrenergic stimulated secretion of melatonin *in vitro*.

Materials and Methods

Chemicals

Quisqualate was obtained from Research Biochemicals Inc. (Natick, MA). Trans-ACPD was a gift from D. Monaghan (Omaha, NE). All other compounds were purchased from Sigma (St. Louis, MO).

Animals

A total of 58 adult (2 - 4 months) male Sprague-Dawley rats (Sasco-King, Oregon, WI) were used in this study. Animals were housed in a fully accredited animal care facility and were used according to the recommendations in the *Guide for the Care and Use of Laboratory Animals* and *The Guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center.* All animals were entrained to a 12:12 hour light-dark cycle (1000 lux). For all experiments, animals had access to food and water *ad libitum.* Animals were sacrificed by rapid decapitation during the middle of the light cycle.

Perifusion

All experiments were performed using an Acusyst-S-APS 10 programmable flow through perifusion sysytem (Endotronics, Coon Rapids, MN). Glands were rapidly dissected and placed in ice cold oxygenated (95% O_2, 5% CO_2) artificial cerebral spinal fluid (CSF) (pH 7.3) during the sampling period. Tissue was then transferred to the perifusion chamber (4X10 mm) where prewarmed (37^{o}C), oxygenated artificial CSF was delivered at a rate of 0.208 ml/minute or 0.037 ml/minute in experiments where LDH was assayed. One pineal gland was placed in each chamber. Pineal glands were

equilibrated by preincubation for a period of 4 hours at which time melatonin output reached basal levels. Tissue was incubated in either the presence of (-)isoproterenol (Iso) (10^{-5}M-10^{-6}M) and L-phenylephrine (PE) (10^{-5}M-10^{-6}M) or L-glutamate alone (10^{-3}M) In experiments that examined the effect of glutamate agonists on the Iso and PE-stimulated secretion of melatonin from pineal glands, the following paradigms were used: L-Glutamate (5 X 10^{-6}M - 5 X 10^{-3}M) or D-glutamate (10^{-3}M) were administered to glands 4 hours after the start of the experiment (4.0 hr) for 60 minutes followed by Iso (10^{-6}M) and PE (10^{-6}M) at 5.0 hr for 25 minutes. Glutamate agonists, kainate, NMDA, and quisqualate (10^{-4}M), were administered to glands at 4.5 hour for 30 minutes folowed by Iso (10^{-6}M) and PE (10^{-6}M) at 5.0 hr for 25 minutes. Trans-ACPD (10^{-4}M) was administered to glands at 4.0 hour for 30 minutes folowed by Iso (10^{-6}M) and PE (10^{-6}M) at 5.0 hr for 25 minutes. D,L-APB (2 X 10^{-4}M) was administered to glands at 4.5 hour for 30 minutes folowed by Iso (10^{-6}M) and PE (10^{-6}M) at 5.0 hr for 25 minutes. Mg^{2+} was omitted and glycine (10^{-6}M) was added to the buffer when experiments included NMDA because Mg^{2+} inhibits and glycine potentiates the action of NMDA at its receptor. Samples of perfusate were collected every 30 minutes by a fraction collector and stored at 4°C until assayed for melatonin content.

Radioimmunoassay

Artificial CSF obtained from *in vitro* perifusion studies was assayed for melatonin with the use of a direct radioimmunoassay based on a modified method of Fraser (Fraser et al. 1983). Briefly, samples (100µl) were incubated with antiserum (sheep antiserum 704-8483, diluted 1:30,000, Guildhay, U.K.) and labeled ([^{3}H] melatonin 81.1 Ci/mmol) overnight at 4°C

in tricine buffered saline (TBS) with 0.1% gelatin (pH 5.5). Bound and free melatonin were separated by the addition of a dextran coated charcoal solution (2.5 g charcoal, 0.25 g dextran/l TBS) to samples. Samples were centrifuged at 1500 X G for 15 minutes and the supernatant decanted into scintillation vials. Bound radioactivity was counted in a Packard tri-carb beta scintillation counter. Unknown sample values were calculated from a standard curve constructed from known amounts of melatonin (0 -100 pg). Lower limit of sensitivity was 2.0 - 3.0 pg/tube. Intra-assay and inter-assay coefficients of variability were 8.0% and 15% respectively.

High Performance Liquid Chromatography (HPLC) of Amino Acids

General methods for HPLC have been described elsewhere (McNulty et al. 1987; McNulty et al. 1990). Briefly, pineal glands were quickly dissected, frozen on dry ice, and stored at -70°C until assayed. Glands were homogenized in cold 0.05 M monobasic phosphate buffer (pH 7.0). Homogenates were centrifuged at 15,000X g for 2 mins and aliquots of supernatant were injected directly into the chromatographic system consisting of a Rainin Rabbit pump, a Rheodyne 7125 inject port with a 100 µl sample loop, and a C18 reverse phase column (10 cm X 4.6 cm, 3 mm particle size, Rainin Instruments, Woburn, MA). Levels of free amino acids were determined using pre-column derivatization with o-phthaldialdehyde (OPA; Sigma) as described previously (McNulty et al. 1990). Fluorescence emitted by the derivatized compounds was measured with a LDC Fluoromonitor III (Milton Roy, Riviera Beach, FL) equipped with standard excitation (340-380 nm) and emission (418-700 nm) filters. The detector was connected to a HP 3390A integrator (Hewlett Packard, Avondale, PA) used to analyze the peaks.

The mobil phase consisted of 0.1 M monobasic sodium phosphate mixed with HPLC-grade methanol (32%) and delivered at a rate of 1.0 ml/min. Identification of eluted compounds were based on retention times, and the concentration of samples were determined by comparison with synthetic standards made daily from stock solutions. Aliquots of supernatant were reserved for the measurement of soluble proteins according to the method of Lowry et al. (1951).

Lactate Dehydrogenase (LDH) Assay

Secretion of LDH, an intracellular enzyme, was measured as an indicator of cell damage or death using a colorimetric kit (Sigma). LDH in the buffer catalyzed the following reaction: pyruvic acid + NADH , lactic acid + NAD. Unconverted pyruvic acid, when treated with 2,4-dinitrophenylhydrazine, produces a phenylhydrazone that is highly colored. LDH activity is inversely proportional to the absorbance of the color formed. Absorbance was measured at a wavelength of 464 nm.

Statistics

All data points are presented as mean $\pm$ SEM. Data obtained from experiments comparing melatonin and LDH release from control and experimental pineal glands over time were analysed with a two-way ANOVA with repeated measures. Total melatonin secreted from each gland 4.5 hours to termination of experiment was calculated and means of control and experimental glands were compared with a Student's T-test. Differences in amino acid content of control and experimental pineal glands were analyzed with a Student's T-test.

Results

Stimulation of pineal glands by adrenergic agonists

Spontaneous release of pineal gland melatonin reached baseline (10-20 pg/min/gland) by 4 hours after the start of the experiment (Fig. 1). At this time most drug admininstration was initiated. A similar time course for the decrease in spontaneous melatonin release has been previously reported (Simonneaux et al. 1989). This unstimulated release of melatonin from the gland was most likely due to cell damage caused by removal of the gland from the brain. LDH release, an indicator of cell damage, had a pattern similar to spontaneous release of melatonin shown in Fig. 2. Differences in the initial release of melatonin by pineal glands between experiments may be due to differences in sampling time, i.e the amount of time glands remained in cold buffer before being transferred to the perifusion apparatus. Pineal glands were stimulated by Iso and PE in a dose dependent manner (10^{-6} - 10^{-5}M) with a detectable increase in melatonin secretion approximately 1.5 hours after Iso and PE administration (Fig. 3). Addition of PE (10^{-6}M) to pineal glands did not potentiate the β-adrenergic-induced (Iso (10^{-6}M) and prazosin (10^{-6}M)) response (Fig. 4).

Effect of L-glutamate on melatonin production

L-Glutamate (10^{-3}M) by itself neither stimulated the secretion of melatonin nor significantly altered baseline levels (Fig. 5). L-Glutamate (10^{-3} M) administered before Iso (10^{-6}M) and PE (10^{-6}M) significantly inhibited melatonin secretion ($p<0.025$ ANOVA), decreasing hormone concentrations by approximately 42% (Fig 6). Lower doses of L-glutamate (5 X 10^{-6} M and 5 X

10^{-4} M) (Figs. 7, 8) had no attenuating effect. A higher dose of glutamate (5 X 10^{-3}M (Fig. 9) did not significantly decrease melatonin secretion ($p<0.065$ ANOVA). D-glutamate (10^{-3}M) was ineffective (Fig. 10).

Melatonin production stimulated by Iso (10^{-6}M) alone was not inhibited by 1 hour pretreatment with L-glutamate (10^{-3}M) (Fig 11). To assure that the alpha-1 receptor was not activated during these experiments, prazosin (10^{-6}M), an $\alpha 1$ antagonist was added to the artificial CSF. To rule out any stimulatory effects of prazosin, the experiment was repeated, but without the addition of prazosin (Fig. 12). L-Glutamate (10^{-3}M), again, did not inhibit the Iso (10^{-6}M) stimulated secretion of melatonin.

Effect of L-glutamate agonists on melatonin secretion

Agonists of the four major glutamate receptor subtypes were assayed for their ability to inhibit Iso (10^{-6}M) and PE (10^{-6}M) stimulated production of melatonin. NMDA, kainate, quisqualate (100μM) (AMPA and metabotropic receptors), D,L-APB (200μM) and trans-ACPD (100μM) (metabotropic receptor) were all ineffective at attenuating the melatonin secretion stimulated by Iso and PE (Figs. 13 - 17).

Timing of L-glutamate administration

L-glutamate administered 2 hour before Iso and PE did not significantly decreased melatonin secretion ($p<0.42$ ANOVA) (Fig. 18). Simultaneous administration of L-glutamate and Iso and PE at 4.0 hour for 25 minutes did not inhibit melatonin secretion (Fig. 19).

Amino acid content of pineal glands administered L-glutamate

The only amino acid found to increase significantly in pineal glands administered L-glutamate (10^{-3}M) was L-glutamate itself, ($p<0.02$ T-test) (Fig 20). Glutamine levels in the tissue were somewhat higher (~ 27%) although not statistically significant.

Discussion

The importance of glutamate as a neurotransmitter within the central nervous system has been clearly established (Mayer and Westbrook 1987). Only recently gaining recognition as a modulator of neuroendocrine function in the mediobasal hypothalamus (Van den Pol et al. 1990), L-glutamate has also been reported to stimulate the release of prolactin from pituitary glands (Login 1990) and the release of catecholamines from isolated adrenal medulla (Nishikawa et al. 1982). L-Glutamate in this study, while having no effect by itself, inhibited melatonin production in adrenergically stimulated pineal glands. From an earlier study by Govitrapong and Ebadi (1988) demonstrating that glutamate inhibits NE-induced SNAT activity it appears that inhibition of melatonin production by glutamate is most likely due to mechanisms involving the SNAT enzyme. The present study extends the observations of Govitrapong and Ebadi (1988) by demonstrating that inhibition of glutamate occurs only if both β-and α-adrenergic receptors are stimulated. If the β-adrenergic receptor is stimulated alone, no inhibition occurs. This suggests an important role for the α-adrenergic receptor in the glutamate induced inhibition of melatonin synthesis.

Because inhibitory effects of glutamate are rare, the possibility that cell death contributed to the decrease in melatonin release was considered. Glutamate-induced toxicity as seen in neurons is very complicated, involves many steps and is not fully understood (Choi and Rothman 1990). When cells are over stimulated by glutamate, i.e. too much is released or it is not removed from the extracellular space, cells begin to swell as an excess amount of sodium enters. This is followed by Cl^- and water and leads to osmotic lysis of the cells (Olney et al. 1986). Glutamate, via the NMDA receptor, can also stimulate excess Ca^{2+} influx into cells. An increase in intracellular Ca^{2+} activates many enzymes including phospholipase A2 which leads to an increase in arachidonic acid (Choi and Rothman 1990). Arachidonic acid can lead to an increase in free radical formation via the enzymes lipoxygenase and cyclooxygenase. Because activation of the α-adrenergic receptor stimulates arachidonic acid formation in the pineal gland, it might be hypothesized that glutamate acting at its pineal binding site synergizes with the α-adrenergic receptor to produce toxic levels of this compound. Studies designed to measure levels of arachidonic acid in glands treated with glutamate and Iso and PE would test this hypothesis.

Another mechanism by which the Iso- and PE-induced production of melatonin may be inhibited by glutamate may be through depolarization of the pinealocyte membrane. It has been demonstrated that agents that cause depolarization of the pinealocyte membrane, e.g. oubain and K^+, lead to an inhibition of the NE-induced membrane hyperpolarization as well as to an inhibition of SNAT activity (Parfitt et al. 1975). Interestingly, a decrease in cAMP formation was not noted in these experiments. In another study in which the effects of αMSH on pineal gland function were examined, it was

noted that depolarization of the membrane by αMSH inhibits the ability of NE to stimulate cAMP formation (Sakai et al. 1976). The mechanism by which membrane depolarization inhibits cAMP or SNAT activity is not known.

In addition to the two possible aforementioned mechanisms of glutamate inhibition of melatonin secretion, another possible mechanism should be considered. While it has been well documented that activation of the α1-adrenergic receptor can potentiate the β-adrenergic induced production of cAMP (Klein et al. 1987), it has been recently been demonstrated that exclusive activation of the α isoform of PKC leads to the inhibition of cAMP formation. The isoforms identified in the pineal gland are the α (80%) and β (20%) forms. Glutamate may interact synergistically with the α1-adrenergic receptor to stimulate production of the α-form of the enzyme which would then inhibit cAMP formation. This hypothesis could be tested by examing the isoforms of PKC that are produced after treatment of the gland with glutamate in conjunction with Iso and PE.

The minimum concentration of glutamate required to inhibit melatonin production was 1mM, approximately 7 times the concentration of glutamate found in plasma (Pratt 1976). When compared to doses known to be toxic in neuronal cell culture, e.g. 100μM for 5 min (Choi and Rothman 1990), this dose appears excessively high. However, whole tissue such as that used in these experiments possesses uptake systems for glutamate that remove excess amino acid from the extracellular space. As demonstrated by the significant increase in glutamate content of the pineal gland 2.5 hours after administration of exogenous glutamate, the pineal gland is capable of such uptake. Interestingly, the amount of glutamate taken up by the gland

did not produce overall levels of glutamate that were significantly higher than those seen in non-perifused samples (18.08 $\pm$ 0.94 and 15.58 $\pm$ 2.86 µg/mg protein, perifused and non-perifused tissue respectively). Other studies such as those employing whole retina have also reported that high concentrations of glutamate (0.3 - 30 mM) are required to evoke a physiological response (Perlman et al. 1987; Zeevalk et al. 1989).

While the mechanism of glutamate's interaction with the Iso and PE stimulated production of melatonin is not clear, it is probably not due to binding of glutamate with any of the currently characterized glutamate receptor subtypes. In agreement with the study in Chapter IV that demonstrated an inability of kainate, NMDA and quisqualate (which has a relatively high affinity for both the AMPA and metabotropic receptors) to inhibit [^{3}H]glutamate binding in the pineal gland, these agonists at 10^{-4}M had no effect on the Iso and PE stimulated production of melatonin. Likewise, trans-ACPD, a ligand specific for the glutamate metabotropic receptor (Palmer et al. 1989) and D,L-APB had no effect. A similar lack of stimulation by glutamate agonists was seen in mammalian (kainic acid) and turtle photoreceptors (Abrams et al. 1989; Tachibana and Kaneko 1988). Because turtle retinal photoreceptors responded to L- and D-aspartate as well as L-glutamate, Tachibana and Keneko (1988) suggested that glutamate exerted its effects on membrane depolarization via the interaction of glutamate with an electrogenic uptake carrier. Other characteristics of the photoreceptor response to glutamate included increased current fluctuations accompanying the glutamate-induced current. Based on this observation, Tachibana and Keneko (1988) suggested that an ion channel may be involved in the glutamate induced response. Whether similar mechanisms are involved in

glutamate's inhibition of melatonin production in the pineal gland is not known.

The observation that glutamate must be administered before Iso and PE in order to have an effect (see Figs. 6, 19) suggests an alternative mechanism for glutamate's action, i.e. the conversion of glutamate to another compound. Intracellular utilization of glutamate is supported by the observation that glutamine content in the pineal increased by 27% after glutamate administration. Similarly, it was reported that when [^{3}H]glutamate was incubated with brain tissue at 30^{o}C for 60 minutes, up to 30% of the radiolabel was not associated with glutamate at the end of the incubation period (Anderson and Vickroy 1990). It is therefore conceivable that the inhibitory effects of glutamate are mediated via its decarboxylation to GABA. While GABA is found in only small amounts in the rat pineal (Mata et al. 1976), this amino acid is able to inhibit melatonin production *in vitro* (Rosenstein et al. 1989). The stimulus for the release of GABA from the pineal is the activation of the α1 adrenergic receptor (Rosenstein, et al. 1989). The conversion of glutamate to GABA within the pineal gland would explain the inability of glutamate to inhibit melatonin production when stimulated by Iso alone. Activation of the α-adrenergic receptor by PE would be required to release GABA from the cells which would then allow it to bind to its membrane receptor, although there is no direct evidence to support this hypothysis. This explanation is not consistent with the observations of Govitrapong and Ebadi (1988) who reported that L-allyl-glycine, a GAD inhibitor, did not inhibit the glutamate induced inhibition of SNAT activity. These authors failed, however, look at the affects of L-allyl-glycine alone on NE-stimulated SNAT activity.

