Demonstration of Amino Acid Neurotransmitter Innervation in Human Pineal Gland

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ABSTRACT: The amino acid neurotransmitters, γ-aminobutyric acid (GABA) and glutamate (Glu), are known to be involved in the physiological functions of the mammalian pineal gland. In order to investigate both of these innervations in the human pineal, the immunohistochemical study was performed on the human pineal glands by using monoclonal antibodies against GABA and antiserum against glutamate as probes. GABA-immunoreactive (IR) cells and nerve fibers were found throughout the gland. Some IR cells resembled neurons with long processes were found occasionally. Only a few Glu-IR nerve fibers but many Glu-IR cell bodies were demonstrated in the human pineal gland. They were arranged with unstained cells into clusters and some of them appeared to be neuron-like cells. Therefore, the present study supports the theory of regulation by both GABA and glutamate amino acid neurotransmitters in the human pineal gland. In addition, the presence of numerous immunoreactive cells indicates paracrine or local circuit regulation in human pineal.

KEYWORDS: GABA , glutamate, human, pineal innervation, immunohistochemistry.

INTRODUCTION

The amino acid neurotransmitters, γ-aminobutyric acid (GABA) and glutamate (Glu), may have physiological effects on the mammalian pineal gland in addition to norepinephrine released from the postganglionic sympathetic neurons of the superior cervical ganglion. Evidence of the presence and role of GABA, an inhibitory neurotransmitter, in the mammalian pineal gland has previously been reported.[1] In addition, the release and uptake sites of GABA were studied in the pineal gland and suggested to be gliocyte cells and 'neuron-like' compartments in the pineal gland. Moreover, GABAergic receptor sites were characterized in bovine[2] and human[3] pineal gland with both high- and low-affinity sites. The localization of GABA in bovine pineal gland was found to be in cells exhibiting morphological characteristics of pinealocytes.[4] Then, again, GABA has been shown to have an inhibitory effect on the norepinephrine (NE)-induced stimulation of the enzyme serotonin N-acetyltransferase (NAT) activity in a dose-dependent fashion in the bovine pineal gland.[5]

The presence of a high concentration of Glu in the mammalian pineal gland has been repeatedly reported.[6] Later, Glu binding sites were pharmacologically characterized in the pineal glands of cow[7] and rat[8]. Furthermore, L-Glu was shown to inhibit NE-stimulated NAT activity[9] and melatonin synthesis.[10] Glu was also localized in the pinealocytes of the mammalian pineal gland by immunohistochemical studies.[11] Until now, the presence and distribution of GABA and Glu have not been studied in the human pineal gland. In the present study, the immuno-peroxidase method was used to immunolocalize the two neurotransmitters performed by using antisera against GABA and glutamate with the avidin-biotin complex method.

MATERIALS AND METHODS

The immunohistochemical study was performed in the human pineal gland using the monoclonal antibody against GABA, (Gb6-11E, provided by Professor Antony O.W. Stretton, Department of Zoology, University of Wisconsin-Madison, Wisconsin, U.S.A.) and antiserum against Glu (supplied by Professor Jon Storm-Mathisen, Institute of Anatomy, University of Oslo, Norway). Six human pineal glands were obtained from the Institute of Forensic Medicine, the Police Hospital, Bangkok, Thailand. The use of human pineals was approved by the Ethics Committee of the Faculty of Medicine, Srinakharinwirot University. After being dissected from the cadavers, they were

immediately fixed by immersion in a solution of 2% glutaraldehyde in 0.1 M Na/K-phosphate buffer (pH 7.4) and transported back to the laboratory. The glands were then cut into 2-mm-thick sagittal slices, postfixed in the same fixative at 4 °C for three weeks and processed for paraffin embedding. The tissue block was cut by an LKB microtome at 5 or 15 μm thick and the sections were then affixed to glass slides.

For immunohistochemical procedures, the dewaxed pineal sections were washed for 2×5 min in 0.1 M PBS and then pretreated in 1% H_2O_2 in PBS for 10 min and followed by incubating in 5% normal swine serum in PBS-A (PBS containing 0.3% Triton X-100, 1% bovine serum albumin) for 30 min. The sections were then incubated for 3 days at 4 °C in the monoclonal antibody against GABA diluted 1:100 or the polyclonal antiserum against Glu diluted 1:1000 in PBS-A. After washing three times in PBS-B (PBS containing 0.1% Triton X-100 and 0.25% bovine serum albumin), the sections were incubated in the biotinylated second antibodies for 60 min at room temperature and then washed in PBS-B for 3×10 min. They were treated with avidin-biotin horseradish peroxidase complex (Vector, USA) diluted 1:250 in PBS for 60 min and then washed sequentially in PBS for 2×10 min and in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min. The tissue sections were further reacted with a solution of 0.025% 3, 3'-diaminobenzidine (DAB) containing 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 30 min. After being rinsed for 2×5 min in distilled water, the sections were counterstained with 0.1% eosin, then dehydrated, cleared in xylene and cover-slipped with Permount®.

For the preabsorption control, the sections were incubated with antisera against GABA diluted 1:100 in GABA-BSA, or with antisera against Glu diluted 1:100 in Glu-BSA. In addition, some pineal sections were stained with 0.1% cresyl violet for Nissl staining.