The present observation that PE did not potentiate the Iso-stimulated secretion of melatonin was an unexpected finding. In the rat pineal gland, PE has been demonstrated to potentiate the Iso-stimulated production of second messenger (cAMP) (Sugden et al. 1986; Vanecek et al. 1985) and SNAT activity (Alphs and Lovenberg 1984; Klein et al. 1983; Sugden et al. 1984). While potentiation of melatonin production by PE has been demonstrated in other species such as hamster and ovine (Howell and Morgan 1991; Santana et al. 1989), it has not been demonstated unequivocally in rat. As reported in both the hamster and ovine pineal gland, however, cAMP/SNAT activity and melatonin production do not always parallel one another (Howell and Morgan 1991; Nilsson and Reiter 1989). This suggests that an increase in cAMP levels and SNAT activity may not necessarily be accompanied by an increase in melatonin production. It is unlikely that the lack of potentiation in the present study was due to a maximal effect on melatonin production because the amount of hormone could be further increased with a higher dose of Iso (10^{-5}M).

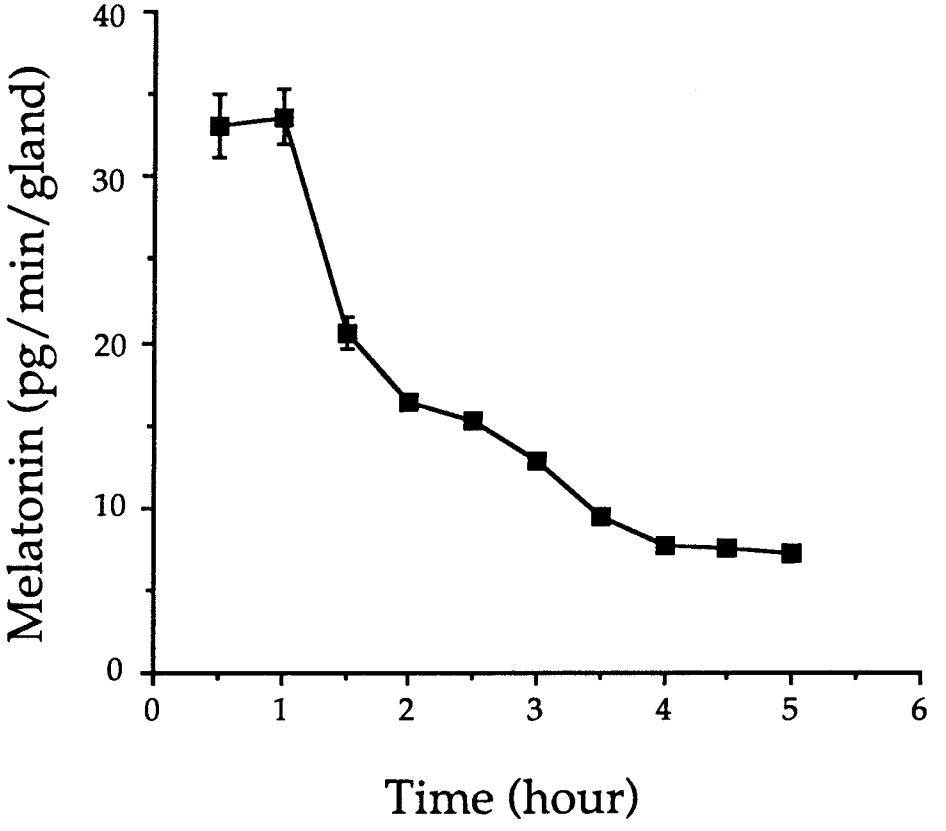


Fig. 1. Spontaneous melatonin release from pineal glands cultured *in vitro*. n = 3 pineal glands.

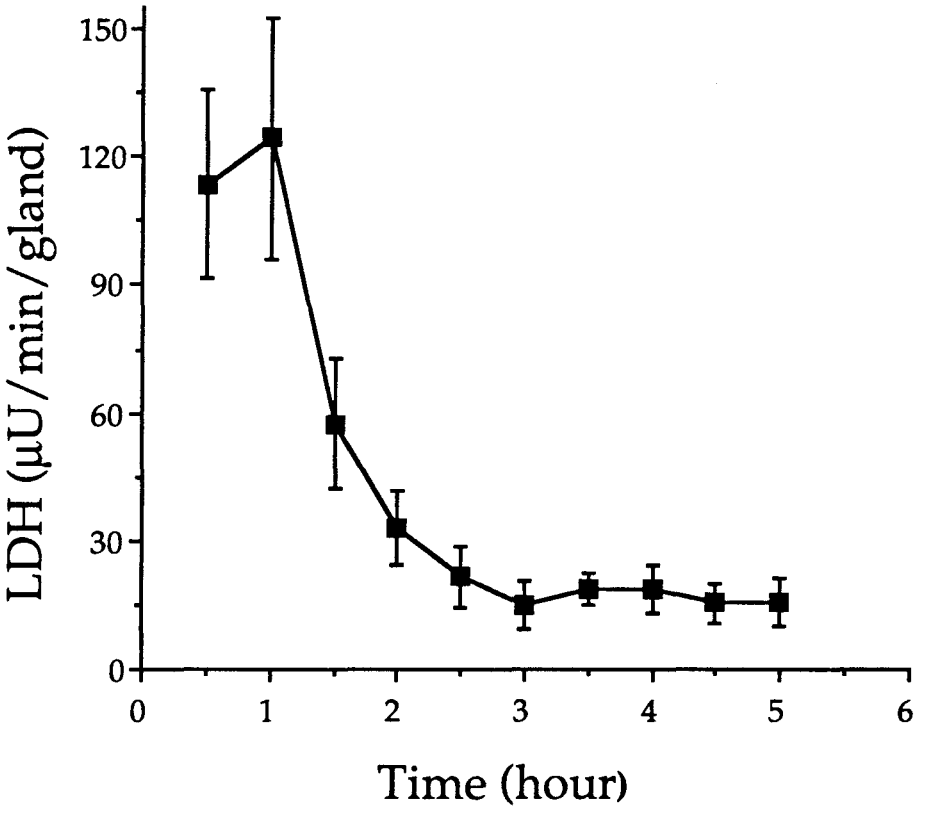


Fig. 2. Spontaneous LDH release from pineal glands cultured *in vitro*. n = 6 pineal glands.

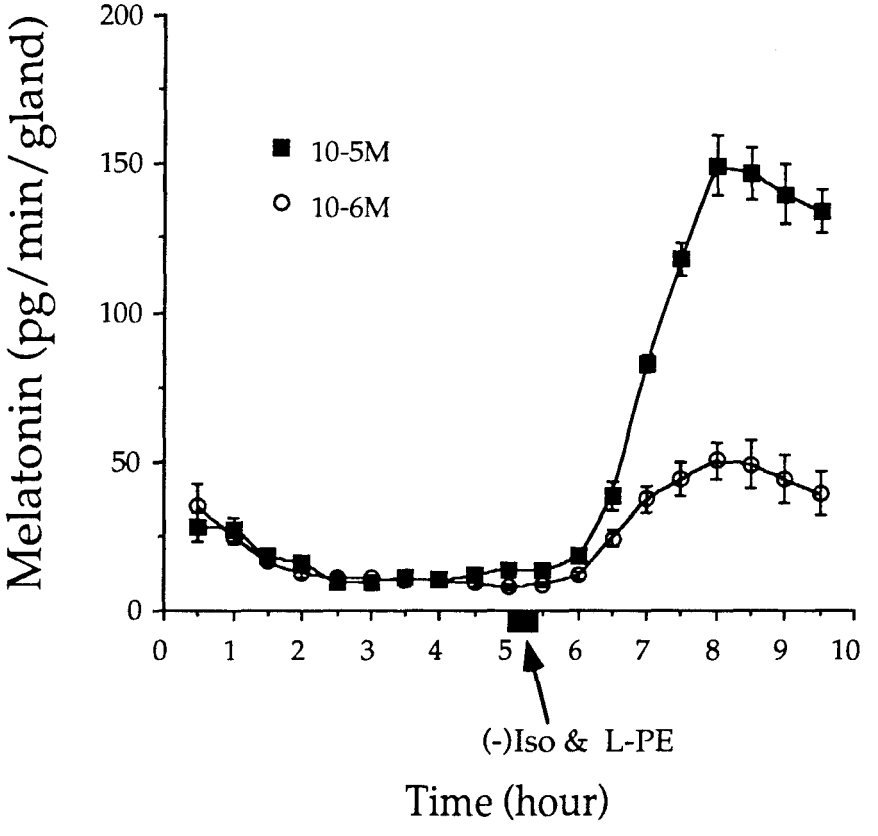


Fig. 3. Dose response of (-)isoproterenol and L-phenylephrine (10^{-5} and10^{-6}M) stimulated melatonin secretion *in vitro*. n = 6 pineal glands.

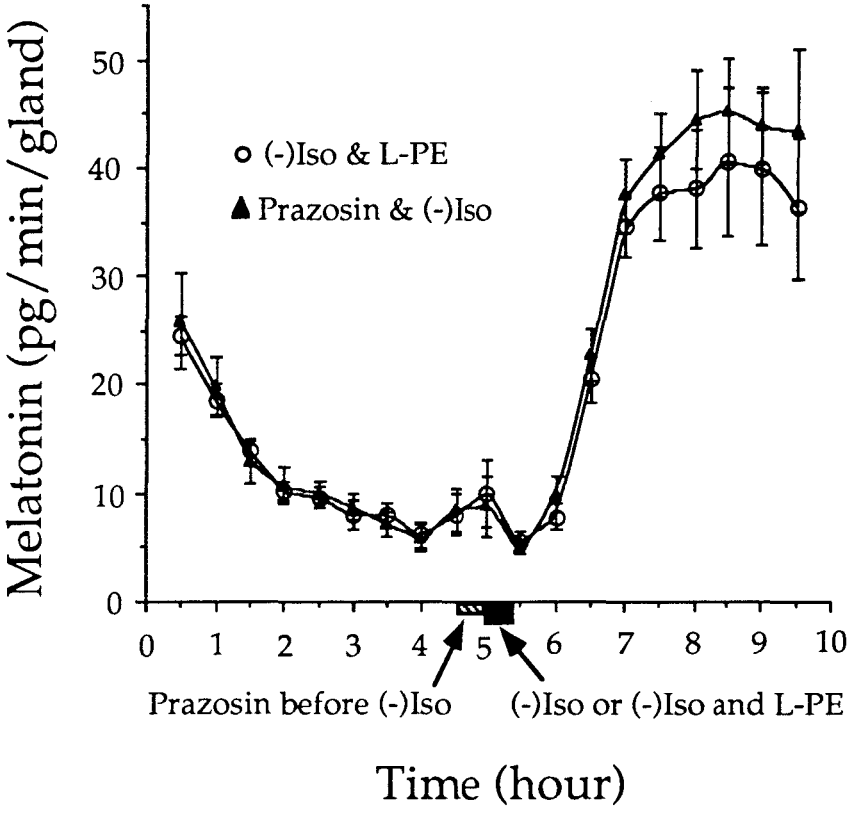


Fig. 4. The amount of melatonin secreted by pineal glands treated with (-)isoproterenol and L-phenylephrine (10^{-6}M) does not differ significantly from the amount of melatonin produced by glands pretreated with prazosin (10^{-6}M) and administered (-)isoproterenol alone (10^{-6}M). n = 6 pineal glands/group.

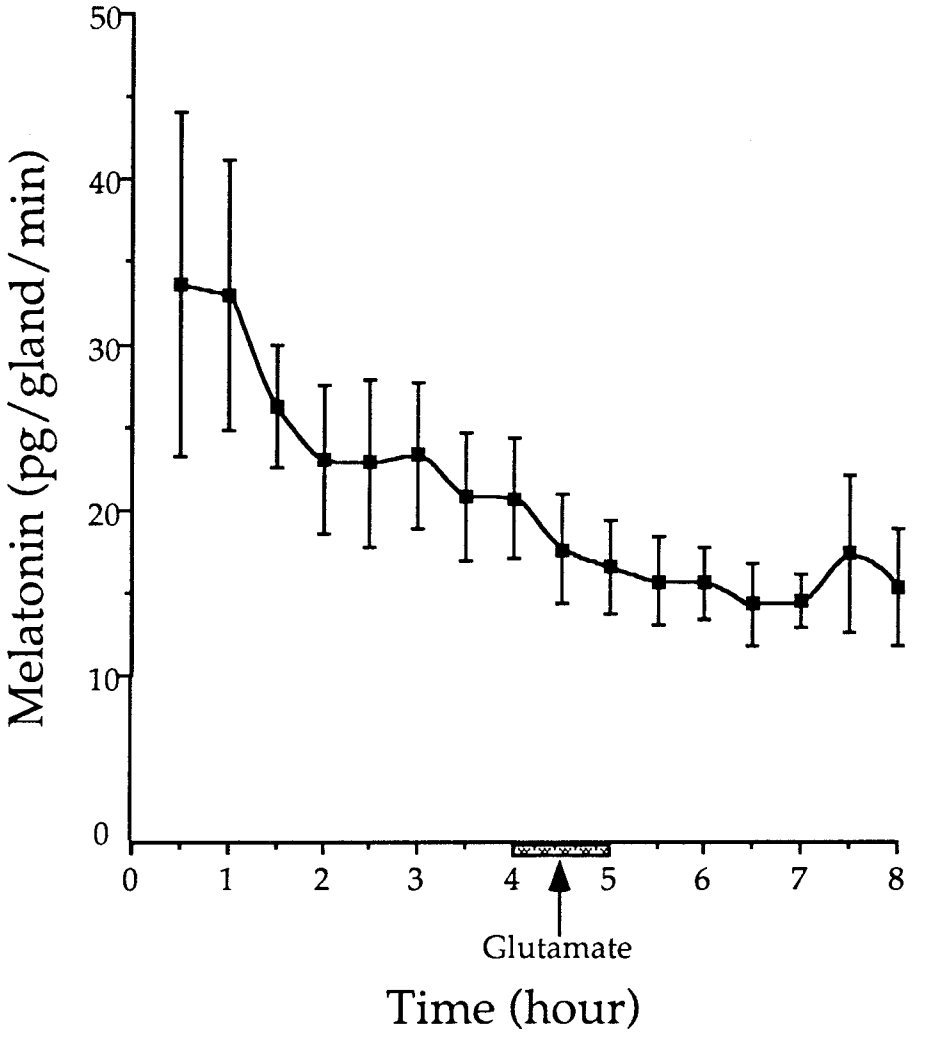


Fig. 5. Glutamate (10^{-3}M) administered to pineal glands by itself has no effect on melatonin secretion or spontaneous release. n = 3 pineal glands.

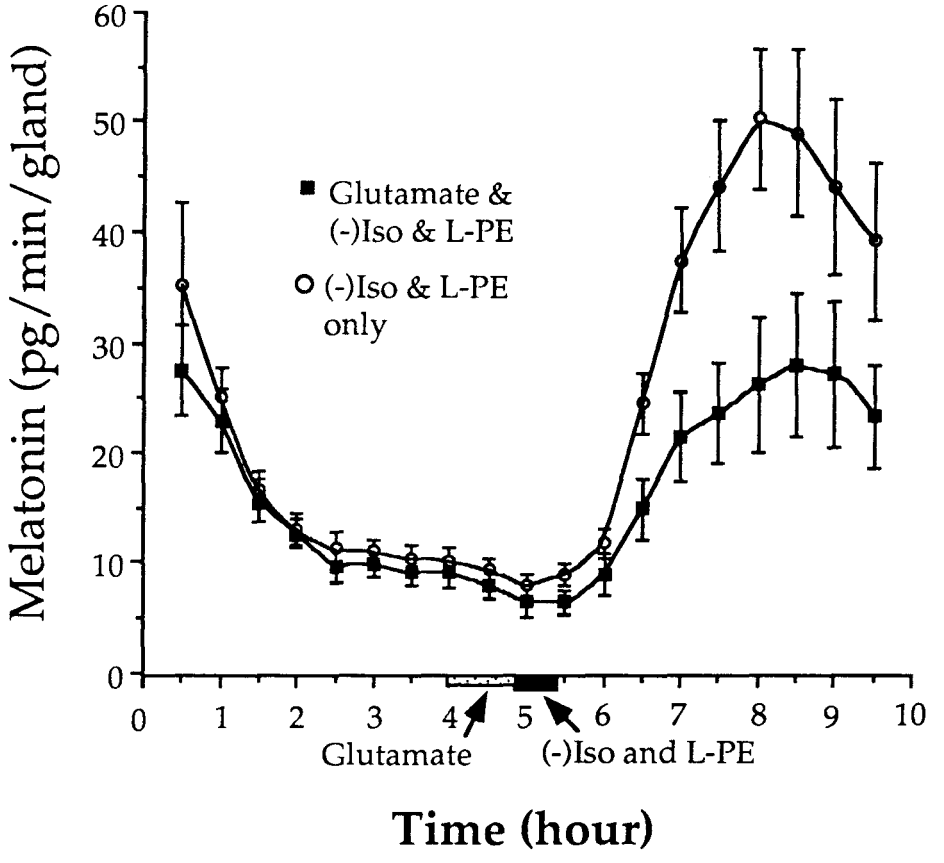
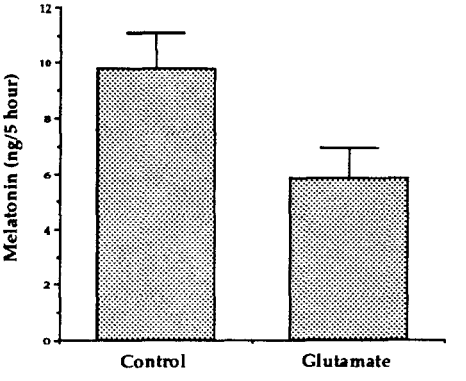


Fig. 6. Pretreatment of pineal glands with 10^{-3}M L-glutamate significantly inhibited melatonin secretion stimulated by 10^{-6}M (-)isoproterenol and L-phenylephrine ($p<0.025$ ANOVA). n = 9 pineal glands/group. Bar graphs represent the sum of melatonin secreted after L-glutamate administration ($p<0.03$ T-test).



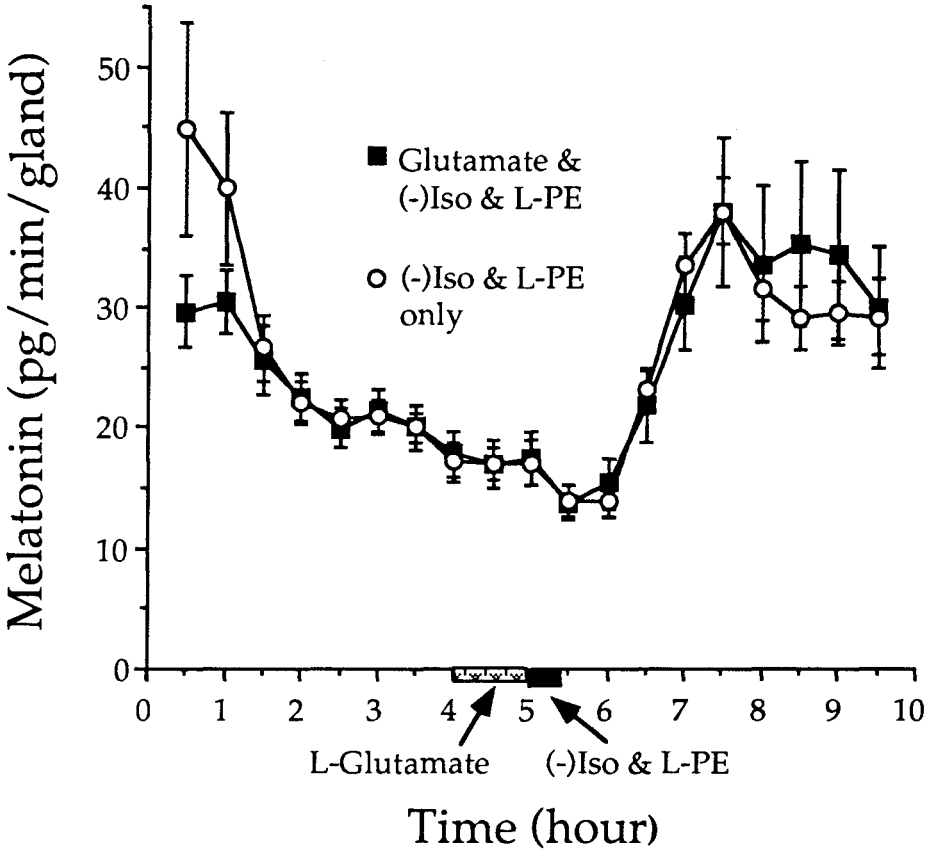


Fig. 7. L-Glutamate (5 X 10^{-6}M) pretreatment of pineal glands does not inhibit the (-)isoproterenol and phenylephrine (10^{-6}M) stimulated secretion of melatonin. n = 9 pineal glands/group.

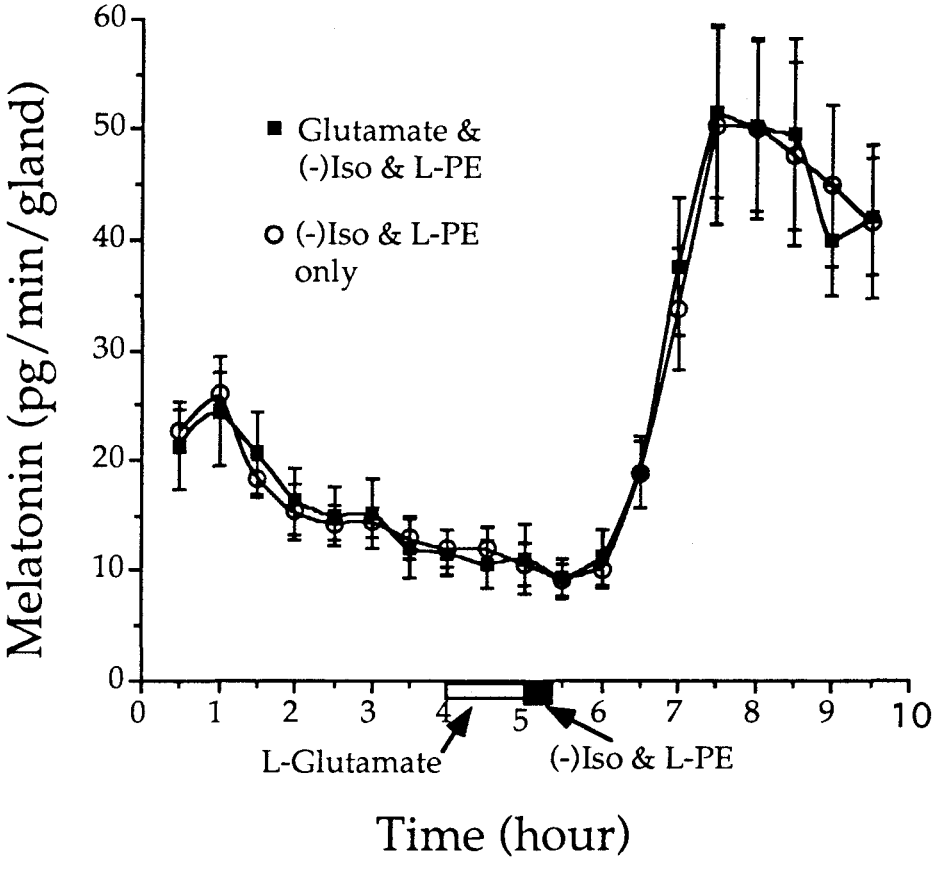


Fig. 8. L-Glutamate (5 X 10^{-4}M) pretreatment of pineal glands does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6}M) stimulated secretion of melatonin. n = 6 pineal glands/group.

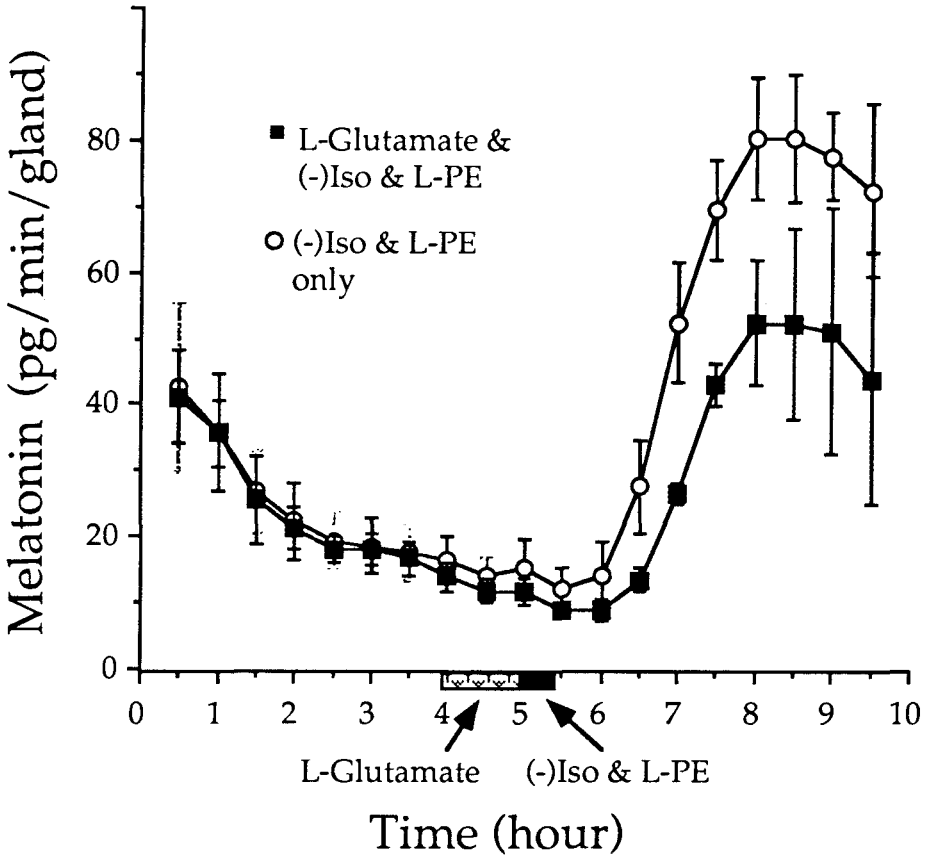
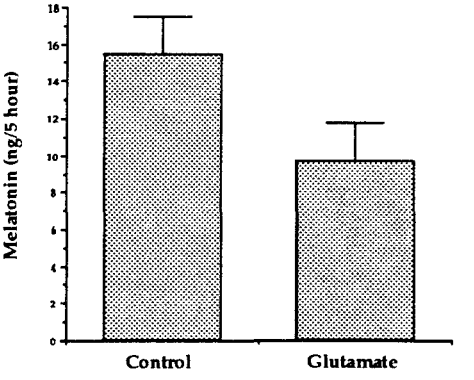


Fig. 9. Pretreatment of pineal glands with 5 X 10^{-3}M L-glutamate did not statistically inhibited melatonin secretion stimulated by 10^{-6}M (-) isoproterenol and L-phenylephrine ($p<0.065$ ANOVA). n = 3 pineal glands /group. Bar graphs represent the sum of melatonin secreted after L-glutamate administration ($p< 0.07$ T-test)



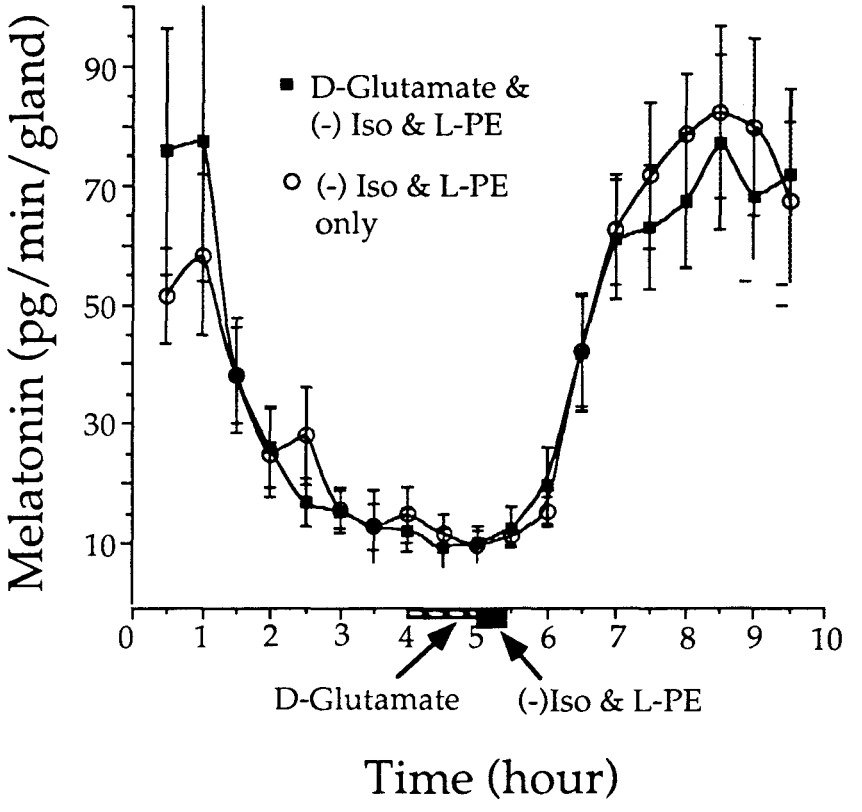


Fig. 10. Pretreatment of pineal glands with D-glutamate (10^{-3}M), the inactive isomer, does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6}M) stimulated secretion of melatonin. n = 6 pineal glands/group.

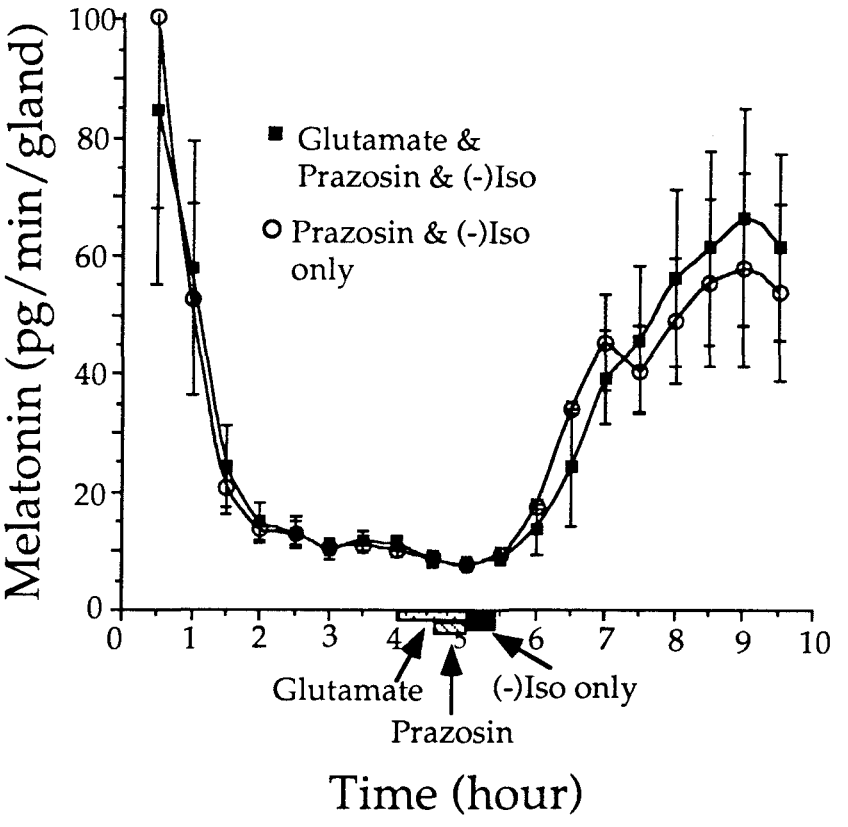


Fig. 11. Pretreatment of pineal glands with 10^{-3}M L-glutamate did not inhibited melatonin secretion stimulated by 10^{-6}M (-)isoproterenol alone. 10^{-6}M Prazosin was added to block the α1-adrenergic receptor. n = 6 pineal glands/group.

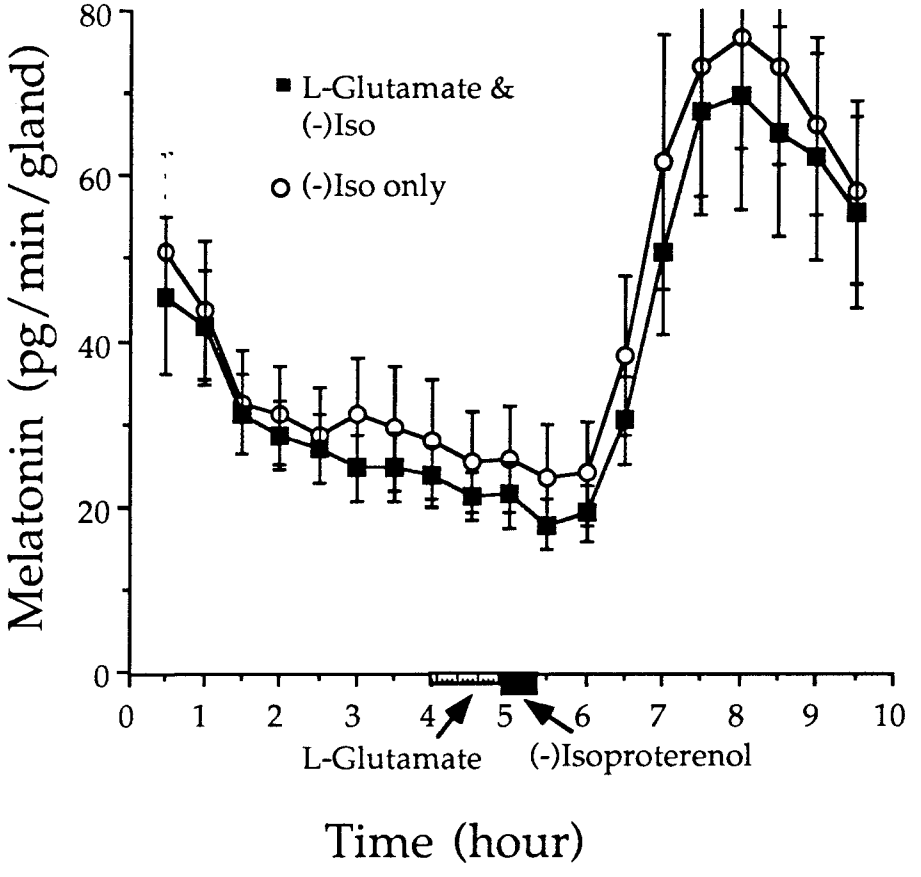


Fig. 12. Pretreatment of pineal glands with L-glutamate (10^{-3}M) does not inhibit the (-)isoproterenol (10^{-6}M) stimulated secretion of melatonin. Prazosin was not added in this experiment. n = 6 pineal glands/group.

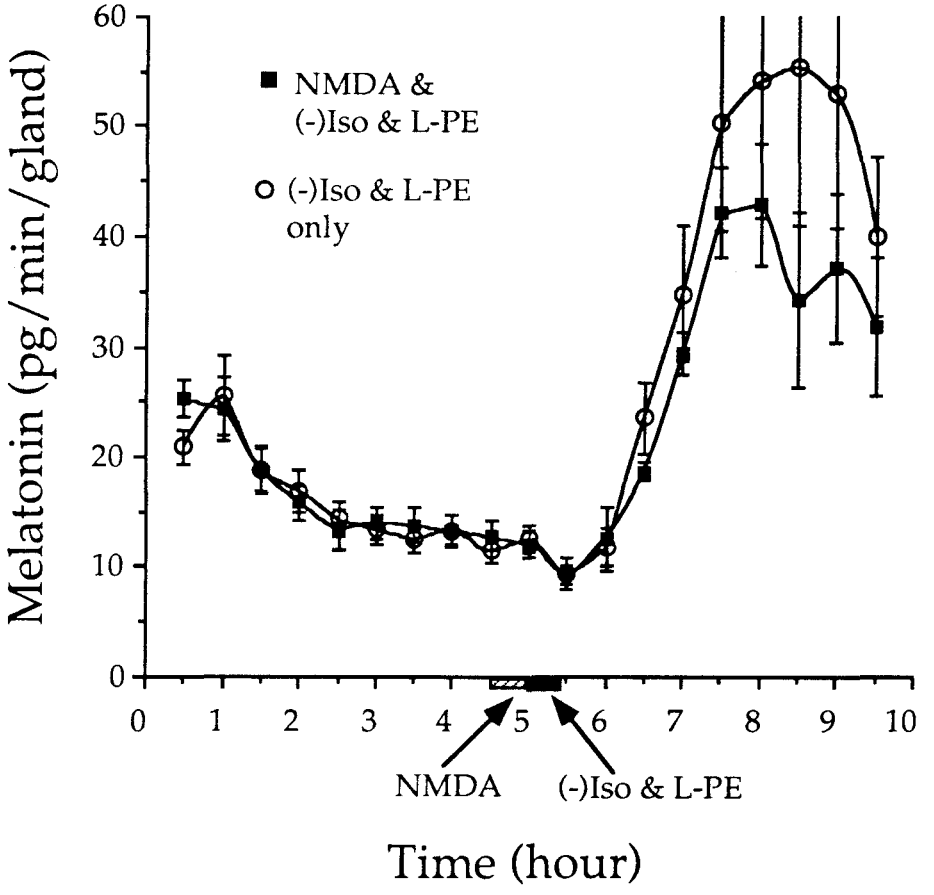
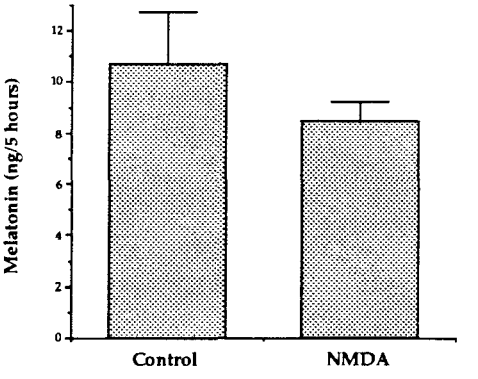


Fig. 13. Pretreatment of pineal glands with NMDA (10^{-4}M) does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6}M) stimulated secretion of melatonin. n = 6 pineal glands/group. Bar graphs represent the sum of melatonin secreted after L-glutamate administration ($p<0.29$ T-test).



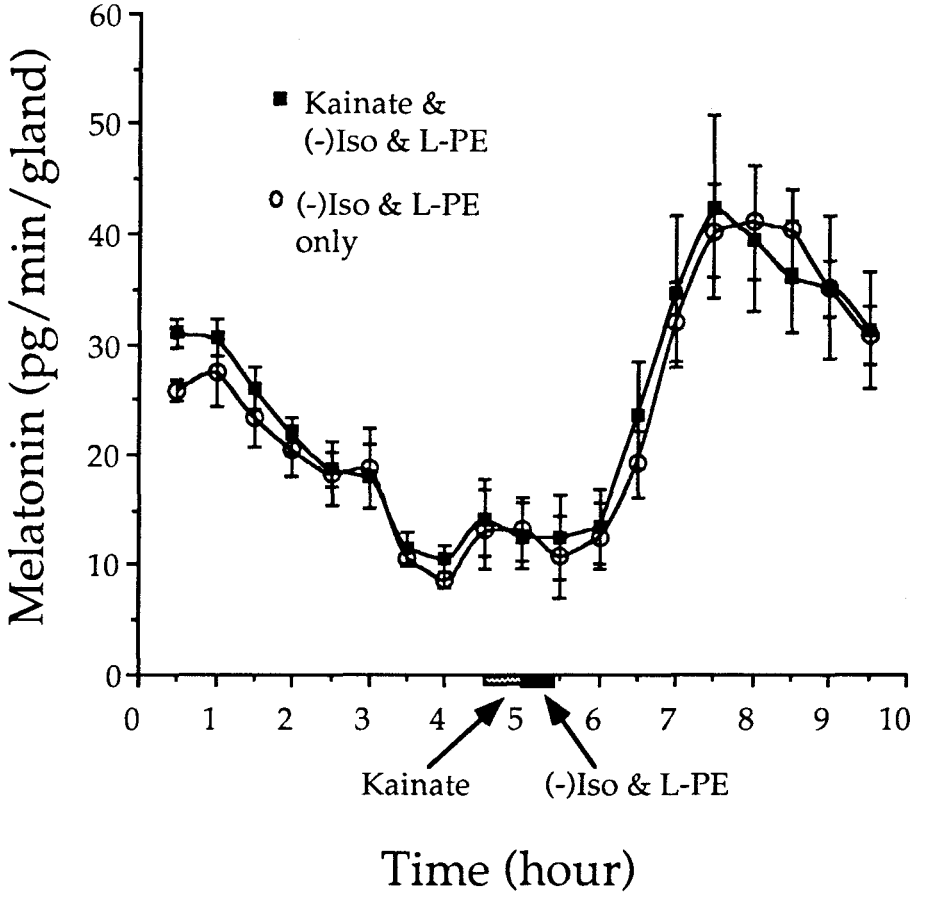


Fig. 14. Pretreatment of pineal glands with kainate (10^{-4} M) does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6} M) stimulated secretion of melatonin. n = 6 pineal glands/group.

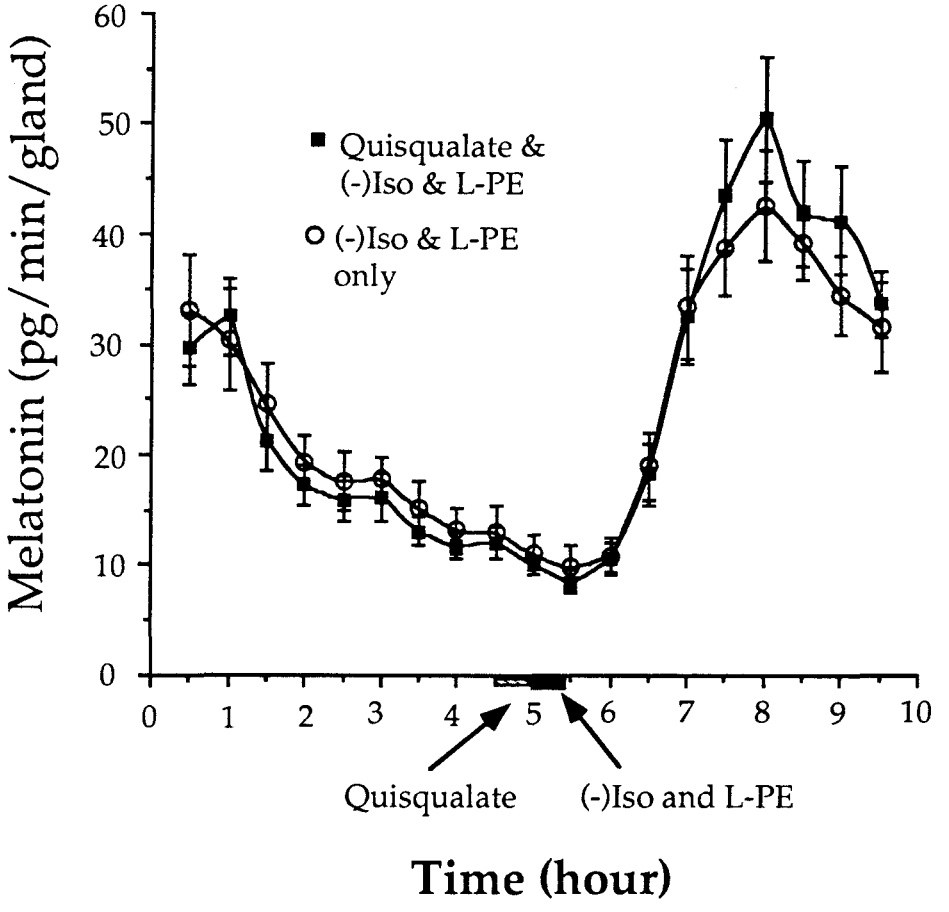


Fig. 15. Pretreatment of pineal glands with quisqualate (10^{-4}M) does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6}M) stimulated secretion of melatonin. n = 6 pineal glands/group.

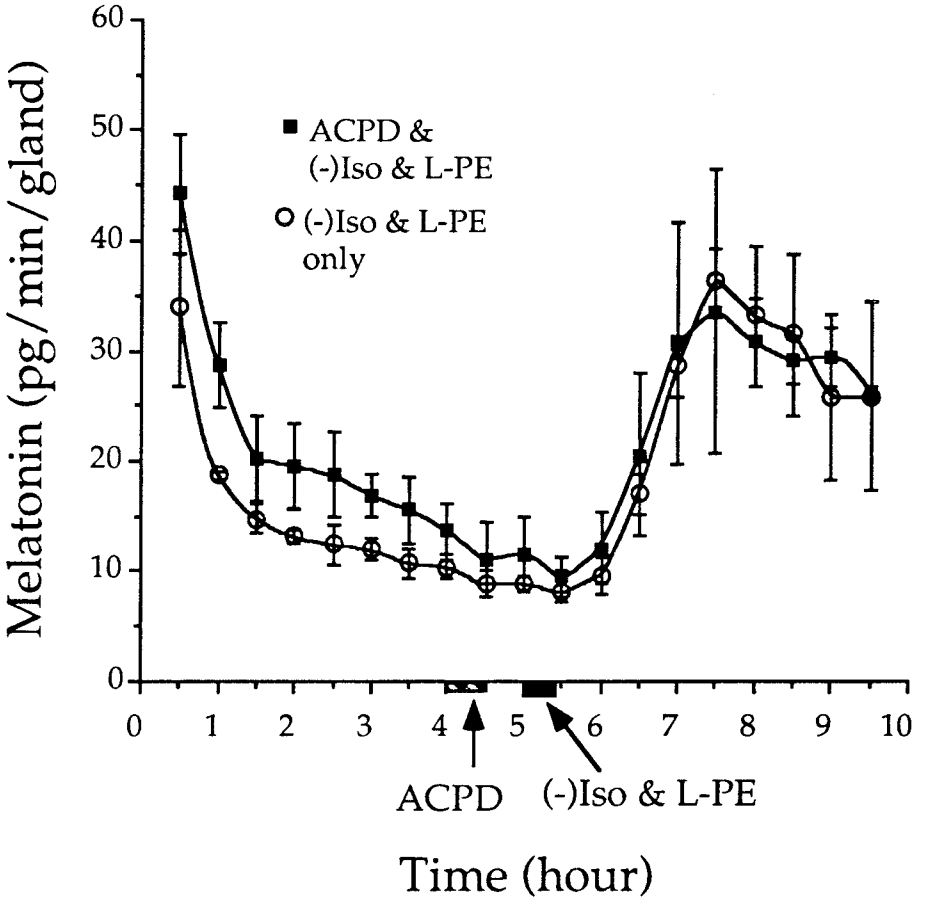


Fig. 16. Pretreatment of pineal glands with trans-ACPD (10^{-4} M) does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6} M) stimulated secretion of melatonin. n = 3 pineal glands/group.

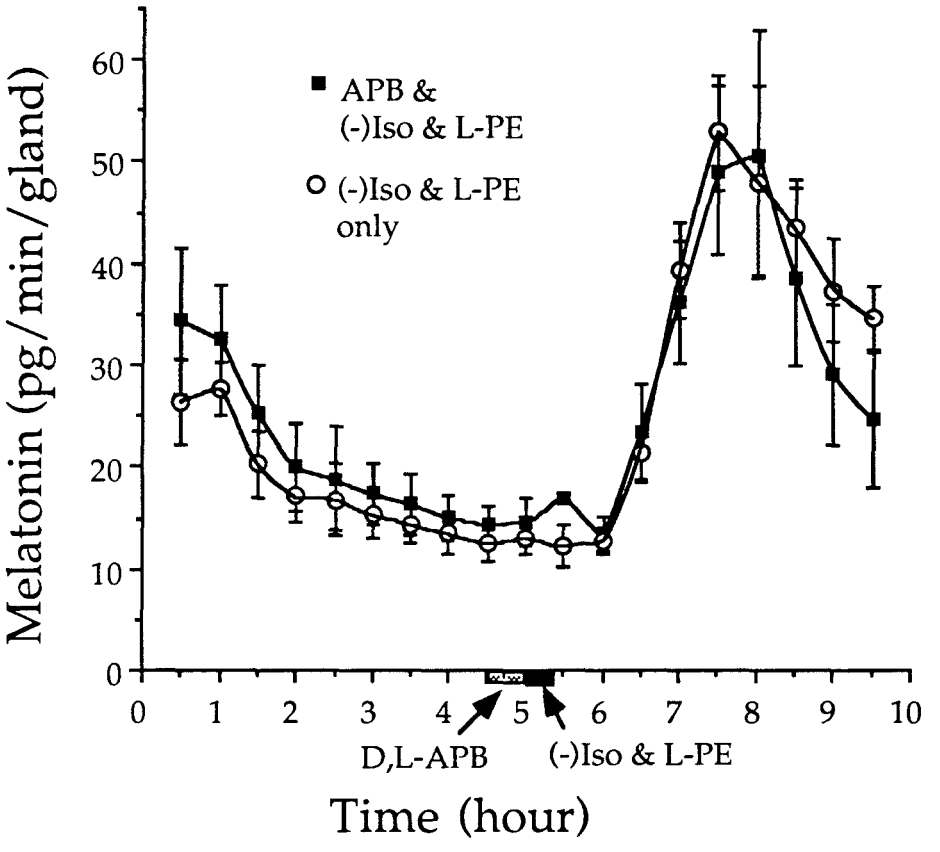


Fig. 17. Pretreatment of pineal glands with D,L-APB (2×10^{-4}M) does not inhibit the (-)isoproterenol and L-phenylephrine (10^{-6}M) stimulated secretion of melatonin. n = 3 pineal glands/group.

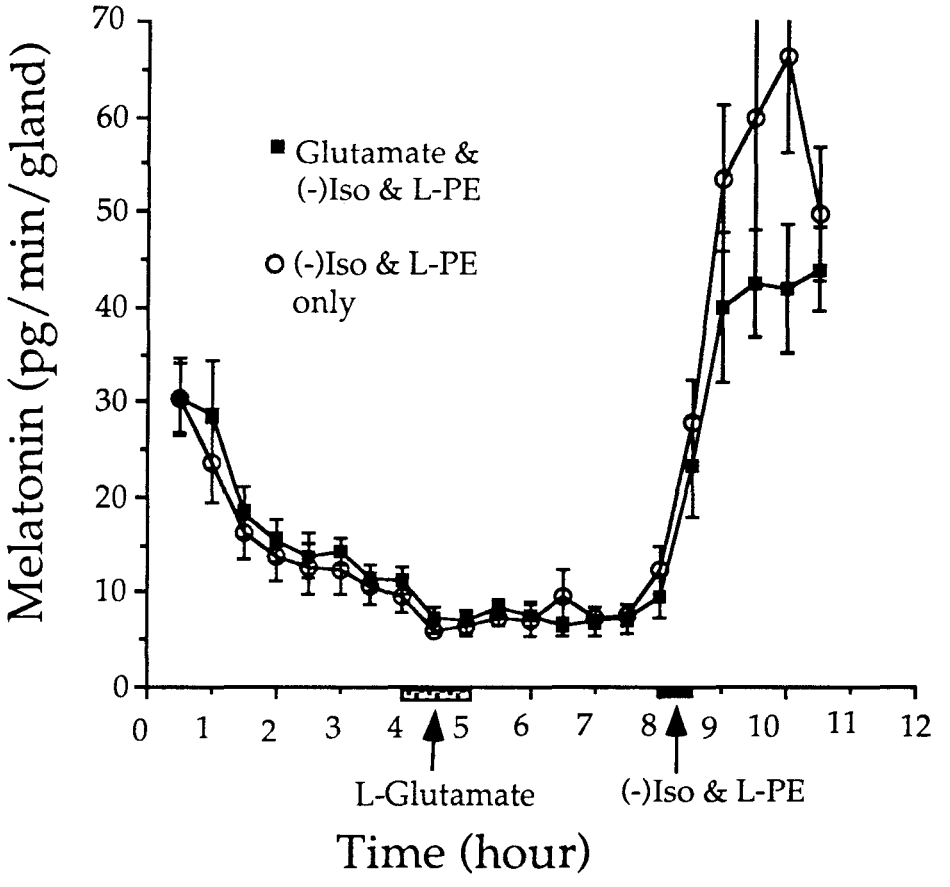
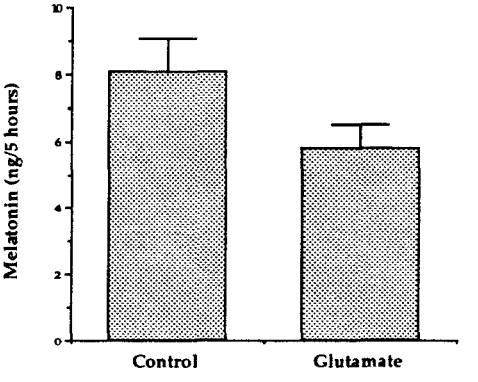


Fig. 18. L-glutamate (10^{-3}M) administered 3 hours prior to (-)isoproterenol and L-phenylephrine (10^{-6}M) does not significantly inhibit melatonin secretion. ($p<0.422$ ANOVA group effect) n = 6 pineal glands/group. Bar graphs represent the sum of melatonin secretion after (-)isoproterenol and L-phenylephrine administration ($p<0.70$ T-test).



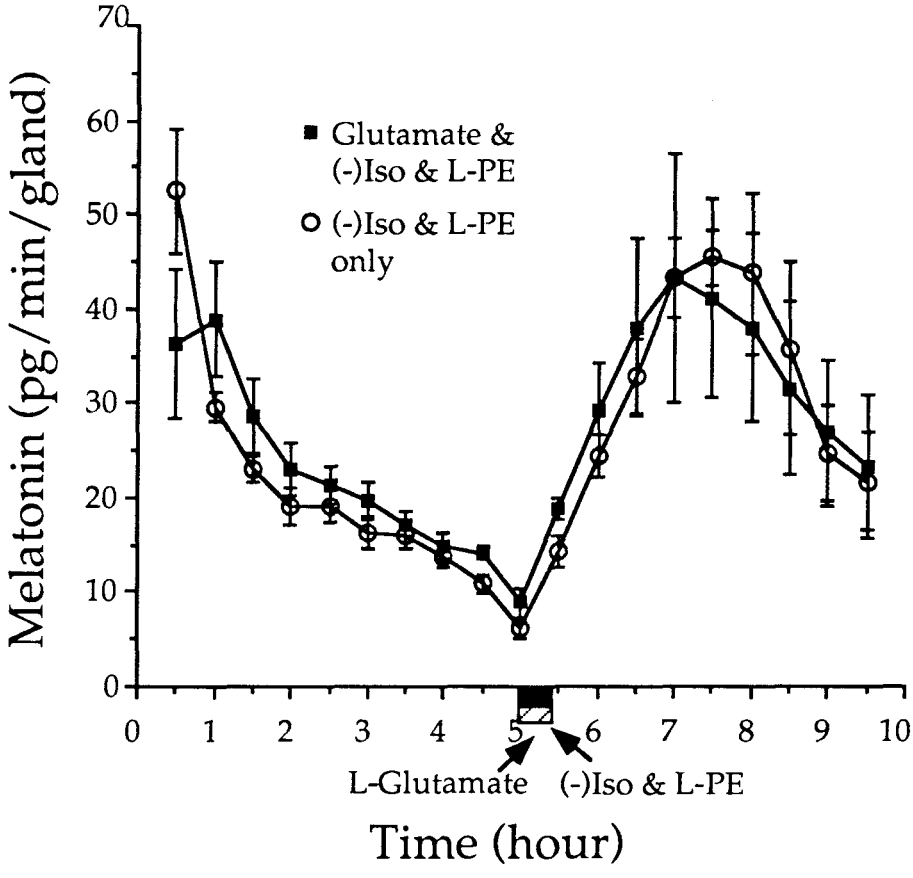


Fig. 19. L-glutamate (10^{-3}M) administered simultaneously with (-)isoproterenol and L-phenylephrine (10^{-6}M - 25 min) does not inhibit melatonin secretion. n = 3 pineal glands/group.

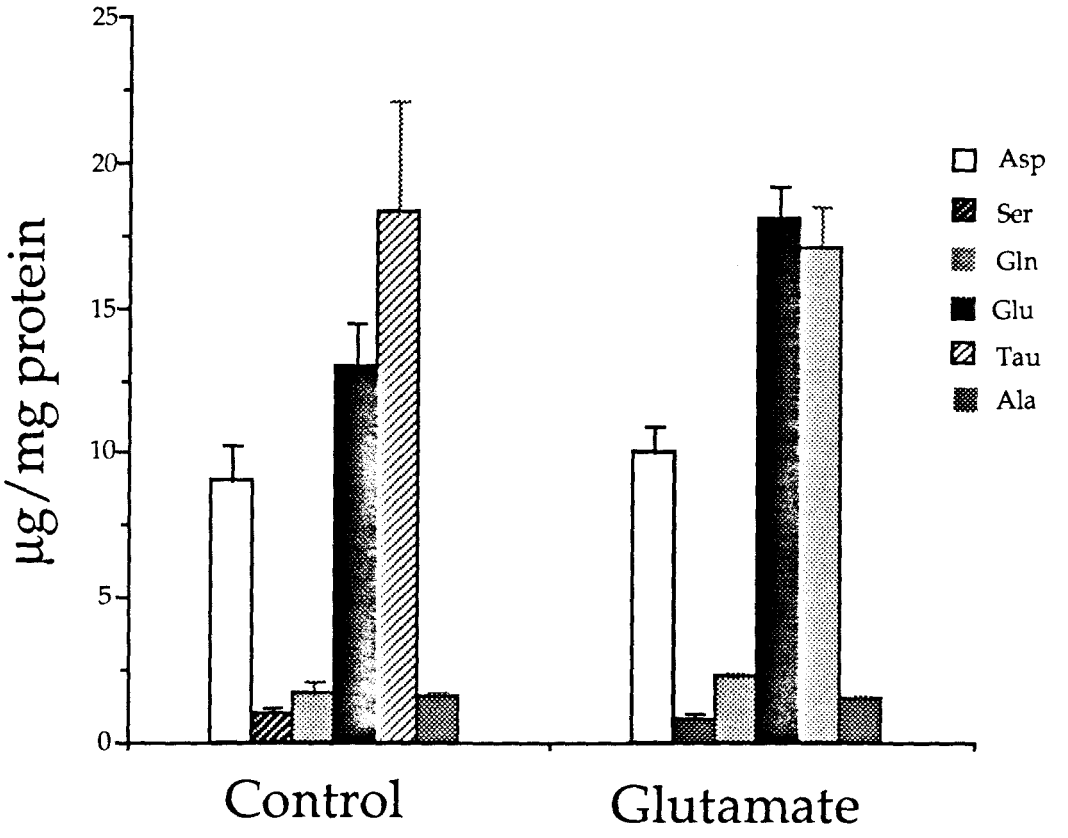


Fig. 20. Amino acid content of pineal glands administered 10^{-3}M L-glutamate at 3.0 hour for 60 min. Perifusion was terminated at 5.5 hour and glands were frozen. Levels of glutamate were significantly higher in the glutamate treated group ($p<0.02$ T-test). Levels of the other amino acids assayed did not increase significantly. n = 4 pineal glands/group.

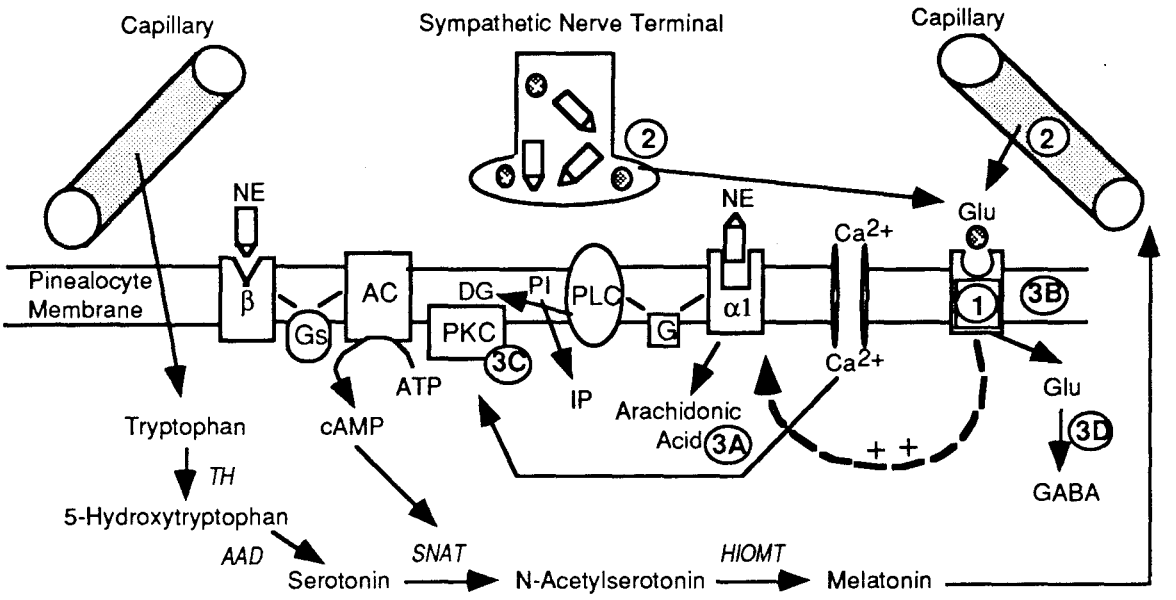
CHAPTER VI

GENERAL DISCUSSION

These studies tested the hypothesis that glutamate modulates melatonin production in the pineal gland of the rat. The role of the adrenergic system in the production of the pineal gland hormone, melatonin, is well known. NE, which is released from sympathetic nerve terminals at night, binds to α- and β-adrenergic receptors to stimulate the production of pinealocyte cAMP. This increase in cAMP stimulates the transcription and translation of the rate limiting enzyme in the synthesis of melatonin, SNAT. Melatonin, which is synthesized from the amino acid tryptophan via multienzymatic pathway, is released from the gland into the blood stream. The proposal of a modulatory role for glutamate in this process *in vivo* requires that several things be demonstrated including (see Fig. 1):

1) cell surface receptors for glutamate (See 1 in Fig. 1)
2) an endogenous source of glutamate for these receptors (See 2 in Fig. 2)
3) the ability of glutamate to influence melatonin production via binding to these receptors (See 3A, 3B, 3C, 3D in Fig. 1 and explanation below)

Fig. 1



1) Glutamate Binding Sites

In chapter IV, a postsynaptic binding site for glutamate in the rat pineal gland is described. The pharmacological profile of this binding site suggests that it does not fit into any of the known categories of glutamate receptor described in brain. In further analyzing the nature of this glutamate binding site, however, it becomes important to determine if a comparison with neuronal glutamate receptors is appropriate. While the pineal gland is considered a part of the brain (epithalamus) and is embryologically derived from neuroectoderm, pinealocytes differ in many ways from neurons. Referred to as paraneurons (Ueck and Wake 1977), pinealocytes, like neurons, exhibit action potentials (Reuss 1987) and synthesize and release a known neurotransmitter, (serotonin is released in response to α1-adrenergic stimulation (Aloyo and Walker 1988). On the other hand, pinealocytes

display many non-neuronal features. For example, there is no evidence for intrapineal synapses like those seen between neurons. In addition, many cytochemical neuronal markers such as neuronal adhesion molecule L1, synaptophysin and neurofilament marker R39 are not present in pinealocytes (Schroder et al. 1990; Vollrath and Schroder 1987). Finally, a suggestion that the receptors present on the gland may not be exactly like receptors present in other parts of the brain comes from a study where it has been demonstrated with the use of photoaffinity labeling that the β-adrenergic receptor present in the rat pineal has a lower molecular weight than the β-adrenergic receptor described in other tissue including brain (Dickinson et al. 1986).

Perhaps it would be more appropriate to compare pinealocytes with retinal photoreceptors based on histochemical, morphological and phylogenetic evidence. Accordingly, the glutamate binding site present in the rat pineal may be more closely related to the glutamate binding site present on photoreceptors. Recent characterization of glutamate receptors in turtle photoreceptors using electrophysiological techniques indicates that these sites are not similar to the glutamate binding sites within the rest of the brain (Tachibana and Kaneko 1988). In the photoreceptor of the turtle, the glutamate binding site has been characterized as a "functional" uptake site. While none of the known glutamate agonists stimulated depolarization in photoreceptors, D-aspartate and L-aspartate, substrates of the glutamate uptake system were effective (Tachibana and Kaneko 1988). Addition of a glutamate uptake inhibitor also reduced the glutamate-induced current. Therefore, the possibility that the binding site described in this study is an uptake site for glutamate must also be considered.

There is no doubt that a glutamate uptake site is present in the pineal gland as extracellular glutamate can be accumulated by this organ (chapter V, Fig. 20). Whether this site is responsible for this uptake or whether there is another, lower affinity uptake site, is unknown. While binding was performed in Na^{2+}, and Cl^- free conditions, it is possible that local tissue concentrations of these ions remained high enough to stimulate glutamate uptake. It would be important to examine the effects of a specific glutamate uptake inhibitor such as L-trans-pyrrolidine-2,4-dicarboxylic acid on the ability of glutamate to inhibit the Iso and PE-stimulated production of melatonin. Based on the diversity of glutamate binding sites defined pharmacologically, e.g. the NMDA, kainate, AMPA, APB, and metabotropic receptors in the CNS, and the glutamate binding sites in photoreceptor and pituitary gland, as well as those defined with the use of molecular techniques, the possibility that the pineal gland of the rat possesses a unique type of glutamate receptor must also be considered.

Assuming that glutamate exerts its effects on pinealocytes via binding to either an uptake site or a receptor, the mechanism for glutamate inhibition of Iso and PE-stimulated melatonin secretion remains an enigma. It was originally hypothesized that glutamate inhibits the α- and β-adrenergic stimulated production of melatonin by inhibiting the α-adrenergic stimulated production of inositol phosphates. It had been demonstrated in brain that glutamate and quisqualate inhibited incorporation of [^{3}H]inositol into the larger phospholipid molecule. If glutamate or quisqualate were administered before NE, it was demonstrated that activation of the α-adrenergic receptor produced less IP3 (Jope and Li 1989). It had been assumed that the β-adrenergic stimulated production of melatonin, like the β-adrenergic

stimulated production of cAMP and SNAT activity, would be potentiated by activation of the α-adrenergic receptor. It was therefore hypothesized that addition of glutamate to pineal glands stimulated with both α- and β-adrenergic agonists would reduce melatonin secretion to the same extent that activation of the α-adrenergic receptor would potentiate β-adrenergic stimulated melatonin production. This idea was not borne out by our data demonstrating lack of potentiation of melatonin production in glands treated with both α and β-adrenergic agonist when compared to glands stimulated with a β-adrenergic agonist alone (chapter V Fig. 4). In addition the role of inositol phospholipid production in melatonin synthesis has been questioned (Sugden et al. 1988; Sugden et al. 1987). Several other mechanisms for glutamate inhibition of Iso- and PE-stimulated secretion of melatonin as discussed in Chapter V include: A) cell death or toxicity; B) pinealocyte depolarization; C) inhibition of cAMP by the α-isoform of PKC; and D) conversion of glutamate to GABA (chapter VI Fig. 1).

2) Endogenous Glutamate

While glutamate can inhibit the secretion of melatonin *in vitro*, a physiological role for this amino acid *in vivo* requires that a regular source of glutamate be available to the gland. Our immunocytochemical data demonstrated that glutamate is not concentrated in nerve terminals in the pineal gland when compared to glutamate immunolabeling over other cell types in the gland suggesting that the SCG is not a source of "transmitter" glutamate. While glutamate may be tonically released and not stored for long periods of time, there are, to my knowledge, no studies that have described a co-localization or co-release of glutamate and NE. Central neuronal

connections do not seem a likely source of glutamate as there is no compelling evidence for the presence of such fibers in the pineal gland of the rat. The possibility that glutamate is blood borne or found in the CSF may be considered as the pineal gland has one of the highest flow rates of blood in the body (on a per weight basis) (Goldman and Wurtman 1964) and is surrounded by CSF. These possibilities, however, both seem unlikely because blood and CSF do not have high levels of glutamate (0.15 mM and 0.4 μM respectively) (Alfredsson et al. 1988; Pardridge 1979) and 0.5 mM glutamate did not inhibit the Iso and PE stimulated production of melatonin *in vitro* (Chapter V, Fig. 8). Another possibility is that the pineal gland itself serves as a source of glutamate. It has been demonstrated in turtle photoreceptors that glutamate is released upon depolarization of the cell (Copenhagen and Jahr 1989). NE, however, hyperpolarizes the pinealocyte membrane (Parfitt et al. 1975; Sakai and Marks 1972). The presence of glutamate binding sites in the pineal gland without a definitive glutaminergic input is an obvious problem. However, in many other brain regions mismatch between neurotransmitters and their receptors have been documented (Herkenham and McLean 1986).

3) Glutamate Modulation of Melatonin Secretion

While glutamate administered before Iso and PE inhibits the amount of melatonin released by the pineal gland *in vitro*, the physiological relevance of this finding must be addressed in light of what has been called the duration hypothysis. It has been suggested that what is physiologicaly important to the animal is not an increase or decrease in the amount of melatonin that it is exposed to, but the amount of time that the animal is exposed to it. For example, the pineal gland of the aged Syrian hamster produces significantly

less melatonin than the pineal gland of a younger animal (Reiter et al. 1980). In spite of the overall decreased levels of melatonin, exposure of these animals to short days will still induce gonadal regression. This indicates that it is the duration of the melatonin signal that induces this change and not the total amount of melatonin produced. Because the production of melatonin in this study was followed for only 4 hours after stimulation by Iso and PE (Chapter V, Fig. 6), it could not be discerned whether one hour of glutamate pretreatment would shorten the overall duration of the melatonin signal.

It is interesting to note that the pineal gland has been suggested by more than one author to serve as a model for events in the central nervous system. In light of the observation made in this study, it is suggested that this idea be approached with caution.

LITERATURE CITED

Abrams L, Politi LE, Adler R (1989) Differential susceptibility of isolated mouse retinal neurons and phototreceptors in kainic acid toxicity. Invest. Ophthalmol. Vis. Sci. 30: 2300-2308

Alfredsson G, Wiessel FA, Lindberg M (1988) Glutamate and glutamine in cerebral spinal fluid and serum from healthy volunteers -analytical aspects. J. Chromatography 424: 378-384

Aloyo VJ, Walker RF (1988) Alpha-adrenergic control of serotonin release from rat pineal glands. Neuroendocrinology 48: 61-66

Alphs L, Lovenberg W (1984) Modulation of rat pineal acetyl-Co A:arylamine N-acetyltransferase induction by alpha adrenergic drugs. J. Pharmacol. Exp. Ther. 230: 431-437

Anderson KJ, Monaghan DT, Bridges RJ, Tavoularis AL, Cotman CW (1990) Autoradiographic characterization of putative excitatory amino acid transport sites. Neurosci. 38: 311-322

Anderson KJ, Vickroy TW (1990) Anatomical and pharmacological heterogeneity of D-[^{3}H]aspartate binding sites. Eur. J. Pharmacol. 185: 119-121

Anis NA, Berry SC, Burton NR, Lodge D (1983) The dissociative anaestetics ketamine and phencyclidine, selectivly reduce excitations of central mammalian neurons by N-methyl-aspartate. Br. J. Pharmacol. 79: 565-575

Asher P, Nowak L (1987) Electrophysiological studies of NMDA receptors. Trends Neurosci. 10: 284-288

Axelrod J, Shein HM, Wurtman RJ (1969) Stimulation of C14-melatonin synthesis from C14-tryptrophan by noradrenaline in rat pineal in organ culture. Proc. Natl. Acad. Sci. 62: 544-549

Axelrod J, Wurtman RJ, Snyder SH (1965) Control of hydroxyindole O-methyltransferase activity in the rat pineal gland by environmental lighting. J. Biol. Chem. 210: 949-954

Bagnara JT, Hadley ME (1970) Endocrinology of the amphibian pineal. Amer. Zool. 10: 201-216

Barbour B, Brew H, Attwell D (1988) Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. Nature 335: 433-435

Baudry M, Evans J, Lynch G (1986) Excitatory amino acids inhibit stimulation of phosphatidylinositol metabolism by aminergic agonists in hippocampus. Nature 319: 329-331

Besson J, Sarrieau A, Vial M, Marie J, Rosselin G, Rostene W (1986) Characterization and autoradiographic distribution of vasoactive intestinal peptide binding sites in the rat central nervous system. Brain Res. 398: 329-336

Bettler B, Boulter J, Hermans-Borhmeyer I, O'Shea-Greenfield A, Deneris ES, Moll C, Borgmeyer U, Hollmann M, Heinemann S (1990) Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. Neuron 5: 583-595

Biscoe TJ, Evans RH, Francis AA, Martin MR, Watkins JC, Davis J, Dray A (1977) D-alpha-aminoadipate as a selective antagonist of amino acid-induced and synaptic excitation of mammalian spinal neurons. Nature 270: 743-745

Boast CA, Gerhardt SC, Pastor G, Lehmann J, Etienne PE, Liebman JM (1988) The N-methyl-D-aspartate antagonists CGS 19755 and CPP reduce brain damage in gerbils. Brain Res. 442: 345-348

Brew H, Attwell D (1987) Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. Nature 327: 707-709

Brownstein M, Axelrod J (1974) Pineal gland: 24-hour rhythm in norepinephrine turnover. Science 184: 163-165

Cantor EH, Greenberg LH, Weiss B (1981) Effect of long-term changes in sympathetic nervous activity on the beta-adrenergic receptor-adenylate cyclase complex of rat pineal gland. Mol. Pharmacol. 19: 21-26

Cardinali DP (1977) Nuclear receptor estrogen complex in the pineal organ. Modulation by sympathetic nerves. Neuroendocrinology 24: 333-346

Carlson MD, Kish PE, Ueda T (1989) Characterization of the solubilized and reconstituted ATP-dependent vesicular glutamate uptake system. J. Biol. Chem. 264: 7369-7376

Cha JJ, Hollingsworth ZR, Greenamyre JT, Young AB (1989) Contamination of commercially available quisqualic acid by glutamate-like and aspartate-like substances. J. Neurosci. Meth. 27: 143-148

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22: 3099-3108

Choi DW (1987) Ionic dependence of glutamate neurotoxicity. J. Neurosci. 7: 369-379

Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu. Rev. Neurosci. 13: 171-182

Cincotta M, Summers RJ, Beart PM (1989) Biochemical characterization of an autoradiographic method for studying excitatory amino acid receptors using L-[^{3}H]glutamate. Anal. Biochem. 177: 150-155

Coan EJ, Collingridge GL (1987) Effects of phencyclidine, SKF 10,047 and related psycholomimetic agents on N-methyl-D-aspartate receptor mediated synaptic responses in rat hippocampal slices. Br. J. Pharmacol. 91: 547-556

Collin J, Brisson P, Falcon J, Voisin P (1986) Multiple cell types in the pineal: functional aspects. In: Pineal and Retinal Relationships. P. J. O'Brien and D. C. Klein (eds) Academic Press, Inc., Orlando, pp 15-32

Collin J-P, Oksche A (1981) Structural and functional relationships in the nonmammalian pineal gland. In: The Pineal Gland: Anatomy and Biochemistry.R. J. Reiter (eds) CRC Press, Inc., Boca Raton, pp 27-67

Collins GGS (1982) Some effects of excitatory amino acid receptor antagonists on synaptic transmission in the rat olfactory cortex slices. Brain Res. 244: 311-318

Copenhagen DR, Jahr CE (1989) Release of endogenous excitatory amino acids from turtle photoreceptors. Nature 341: 536-539

Craft CM, Morgan WW, Jones DJ, Reiter RJ (1985) Hamster and rat pineal gland beta-adrenoceptor characterization with iodocyanopindolol and the effect of decreased catecholamine synthesis on the receptor. J. Pineal Res. 2: 51-66

Craft CM, Morgan WW, Reiter RJ (1984) 24-Hour changes in catecholamine synthesis in rat and hamster pineal glands. Neuroendocrinology 38: 193-198

Curtis DR, Davis R (1962) Pharmacological studies upon neurons of the lateral geniculate nucleus of the cat. J. Pharmacol. 18: 217-246

Curtis DR, Koizumi K (1961) Chemical transmitter substances in the brain stem of the cat. J. Neurophysiology 24: 80-90

Curtis DR, Phillis JW, Watkins JC (1959) Chemical excitation of spinal neurons. Nature 183: 611-612

Curtis DR, Watkins JC (1960) The excitation and depression of spinal neurons by structurally related amino acids. J. Neurochem. 6: 117-141

Dafny N (1983) Evidence that the rat pineal has neuronal connections via the pineal stalk. Exp. Neurol. 79: 858-861

Davies JD, Francis AA, Jones AW, Watkins JC (1981) 2-amino-5-phosphonovalerate (2-AP5), a potent and selective antagonist of amino acid-induced and synaptic excitation. Neurosci. Lett. 221: 77-81

Davies JD, Watkins JC (1982) Actions of D- and L-forms of 2-amino-5-phosphonovalerate and 2-amino-4-phosphonobutyrate in the cat spinal cord. Brain Res. 235: 378-386

Deguchi T, Axelrod J (1972) Control of circadian change in serotonin N-acetyltransferase activity in the pineal organ by the beta-adrenergic receptor. Proc. Natl. Acad. Sci. 69:2547-2550

Dickinson KEJ, Leeb-Lundberg LMF, Strasser RH, Caron MG, Lefkowitz RJ (1986) Identification of the subunit structure of rat pineal adrenergic receptors by photoaffinity labeling. J. Neurochem. 46: 1153-1160

Dudek SM, Bear MF (1989) A biochemical correlate of the critical period for synaptic modification in the visual cortex. Science 246: 673-675

Duggan AW (1974) The differential sensitivities to L-glutamate and L-aspartate of spinal interneurons and Renshaw cells. Exp. Brain Res. 19: 522-528

Ebadi M (1987) Indolic, aminergic and peptidergic receptors and their modulation of pineal function. In: Fundamentals and clinics in pineal research. G. P. Trentini, C. DeGaetani and P. Pevet (eds) Raven Press, New York, pp 91-110

Ebadi M, Chan A (1980) Characteristics of GABA binding sites in bovine pineal gland. Brain Res. Bull. 5: 179-187

Ebadi M, Govitrapong P (1986) Orphan transmitters and their receptor sites in the pineal gland. In: Pineal Research Reviews. R. J. Reiter (ed) Alan R. Liss, New York, pp 1-54

Ebadi M, Govitrapong P, Murrin LC (1986) The presence and function of glutamate receptors in the pineal gland. In: Advances in pineal research. R. J. Reiter and M. Karasek (eds) John Libbey & Co. Ltd., London, pp 99-109

Egebjerg J, Bettler B, Hermans-Borgmeyer I, Heinemann S (1991) Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. Nature 351: 745-748

Elliott JA (1976) Circadian rhythms and photoperiodic time measurement in mammals. Fed. Proc. 35: 2339-2346

Evans RM, Francis AA, Jones AW, Smith DAS, Watkins JC (1982) The effects of a series of -phosphonic-carbonxylic amino acids on electrically evoked and amino acid induced responses in isolated spinal cord preparation. Br. J. Pharmacol. 75: 65-75

Evans RM, Francis AA, Watkins JC (1977) Selective antagonism by Mg^{2+} of amino acid-induced depolarization of spinal neurons. Experientia 33: 489-491

Fagg GF, Mena EE, Monaghan DT, Cotman CW (1983) Freezing eliminates a specific population of L-glutamate receptors in synaptic membranes. Neurosci. Lett. 38: 157-162

Fink-Jensen A, Moller M (1990) Direct projections from the anterior and tuberal regions of the lateral hypothalamus to the rostral part of the

pineal complex of the rat. An anterograde neuron-tracing study by using *Phaseolus vulgaris* leucoagglutinin. Brain Res. 522: 337-341

Forsythe ID, Clements JD (1988) Glutamate autoreceptors reduce EPSP's in cultured hippocampal neurons. Soc. Neurosci. Abstr. 14: 791

Foster AC, Fagg GE (1984) Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. Brain Res. Rev. 7: 103-164

Frandsen A, Krogsgaard-Larsen P, Schousboe A (1990) Novel glutamate receptor antagonists selectively protect against kainic acid neurotoxicity in cultured cerebral cortex neurons. J. Neurochem. 55: 1821-1823

Fraser S, Cowen P, Franklin M, Franey C, Arendt J (1983) Direct radioimmunoassay for melatonin in plasma. Clin. Chem. 29: 396-397

Gilbey MP, Peterson DF, Coote JH (1982) Some characteristics of sympathetic preganglionic neurons in the rat. Brain Res. 241: 43-48

Goldman H, Wurtman RJ (1964) Flow of blood to the pineal body of the rat. Nature 203: 87-88

Gonzalez-Brito A, Reiter RJ (1987) Mammalian pineal beta-adrenergic receptors: their regulation and physiological significance. In: Fundamentals and Clinics in Pineal Research. G. P. Trentini, C. DeGaetani and P. Pevet (eds) Raven Press, New York, pp 121-124

Govitrapong P, Ebadi M (1988) The inhibition of pineal arylalkylamine N-acetyltranferase by glutamic acid and its analogues. Neurochem. Int. 13: 223-230

Govitrapong P, Ebadi M, Murrin LC (1986) Identification of a Cl^-/Ca^{2+} - dependent glutamate (quisqualate) binding site in bovine pineal organ. J. Pineal Res. 3: 223-234

Govitrapong P, Hama Y, Pfeiffer R, Ebadi M (1989) Status of dopamine in bovine pineal glands and the stimulation of N-acetyltransferase activity by D2-dopaminergic receptor agonists in the rat pineal glands in culture. J. Pineal Res. 6: 17-31

Govitrapong P, Murrin LC, Ebadi M (1984) Characterization of dopaminergic receptor sites in bovine pineal gland. J. Pineal Res. 1: 215-226

Greenamyre JT, Young AB (1989) Excitatory amino acids and Alzheimer's disease. Neurobiol. Age. 10: 593-602

Gusovsky F, Gutkind JS (1991) Selective effects of activation of protein kinase C isozymes on cyclic AMP accumulation. Mol. Pharmacol. 39: 124-129

Haldemann S, McLennan H (1972) The antagonistic action of glutamic acid diethyl ester towards amino acid induced and synaptic excitation of central neurons. Brain Res. 45: 393-400

Harris EW, Ganong AH, Monaghan DT, Watkins JC, Cotman CW (1986) Action of 3-(C+/-)-2-carboxypiperazin-4-yl)-propl-1-phosphonic acid (CPP): A new and highly potent antagonist of N-methyl-D-aspartate receptors in the hippocampus. Brain Res. 382: 174-177

Hauser G, Shein HM, Eichberb J (1974) Relationship of alpha-adrenergic receptor in rat pineal gland to drug induced stimulation of phospholipid metabolism. Nature 252: 482-483

Hayashi T (1954) Effects of sodium glutamate on the nervous system. Keio. J. Med. 3: 183-192

Hedger JH, Webber RH (1976) Anatomical study of the cervical sympathetic trunck and ganglia in the albino rat (Mus norvegicus albinus). Acta Anat. 96: 206-217

Henley JM, Johnston GAR (1991) Vintage amino acid meeting describes new tools for amino acid research, subtypes of metabotropic receptors. Trends Pharmacol. 12: 357-359

Herkenham M, McLean S (1986) Mismatches between receptor and transmitter localizations in brain. In: Quantitative Receptor Autoradiography. C. A. Boast, E. W. Snowhill and C. A. Altar (eds) Alan R. Liss, pp 137-171

Ho AK, Chik C, Klein DC (1988) Permissive role of calcium in α1-adrenergic stimulation of pineal phosphatidylinositol phosphodiesterase (phospholipase C) activity. J. Pineal Res. 5: 553-564

Ho AK, Chik CL, Weller JL, Cragoe EJJ, Klein DC (1989) Evidence of a1-adrenergic - protein kinase C - Na^+/H^+ antiporter-dependent increase in pinealocyte intracellular pH. J. Biol. Chem. 264: 12983-12988

Ho AK, Thomas TP, Anderson WB, Klein DC (1988) Protein kinase C: subcellular redistribution by increased Ca^{2+} influx. J. Biol. Chem. 263: 282-9297

Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. Science 252: 851-853

Hollmann M, O'Shea-Greenfield A, Rogers SW, Heineman S (1989) Cloning by functional expression of a member of the glutamate receptor family. Nature 342: 643-648

Honore T, Davies SN, Drejir J, Fletcher EJ, Jacobsen P (1988) Quinoxalinedione: potent competative non-NMDA glutamate receptor antagonists. Science 241: 701-703

Honore T, Lauridsen J, Krogsgaard-Larsen P (1982) The binding of [^{3}H]AMPA, a structural analogue of glutamate, to rat brain membrans. J. Neurochem. 38: 173-178

Howell HE, Morgan PJ (1991) β-adrenergic stimulation increases cAMP and melatonin production in ovine pinealocyte cultures. J. Pineal Res. 10: 122-129

Jackson RL, Lovenberg W (1971) Isolation and characterization of multiple forms of hydroxyindole-O-methyltransferase. J. Biol. Chem. 246: 4280-4285

Jansen KLR, Dragunow M, Faull RLM (1990) Sigma receptors are highly concentrated in the rat pineal gland. Brain Res. 507: 158-160

Ji Z, Aas J-E, Laake J, Walberg F, Ottersen OP (1991) An electron microscopic immunogold analysis of glutamate and glutamine in terminals of rat spinocerebellar fibers. J. Comp. Neurol. 307: 296-310

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325: 529-531

Jope RS, Li X (1989) Inhibition of inositol phospholipid synthesis and norepinephrine-stimulated hydrolysis in rat brain slices by excitatory amino acids. Biochem. Pharmacol. 38: 589-596

Kappers AJ (1960) The development, topographical relations and innervation of the epiphysis cerebri in the albino rat. Z. Zellforsch 52: 163-215

Kappers AJ (1964) Survey of the innervation of the pineal organ in vertebrates. Am. Zoologist 4: 47-51

Kappers JA (1960) Innervation of the epiphysis cerebri in the albino rat. Anat. Rec. 136: 220-221

Kappers JA (1965) Survey of the innervation of the epiphysis cerebri and the accessory pineal organs of vertebrates. Prog. Brain Res. 10: 87-153

Karasek M (1983) Ultrastructure of the mammalian pineal gland: its comparative and functional aspects. Pineal Res. Rev. 1: 1-48

Kebabian JW, Zatz M, Romero JA, Axelrod J (1975) Rapid changes in rat pineal β-adrenergic receptor: alterations in l-[^{3}H]alprenolol binding and adenylate cyclase. Proc. Natl. Acad. Sci. 72: 3735-3739

Keinanen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. Science 249: 556-560

King TS, Steger RW, Steinlechner S, Reiter RJ (1984) Day-night differences in the estimated rates of 5-hydroxytryptamine turnover in the rat pineal gland. Exp. Brain Res. 54: 432-436

Kleckner NW, Dingledine R (1988) Requirement for glycine in activation of NMDA- receptors expressed in Xenopus oocytes. Science 241: 835-837

Klein DC (1985) Photoneural regulation of the mammalian pineal gland. In: Photoperiodism, Melatonin and the Pineal. D. Evered and S. Clark (eds) Pitman, London, pp 38-56

Klein DC, Auerbach DA, Namboodiri MAA, Wheler GHT (1981) Indole metabolism in the mammalian pineal gland. In: The Pineal Gland: Anatomy and Biochemistry. RJ Reiter (ed) CRC Press, Inc., Boca Raton, pp 199-227

Klein DC, Buba MJ, Kapoor CL, Krishna G (1978) Pineal serotonin N-acetyltransferase activity: abrupt decrease in adenosine 3', 5'-monophosphate may be signal for "turnoff". Science 199: 309-311

Klein DC, Smoot R, Weller JL, Higa S, Markey SP, Creed GJ, Jacobowitz DM (1983) Lesions of the paraventricular nucleus area of the hypothalamus

disrupt the suprachiasmatic - spinal cord circuit in the melatonin rhythm generating system. Brain Res. Bull. 10: 647-652

Klein DC, Sugden D, Weller JL (1983) Postsynaptic alpha-adrenergic receptors potentiate the beta-adrenergic stimulation of pineal serotonin N-acetyltransferase. Proc. Natl. Acad. Sci. 80: 599-603

Klein DC, Weller J, Sugden AL, Sugden D, Vanecek J, Chik CL, Ho AK (1987) Integration of multiple receptor mechanisms regulating pineal cyclic nucleotides. In: Fundamentals and Clinics in Pineal Research. GP Trentini, C DeGaetani and P Pevet (eds) Raven Press, New York, pp 111-119

Klein DC, Weller JL (1970) Indole metabolism in the pineal gland: a circadian rhythm in N-acetyltransferase. Science 169: 1093-1095

Korf HW, Foster RG, Ekstrom P, Schalken JJ (1985) Opsin-like immunoreactivity in the retinae and pineal organs of four mammalian species. Cell Tiss. Res. 242: 645-648

Korf HW, Moller M, Gery I, Zigler JS, Klein DC (1985) Immunocytochemical demonstration of retinal S-antigen in the pineal organ of four mammalian species. Cell Tiss. Res. 239: 81-85

Krnjevic K, Phillis JW (1963) Iontophoretic studies of neurons in the mammalian cerebral cortex. J. Physiol. 165: 274-304

Kumar KN, Tilakaratne N, Johnson PS, Allen AE, Michaelis EK (1991) Cloning of cDNA for the glutamate-binding subunit of an NMDA receptor complex. Nature 354: 70-73

Kus L, Handa RJ, McNulty JA (1990) Characterization of a Na^+, Cl^-, and Ca^{2+}-independent [^{3}H]glutamate binding site in rat pineal. Soc. Neurosci. Abstr. 16: 869

Kus L, Handa RJ, McNulty JA (1991) Glutamate inhibition of melatonin production in the rat pineal *in vitro*. Anat. Rec. 229: 47A

Laake JH, Gundersen V, Nordbo G, Ottersen OP, Storm-Mathisen J (1986) An antiserum against glutamine. In: Excitatory Amino Acids. P. J. Roberts, J. Storm-Mathisen and H. F. Bradford (eds) Macmillan, London, pp 448-450

Lanthorn TM, Ganong AM, Cotman CW (1984) 2-amino-4-phosphonobutyrate selectively blocks mossey fiber-CA3 responses in guinia pig but not rat hippocampus. Brain Res. 290: 174-178

Leep-Lundberg LMF, Cotecchia S, DeBlasi A, Caron MG, Lefkowitz RJ (1986) Regulation of adrenergic receptor function by phosphorylation I. Agonist-promoted desensitization and phosphorylation of α1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT,MF-2 smooth muscle cells. J. Biol. Chem. 262: 3098-3105

Lehmann J, Chapman AG, Meldrum BS, Hutchison A, Tsai C, Wood PL (1988) CGS 19755 is a potent and competitive antagonist at NMDA-type receptors. Eur. J. Pharmacol. 154: 89-93

Lieberman HR, Waldhauser F, Garfield G, Wurtman RJ (1984) The effect of melatonin on human mood and performance. Brain Res. 323: 201-207

Liu B, Burbach JPH (1987) Detection and high performance liquid chromatography identification of the summer rises of vasopressin and oxytocin immunoreactivity in the rat pineal gland. Endocrinology 121: 1716-1720

Liu B, Burbach PH (1988) Circadian variation of vasopressin level and vasopressin-converting aminopeptidase activity in the rat pineal gland. Peptides 9: 973-978

Login IS (1990) Direct stimulation of pituitary prolactin release by glutamate. Life Sci. 47: 2269-2275

Lowry AJ, Sack RL, Singer CM (1985) Melatonin, light and chronobiological disorders. In: Photoperiodism, Melatonin, and the Pineal. D. Evered and S. Clark (eds) Pitman, London, pp 231-252

Lowry OH, Rosebrough AL, Farr AL, Randall RJ (1951) Protein measurement with a folin phenol reagent. J. Biol. Chem. 193: 265-275

Lucas DR, Newhouse JP (1957) The toxic effects of sodium L-glutamate on the inner layers of the retina. A.M.A. Arch. Opthalmol. 58: 193-201

Lynch HJ, Jimerson DC, Ozaki Y, Post RM, Bunney WE, Wurtman RJ (1978) Entrainment of rhythmic melatonin secretion from the human pineal to a 12-hour phase shift in the light-dark cycle. Life Sci. 23: 1557-1564

Lynch MA, Errington ML, Clements MP, Bliss TV, Redini-Del Negro C, Laroche S (1990) Increases in glutamate release and phosphoinositide metabolism associated with long-term potentiation and classical conditioning. Prog. Brain Res. 83: 251-256

Martin C, Meissl H (1990) Characterization of the light response in the pineal gland of intact and sympathectomized rats. J. Neural Trans. 79: 81-91

Masu M, Tanabe Y, Tsuchida K, Shigemoto R, Nakanishi S (1991) Sequence and expression of a metabotropic glutamate receptor. Nature 349: 760-765

Mata MM, Schrier BK, Klein DC, Weller JL, Chiou CY (1976) On GABA function and physiology in the pineal gland. Brain Res. 118: 383-394

Mattson M, Dou P, Kater SB (1988) Outgrowth-regulatory actions of glutamate in isolated pyramidal neurons. J. Neurosci. 8: 2087-2100

Mayer ML, Westbrook GL (1987) The physiology of excitatory amino acids in the vertebrate central nervous system. Prog. Neurobiol. 28: 197-276

Mayer ML, Westbrook GL, Guthrie PP (1984) Voltage dependent block by Mg^{++} of NMDA responses in spinal cord neurons. Nature 309: 261-263

McCulloch RM, Johnston GAR, Gaure CJA, Curtis DR (1974) The differential sensitivities of spinal cord interneurons and Renshaw cells to kainate and N-methyl-D-aspartate. Exp. Brain Res. 21: 515-518

McGeer EG, McGeer PL (1976) Duplication of biochemical changes in Huntington's chorea by intrastriatal injections of glutamic acid and kainic acids. Nature 263: 517-519

McNulty JA (1984) Functional morphology of the pineal complex in cyclostomes, elasmobranchs, and bony fishes. Pineal Res. Rev. 2: 1-40

McNulty JA, Fox LM, Kus L (1989) Free amino acids in the pineal gland: circadian changes and effects of superior cervical ganglionectomy. Soc. Neurosci. Abstr. 15: 951

McNulty JA, Fox LM, Lisco SJ (1987) Pinealocyte dense-cored vesicles ans synaptic ribbons: a correlative ultrastuctural-biochemical investigation in rats and mice. J. Pineal Res. 4: 45-59

McNulty JA, Kus L, Ottersen OP (1992) Pineal gland amino acids in the rat: Immunocytochemistry and circadian biochemical analysis after superior cervical ganglionectomy. Cell Tissue Res. In press

McNulty JA, McReynolds HD, Bowman DC (1990) Pineal gland free amino acid and indoles during postnatal development of the rat: correlations in individual glands. J. Pineal Res. 9: 65-73

McNulty JA, Relfson M, Fox LM, Kus L, Handa RJ, Schneider GB (1990) Circadian analysis of mononuclear immune cells in the rat following pinealectomy and superior cervical ganglionectomy. Brain Behav. Immun. 4: 292-307

Meister A (1979) Biochemistry of glutamate: glutamine and glutathione. In: Glutamic Acid: Advances in Biochemistry. L. J. J. Filer, M. R. Kare, S. Garattini, W. A. Reynolds and R. J. Wurtman (eds) Raven Press, New York, pp 69-84

Michaelis EK, Michaelis ML, Boyarski LL (1974) High affinity glutamate binding to brain synaptic membranes. Biochem. Biophys. Acta. 367: 338-348

Mikkelsen JD, Moller M (1990) A direct neural projection from the intergeniculate leaflet of the lateral geniculate nucleus to the deep pineal gland of the rat, demonstrated with *Phaseolus vulgaris* leucoagglutinin. Brain Res. 520: 342-346

Miller RJ (1991) The revenge of the kainate receptor. Trends Neurosci. 14: 447-497

Minneman KP, Iverson LL (1976) Diurnal rhythm in rat pineal cyclic nucleotide phosphodiesterase activity. Nature 260: 59-61

Monaghan DT, Bridges RJ, Cotman CW (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 29: 365-402

Monaghan DT, Cotman CW (1982) The distribution of [^{3}H]kainic acid binding sites in rat CNS as determined by autoradiography. Brain Res. 252: 91-100

Monaghan DT, Yao D, Cotman CW (1984) Distribution of [^{3}H]AMPA binding sites in rat brain as determined by quantitative autoradiography. Brain Res. 324: 160-164

Moore RJ (1973) Retinohypothalamic projection in mammals: a comparative study. Brain Res. 49: 403-409

Moore RJ, Lenn NJ (1972) A retinohypothalamic projection in the rat. J. Comp. Neurol. 146: 1-14

Moore RY (1978) The innervation of the mammalian pineal gland. Prog. Reprod. Biol. 4: 1-29

Moore RY, Heller A, Wurtman RJ, Axelrod J (1964) Visual pathway mediating pineal responses to environmental light. Science 155: 230-232

Moore RY, Klein DC (1974) Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity. Brain Res. 71: 17-33

Moore RY, Rapport RL (1971) Pineal and gonadal function in the rat following cervical sympathectomy. Neuroendocrinol. 7: 361-374

Morton DJ (1987) Both hydroxy- and methoxyindoles modify basal temperature in the rat. J. Pineal Res. 4: 1-5

Moujir F, Sanchez-Franco F, Santana C, Cacicedo L, Alonso R (1990) Immunoreactive levels of pineal arginine vasopressin change during the rat estrous cycle. J. Pineal Res. 8: 359-366

Muller F, Wassle H, Voigt T (1988) Pharmacological modulation of the rod pathway in the cat retina. J. Neurophysiol. 59: 1657-1672

Murphy TH, Miyamoto M, Sastre A, Schnarr RL, Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2: 1547-1558

Nicklas WJ, Zeevalk G, Hyndman A (1987) Interactions between neurons and glia in glutamate/glutamine compartmentation. Biochem. Soc. Trans. 15: 208-210

Nicoletti F, Iadarola MJ, Wroblewski JT, Costa E (1986) Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism:

developmental changes and interaction with α_1-adrenoceptors. Proc. Natl. Acad. Sci. 83: 1931-1935

Nicoletti F, Meek JF, Iadarola M, Chuang DM, Roth BL, Costa E (1986) Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. J. Neurochem. 46: 40-46

Nicoletti F, Wroblewski JT, Novelli A, Alho H, Guidotti A, Costa E (1986) The activation of inositol phospholipid metabolism as a signal transducing system for excitatory amino acids in primary cultures of cerebellar granule cells. J. Neurosci. 6: 1905-1911

Nilsson KJ, Reiter RJ (1989) *In vivo* stimulation of Syrian hamster pineal melatonin levels by isoproterenol plus phenylephrine is not accompanied by a commensurate large increase in N-acetyltransferase activity. Neuroendocrinol. Lett. 11: 63-68

Nir I, Briel G, Dames W, Neuhoff V (1973) Rat pineal free amino acids diurnal rhythm and effect of light. Arc. Intn. Phys. Biochim. 81: 617-627

Nishikawa T, Morita K, Kinjo K, Tsujimoto A (1982) Stimulation of catecholamine release from isolated adrenal glands by some amino acids. Jap. J. Pharmacol. 32: 291-297

Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334: 661-665

Noble EP, Sincini E, Bergmann D, Bruggencate G (1989) Excitatory amino acids inhibit stimulated phosphoinositide hydrolysis in the rat prefrontal cortex. Life Sci. 44: 19-26

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurons. Nature 307: 462-465

Nowak R, Rajkumar RR, Webley GE, Rodway RG (1990) Effect of prolonged exposure to exogenous melatonin on the onset and end of the breeding season and on the growth rate of ewe lambs. Br. Vet. J. 146: 17-23

Oksche A (1965) Survey of the development and comparative morphology of the pineal organ. In: Progress in Brain Research. J. A. Kappers and J. P. Schade (eds) Elsevier, Amsterdam, pp 3-29

Oksche A, Hartwig HG (1979) Pineal sense organs: components of photoneuroendocrine systems. In: Progress in Brain Research. A. J. Kappers and P. Pevet (eds) Elsevier, Amsterdam, pp 113-130

Olcese J (1991) Neuropeptide Y: an endogenous inhibitor of norepinephrine-stimulated melatonin secretion in the rat pineal gland. J. Neurochem. 57: 943-947

Olney JW, Price MT, Samson L, Labruyere J (1986) The role of specific ions in glutamate neurotoxicity in a neural cell line. Neurosci. Lett. 65: 65-71

Olsen RW, Szamraj O, Houser CR (1987) [^{3}H]AMPA binding to glutamate receptor subpopulation in rat brain. Brain Res. 402: 243-254

Oren DA, Brainard GC, Johnston SH, Joseph-Vanderpool JR, Sorek E, Rosenthal NE (1991) Treatment of seasonal affective disorder with green light and red light. Amer. J. Psych. 148: 509-511

Ottersen OP (1987) Postembedding light- and electron microscopic immunocytochemistry of amino acids: description of a new model system allowing idential conditions for specificity testing and tissue processing. Exp. Brain Res. 69: 167-174

Ottersen OP (1988) Quantitative assessment of taurine-like immunoreactivity in different cell types and processes in rat cerebellum: an electronmicroscopic study based on a postembedding immunogold labelling procedure. Anat. Embryol. 178: 407-421

Palmer E, Monaghan DT, Cotman CW (1989) Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. Eur. J. Pharmacol. 166: 585-587

Pang SF, Yip PCY (1988) Secretory patterns of pineal melatonin in the rat. J. Pineal Res. 5: 279-292

Pangerl B, Pangerl A, Reiter RJ (1990) Circadian variations of adrenergic receptors in the mammalian pineal gland: A review. J. Neural Trans. 81: 17-29

Pardridge WM (1979) Regulation of amino acid availability to brain: selective control mechanisms for glutamate. In: Glutamic Acid: Advances in Biochemistry and Physiology. L. JJ Filer, MR Kare, S Garattini, WA Reynolds and RJ Wurtman (eds) Raven Press, New York, pp 125-137

Parfitt A, Weller JL, Klein DC (1975) Blockade by ouabain or elevated potassium ion concentration of the adrenergic and adenosine cyclic 3'5'-monophosphate-induced stimulation of pineal serotonin N-acetyltransferase activity. Mol. Pharmacol. 11: 241-255

Patrickson JW, Smith TE (1987) Innervation of the pineal gland in the rat: an HRP study. Exp. Neurol. 95: 207-215

Patrickson JW, Smith TE (1987) Sympathetic projections to the epithalamus. Exp. Neurol. 97: 413-417

Perlman I, Normann RA, Anderton PJ (1987) The effects of prolonged superfusions with acidic amino acids and their agonists on field potentials and horizontal cell photoresponses in the turtle retina. J. Neurophys. 57: 1022-1032

Peters S, Koh T, Choi D W. (1987) Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons. Science 236: 589-592

Pevet P (1983) Is 5-methoxytryptamine a pineal hormone? Psychoneuroendocrinology 8: 61-73

Phillis JW, Krnjevie K (1961) Sensitivity of cortical neurons to acetylcholine. Experentia 17: 469

Pratt OE (1976) The transport of metabolizable substances into the living brain. Adv. Exp. Biol. Med. 69: 55-75

Purpura DP, Girado M, Smith TG, Callan DA, Grundfest H (1959) Structure-activity determinants of pharmacological effects of amino acids and related compounds on central synapses. J. Neurochem. 3: 238-268

Purves D, Lichtman JW (1985) Priciples of Neural Development, edn. Sinauer Assoc. Inc., Sunderland

Quay WB (1963) Circadian rhythm in rat pineal serotonin and its modifications by estrous cycle and photoperiod. Gen. Comp. Endocrinol. 3: 473-479

Rando TA, Bowers CW, Zigmond RE (1981) Localization of neurons in the rat spinal cord which project to the superior cervical ganglion. J. Comp. Neurol. 196: 73-83

Ransom RW, Stec NL (1988) Cooperative modulation of [^{3}H]-MK801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. J. Neurochem. 51: 830-836

Reiter RJ (1972) Surgical procedures involving the pineal gland which prevent gonadal degeneration in adult male hamsters. Ann. Endocrinol. 33: 571-581

Reiter RJ (1980) The pineal and its hormones in the control of reproduction in mammals. Endo. Rev. 1: 109-131

Reiter RJ (1991) Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocrine Rev. 12: 151-180

Reiter RJ, Richardson BA, Johnson LY, Ferguson BN, Dinh DT (1980) Pineal melatonin rhythm: reduction in aging Syrian hamsters. Science 210: 1372-1373

Reppert SM, Weaver DR, Rivkees SA, Stopa EG (1988) Putative melatonin receptors in a human biological clock. Science 242: 78-81

Reuss S (1987) Electrical activity of the mammalian pineal gland. Pineal Res. Rev. 5: 153-189

Reuss S, Moller M (1986) Direct projections to the rat pineal gland via the stria medularis thalami. Cell Tiss. Res. 244: 691-694

Roberts PJ (1974) Glutamate receptors in the rat CNS. Nature 252: 399-401

Romero JA, Zatz M, Axelrod J (1975) Beta-adrenergic stimulation of pineal N-acetyltransferase: adenosine 3':5'-cyclic monophosphate stimulates both RNA and protein synthesis. Proc. Natl. Acad. Sci. 72: 2107-2111

Ronnekleiv OK, Kelly MJ, Wuttke W (1980) Single unit recording in the rat pineal gland: evidence for habenulo-pineal neural connections. Exp. Brain Res. 39: 187-192

Ronnekleiv OK, Moller M (1979) Brain-pineal nervous connections in the rat: an ultrastructural study following habenular lesion. Exp. Brain Res. 37: 551-562

Rosenstein RE, Chuluyan HE, Pereyra EN, Cardinali DP (1989) Release and effect of gamma-aminobutyric acid (GABA) on rat pineal melatonin production *in vitro*. Cell. Mol. Neurobiol. 9: 207-219

Rothman SM (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. J. Neurosci. 6: 1483-1489

Sakai K, Fafeur V, Vulliez-Le Normand B, Dray F (1988) 12-Hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-HPETE stimulate melatonin synthesis in rat pineals. Prostaglandins 35: 969-976

Sakai KK, Marks BH (1972) Adrenergic effects on pineal cell membrane potential. Life Sci. 11: 285-291

Sakai KK, Schneider D, Felt B, Marks BH (1976) The effect of αMSH on β-adrenergic receptor mechanisms in the rat pineal. Life Sci. 19: 1145-1150

Santana C, Guerrero JM, Reiter RJ, Menendez-Pelaez A (1989) Role of postsynaptic α-adrenergic receptors in the β-adrenergic stimulation of melatonin production in the Syrian hamster pineal gland in organ culture. J. Pineal Res. 7: 13-22

Sarantis M, Everett K, Attwell D (1988) A presynaptic action of glutamate at the cone output synapse. Nature 332: 451-453

Scatchard G (1949) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51: 660-672

Schoepp D, Bockaert J, Sladeczek F (1990) Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. Trends Pharmacol. 11: 508-515

Schoepp DD, Johnson BG (1988) Excitatory amino acid agonist-antagonist interaction at 2-amino-4-phosphonobutyric acid-sensitive quisqualate receptors coupled to phosphoinositide hydrolysis in slices of rat hippocampus. J. Neurochem. 50: 1605-1613

Schroder H, Bendig A, Dahl D, Groschel-Stewart U, Vollrath L (1990) Neuronal markers in the rodent pineal gland-an immunohistochemical investigation. Histochem. 94: 309-314

Silverman AJ, Hoffman DS, Zimmerman EA (1981) The descending afferent connections of the paraventricular nucleus of the hypothalamus. Brain Res. Bull. 6: 47-61

Simonneaux V, Ebadi M, Byluind DB (1991) Identification and characterization of α_{2D}-adrenergic receptors in bovine pineal gland. Mol. Pharmacol. 40: 235-241

Simonneaux V, Murrin LC, Ebadi M (1990) Characterization of D1 dopamine receptors in the bovine pineal gland with [^{3}H]SCH 23390. J. Pharmacol. Exp. Ther. 253: 214-220

Simonneaux V, Ouichou A, Burbach JPH, Pevet P (1990) Vasopressin and oxytocin modulation of melatonin secretion from rat pineal glands. Peptides 11: 1075-1079

Simonneaux V, Ouichou A, Pevet P (1990) Vasoactive intestinal peptide stimulates melatonin release from perifused pineal glands of rats. J. Neural Transm. [Gen Sect] 79: 69-79

Simonneaux V, Ouichou A, Pevet P, Masson-Pevet M, Vivien-Roels B, Vaudry H (1989) Kinetic study of melatonin release from rat pineal glands using a perifusion technique. J. Pineal Res. 7: 63-83

Sitaram BR, Lees GL (1978) Diurnal rhythms and turnover of tryptophan hydroxylase in pineal gland of the rat. J. Neurochem. 31: 1021-1026

Sladeczek F, Pin JP, Recasens M, Bockaert J, Weiss S (1985) Glutamate stimulates inositol phosphate formation in striatal neurons. Nature 317: 717-719

Sladeczek F, Recasens M, Bockaert J (1988) A new mechanism for glutamate receptor action: phosphoinositide hydrolysis. Trends Neurosci. 11: 545-549

Slaughter MM, Miller RF (1981) 2-Amino-4-phosphonobutyric acid: a new pharmacological tool for retina research. Science 211: 182-185

Sloviter RS (1983) "Epileptic" brain damage in rats induced by sustained electrical stimulation of the perforant path. I. Acute electrophysiological and light microscopic studies. Brain Res. Bull. 10: 675-697

Slusher BS, Robinson MB, Tsai G, Simmons ML, Richards SS, Coyle JT (1990) Rat brain N-acetylated a-linked acidic dipeptidase activity. J. Biol. Chem. 265: 21297-21301

Smith TL, J. E, Hauser G (1979) Postsynaptic localization of the alpha receptor-mediated stimulation of phosphatidylinositol turnover in pineal gland. Life Sci. 24: 2179-2184

Snyder SH, Axelrod J, Zweig M (1967) Circadian rhythm in the serotonin content of the pineal gland: regulating factors. J. Pharmacol. Exp. Ther. 158: 206-213

Snyder SH, Zweig M, Axelrod J, Fischer JE (1965) Control of the circadian rhythm in serotonin content of the rat pineal gland. Proc. Natl. Acad. Sci. 53: 301-305

Sommer B, Keinanen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Kohler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: A cell-specific functional switch in glutamate-operated channels of the CNS. Science 249: 1580-1585

Somogyi P, Halasy K, Somogyi J, Storm-Mathisen J, Ottersen OP (1986) Quantification of immunogold labelling reveals enrichment of glutamate in mossy and parallel fibre terminals in cat cerebellum. Neurosci. 19: 1045-1050

Spencer PS, Ludolph A, Dwived MP, Roy DN, Hugon J, Schamburg HH (1986) Lathyrism: evidence for role of the neuroexcitatory amino acid BOAA. Lancet 2: 1066-1067

Stankov B, Lucini V, Mariani M, Scaglione F, Demartini G, Fraschini F (1990) Alpha-1 adrenoceptor involvement in the control of melatonin secretion in the golden hamster. J. Pineal Res. 9: 21-28

Stanley LC, Horikawa K, Powell EW (1987) Innervation of the superficial pineal of the rat using retrograde tracing methods. Am. J. Anat. 180: 249-254

Stauch BL, Robinson MB, Forloni G, Tsai G, Coyle JT (1989) The effects of N-acetylated alpha-linked acidic dipeptidase (NAALADase) inhibitors on [^{3}H]NAAG catabolism *in vivo*. Neurosci. Lett. 100: 295-300

Stehle J, Reuss S, Riemann R, Seidel A, Vollrath L (1991) The role of arginine-vasopressin for pineal melatonin synthesis in the rat: involvement of vasopressinergic receptors. Neurosci. Lett. 123: 131-134

Storm-Mathisen J, Lekness AK, Bore AT, Vaaland JL, Edminson P, Haug F-MS, Ottersen OP (1983) First visualization of glutamate and GABA in neurons by immunocytochemistry. Nature 301: 517-520

Storm-Mathisen J, Ottersen OP (1990) Immunocytochemistry of glutamate at the synaptic level. J. Histo. Cyto. 38: 1733-1743

Sugden D (1979) Circadian changes in rat pineal tryptophan content: lack of correlation with serum tryptophan. J. Neurochem. 33: 811-813

Sugden D (1989) Melatonin biosynthesis in the mammalian pineal gland. Experientia 45: 922-932

Sugden D (1990) β-adrenergic regulation of cyclic GMP in rat pinealocytes. Biochem. Biophys. Res. Comm. 167: 835-841

Sugden D (1991) Down-regulation of pinealocyte protein kinase C: effect on α1-adrenergic potentiation of b-adrenoceptor stimulation of cyclic AMP accumulation and induction of serotonin N-acetyltransferase activity. J. Neurochem. 57: 216-221

Sugden D, Ho AK, Sugden LA, Klein DC (1988) Negative feedback mechanisms: evidence that desensitization of pineal α1-adrenergic responses involves protein kinase C. Endocrinology 123: 1425-1432

Sugden D, Klein DC (1984) Rat pineal α_1-adrenoceptors: identification and characterization using [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone. Endocrinology 114: 435-440

Sugden D, Klein DC (1985) Regulation of rat pineal α1-adrenoceptors. J. Neurochem. 44: 63-67

Sugden D, Klein DC (1988) Activators of protein kinase C act at a postreceptor site to amplify cyclic AMP production in rat pinealocytes. J. Neurochem. 50: 149-155

Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB (1985) Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 314: 359-361

Sugden D, Weller JL, Klein DC, Kirk KL, Creveling CR (1984) Alpha-adrenergic potentiation of beta-adrenergic stimulation of rat pineal N-acetyltransferase. Biochem. Pharmacol. 33: 3947-3950

Sugden LA, Sugden D, Klein DC (1986) Essential Role of Calcium influx in the adrenergic regulation of cAMP and cGMP in rat pinealocytes. J. Biol. Chem. 261: 11608-11612

Sugden LA, Sugden D, Klein DC (1987) α1-adrenoceptor activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. J. Biol. Chem. 262: 741-745

Suranyi-Cadotte B, Lal S, Nair NPV, Lafaille F, Quirion R (1987) Coexistence of central and peripheral benzodiazepine binding sites in the human pineal gland. Life Sci. 40: 1537-1543

Swann AC, Steketee JD (1989) Subacute noradrenergic agonist infusions *in vivo* increase Na^+, K^+-ATPase and ouabain binding in rat cerebral cortex. J. Neurochem. 52: 1598-1604

Swanson LW, Kuypers HGJM (1980) A direct projection from the ventromedial nucleus and retrochiasmatic area of the hypothalamus to the medulla and the spinal cord of the rat. Neurosci. Lett. 17: 307-312

Tachibana M, Kaneko A (1988) L-glutamate-induced depolarization in solitary photoreceptors: A process that may contribute to the interaction between photoreceptors *in situ*. Proc. Natl. Acad. Sci. 85: 5315-5319

Tamarkin L, Hollister CW, Lefebvre NG, Goldman BD (1977) Melatonin induction of gonadal quiescence in pinealectomized Syrian hamsters. Science 198: 935-955

Taylor RL, Albuquerque MLC, Burt DR (1980) Muscarinic receptors in pineal. Life Sci. 26: 2195-2200

Thompson C, Stinson D, Smith A (1990) Seasonal affective disorder and season-dependent abnormalities of melatonin suppression by light. Lancet 336: 703-706

Torp R, Andine P, Hagberg H, Karagulle T, Blackstad TW, Ottersen OP (1991) Cellular and subcellular redistribution of glutamate-, glutamine-, and taurine-like immunoreactivities during forebrain ischemia: a semiquantitative electron microscopic study in the rat hippocampus. Neurosci. 41:433-447

Ueck M, Wake K (1977) The pinealocyte - a paraneuron? Arch. Histol. Jap. 40, Suppl.: 261-278

Ulshafer RJ, Sherry DM, Dawson R Jr., Wallace DR (1990) Excitatory amino acid involvement in retinal degeneration. Brain Res. 531: 350-354.

Unnerstall JR, Wamsley JK (1983) Autoradiographic localization of high-affinity [^{3}H]kainic acid binding sites in the rat forebrain. Eur. J. Pharmacol. 86: 361-371

Vacas MI, Lowenstein PR, Cardinali DP (1980) Dihydroergocryptine binding sites in bovine and rat pineal glands. J. Auto. Nerv. Syst. 2: 305-313

Vacas MI, Sarmiento MI, Pereyra EN, Cardinali DP (1989) Effect of adenosine on melatonin and norepinephrine release in rat pineal explants. Acta Physiol. Pharmacol. Latino. 39: 189-195

Van den Pol AN, Wuarin J, Dudek FE (1990) Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. Science 250: 1276-1277

Van Harreveld A (1959) Compounds in brain extracts causing spreading depression of cerebral cortical activity and contraction of crustacean muscle. J. Neurochem. 3: 300-315

Van Veen T, Ostholm T, Gierschik P, Spiegel A, Somers R, Korf HW, Klein DC (1986) α-Transducin immunoreactivity in retinae and sensory pineal organs of adult vertebrates. Proc. Natl. Acad. Sci. 83: 912-916

Van Winkle LJ (1988) Amino acid transport in developing animal oocyte and early conceptuses. Biochim. Biophys. Acta 947: 173-208

Vanecek J, Sugden D, Weller J, Klein DC (1985) Atypical synergistic α1- and β-adrenergic regulation of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in rat pinealocytes. Endocrinology 116: 2167-2173

Vaughan GM (1984) Melatonin in humans. In: Pineal Research Reviews. R. J. Reiter (ed). Alan R. Liss, New York, pp 239-247

Voisin P, Namboodiri MAA, Klein DC (1984) Arylamine N-acetyltransferase and arylalkylamine N-acetyltransferase in the mammalian pineal gland. J. Biol. Chem. 259: 10913-10918

Vollrath L, Schroder H (1987) Neuronal properties of mammalian pinealocytes? In: Fundamentals and Clinics in Pineal Research. G. P.

Trentini, C. DeGaetani and P. Pevet (eds) Raven Press, New York, pp 13-23

Vriend J, Sackman JW, Reiter RT (1977) Effect of blinding, pinealectomy, and superior cervical ganglionectomy on free thyroxine index of male Syrian hamsters. Acta Endocrinology 81: 758-762

Waelsch H (1951) Glutamic acid and cerebral function. Adv. Protein Chem. 6: 299-331

Wang JKT, Andrews H, Thukral V (1992) Presynaptic glutamate receptors regulate noradrenaline release from isolated nerve terminals. J. Neurochem. 58: 204-211

Watkins JC (1962) The synthesis of some amino acids possessing neuropharmacological activity. J. Med. Pharm. Chem. 5: 1187-1199

Watkins JC, Krogsgaard-Larsen P, Honore T (1990) Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. Trends Pharmacol. Sci. 11: 25-33

Wayne NL, Malpaux B, Karsch FJ (1990) Photoperiodic requirements for timing onset and duration of the breeding season of the ewe: synchronization of an endogenous rhythm of reproduction. J. Comp. Physiol. A 166: 835-842

Webb SM, Champney TH, Steger RW, Bartke A, Reiter RJ (1984) Immunoreactive somatostatin in the pineal gland of different rodent species: circadian rhythm, effects of superior cervical ganglionectomy, pineal indole administration and lighting conditions. Biomed. Res. 6: 473-480

Weissman BA, Skolnick P, Klein DC (1984) Regulation of "peripheral-type" binding sites for benzodiazepines in the pineal gland. Pharmacol. Biochem. Behav. 21: 821-824

Werner P, Voigt M, Keinanen K, Wisden W, Seeburg PH (1991) Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. Nature 351: 742-744

Westbrook GL, Mayer ML (1987) Micromolecular concentrations of Zn^{++} antagonize NMDA and GABA responses of hippocampal neurons. Nature 328: 640-646

Wheler GHT, Weller JL, Klein DC (1979) Taurine: stimulation of pineal N-acetyltransferase activity and melatonin production via a beta-adrenergic mechanism. Brain Res. 166: 65-74

Williams LM, Morgan PJ (1988) Demonstration of melatonin-binding sites of the pars tubaralis of the rat. J. Endocrinol. 119: R1

Winters WD, Yuwiler A, Oxenkrug GF (1991) The effects of benzodiazepines on basal and isoproterenol-stimulated N-acetyltransferase activity by the rat pineal gland, *in vivo* and *in vitro*. J. Pineal Res. 10: 151-158

Wong EHF, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iverson LL (1986) The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. Proc. Natl. Acad. Sci. 83: 7104-7108

Woodruff GN, Foster AC, Gill R, Kemp JA, Wong EHF, Iverson LL (1987) The interaction between MK-801 and receptors for N-methyl-D-aspartate: functional consequences. Brain Res. 26: 903-909

Wurtman RJ, Shein HM, Larin F (1971) Mediation by β-adrenergic receptors of effect of norepinephrine on pineal synthesis of [^{14}C]serotonin and [^{14}C]melatonin. J. Neurochem. 18: 1683-1687

Yoneda Y, Ogita K (1989) Solubilization of quisqualate-sensitive [^{3}H]glutamate binding activity from rat retina. J. Neurochem. 52: 1501-1507

Yoneda Y, Ogita K (1989) Solubilization of stereospecific and quisqualate-sensitive activity of [^{3}H]glutamate binding in the pituitary of the rat. Neuropharmacol. 28: 611-616

Yoshida Y, Huang FC, Nakabayashi H, Huang KP (1988) Tissue distribution and development expression of protein kinase C isozymes. J. Biol. Chem. 263: 9868-9873

Zeevalk GD, Hyndman AG, Nicklas WJ (1989) Excitatory amino acid-induced toxicity in chick retina: amino acid release, histology, and effects of chloride channel blockers. J. Neurochem. 53: 1610-1619

The dissertation submitted by Laura Kus has been read and approved by the following committee:

Dr. John A. McNulty, Chairperson
Professor, Department of Cell Biology, Neurobiology and Anatomy
Loyola University Chicago

Dr. Robert J. Handa
Assistant Professor, Department of Cell Biology, Neurobiology and Anatomy
Loyola University Chicago

Dr. Edward J. Neafsey
Associate Professor, Department of Cell Biology, Neurobiology and Anatomy
Loyola University Chicago

Dr. George Battaglia
Assistant Professor, Department of Pharmacology
Loyola University Chicago

Dr. Nicholas V. Emanuele
Professor, Department of Medicine
Hines V.A. Hospital

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

20 Apr 92
Date

Director's Signature