RESULTS

The human pineal glands were cone-shaped and extended from the habenular to the posterior commissures (Fig 1). They were covered by the pial capsule which penetrated into the gland as the pial septae, dividing the parenchyma into small lobules which looked like follicles. Some "brain sand" was also seen within the gland.

Immunohistochemical Localization of GABA in the Human Pineal Gland

By the use of monoclonal antibody against GABA, both immunoreactive (IR) cells and nerve fibers were demonstrated within the gland (Fig 1C). A moderate amount of GABA-IR cells (2% of total cell population)

was present throughout the gland . Most of them were oval or irregular in shape and 5-7 μm in diameter (Figs 2A, 2B). Furthermore, there were other IR cells with morphology appearing like neurons (6 - 10 μm in diameter) with processes (Figs 2B, 2C). However, the number of positive neuronal-like cells was fewer than that of GABA-IR cells (0.25% of total cell population).

A small number of GABA-IR nerve fibers were widely-distributed in the gland, both in the perivascular space (Fig 2D) and intraparenchymally between the pinealocytes (Figs 3A, 3B). They exhibited various shapes, i.e., smooth, coiled, and endowed with bouton-like dots. Some fibers were endowed with huge varicosities exhibiting a peptidergic fiber-like morphology (Fig 3B). In addition, at the base of the gland, many GABA-IR cells were observed, intermingled with some GABA-IR nerve fibers (Fig 1C). Immunostaining of the
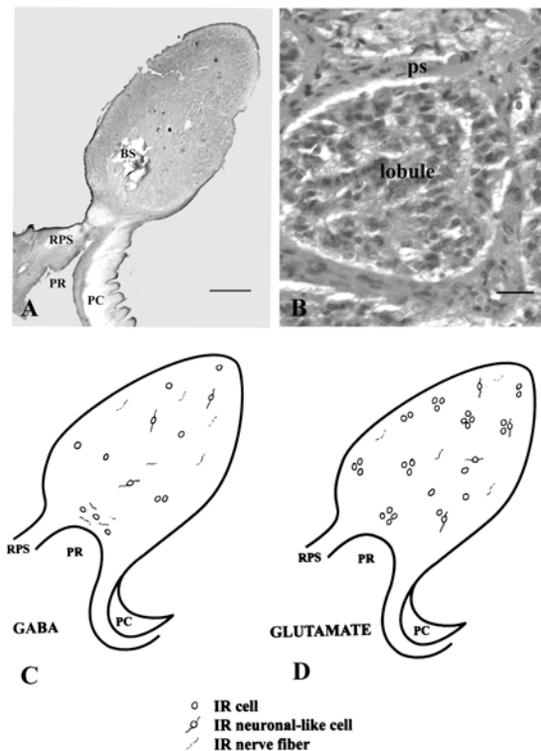

Fig 1. The photomicrographs of the human pineal stained with crysel violet (A and B). A) The picture of the whole gland at low power showing the brain sand (BS) within the gland. B) The micrograph at higher magnification shows that the pinealocytes arrange themselves as a lobule which surrounded by the pial septum (ps). C) and D) Drawing of a sagittal section of a human pineal gland showing the densities and localization of GABA- and glutamate-immunoreactivities. RPS, rostral pineal stalk; PR, pineal recess; PC, posterior commissure. Bar, 1 mm (A), 20 μm (B).
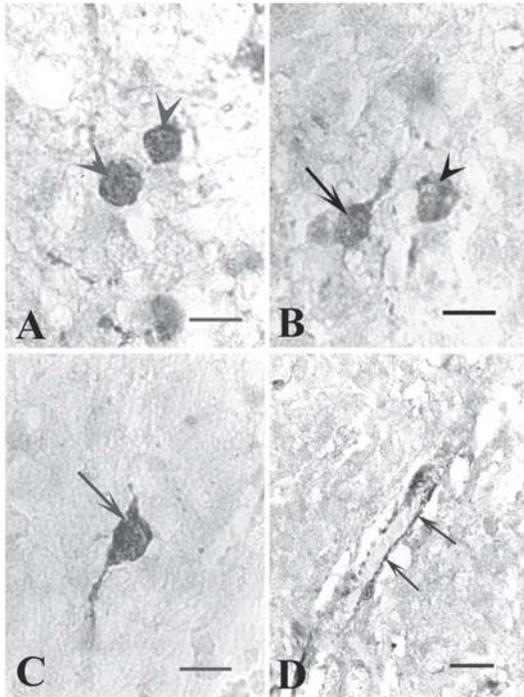
Fig 2. GABA immunoreactivity in the human pineal gland. A)
Two GABA-IR cells (arrowheads). B) Both GABA-IR cell
(arrowhead) and neuronal-like cell (arrow). C) GABA-IR
neuronal-like cell (arrow) with its processes. D) GABA-
IR nerve fiber (arrows) along a vessel. Bar, 10 μm (A, B,
C), 20 μm (D).

antiserum against GABA was completely abolished by
liquid phase absorption of the antiserum with GABA.

Immunohistochemical Localization of Glu in the Human Pineal Gland

By using the antiserum against Glu, a large num-
ber of Glu-IR cells (25% of total cell population) and a
very few IR nerve fibers were demonstrated in the
human pineal gland (Fig 1D). These Glu-IR cells were
rather oval and 4-6 mm in diameter. They were divided
into deeply-stained and weakly-stained cells, and were
intermingled with unstained cells (Figs 4A, 4B). In ad-
dition, some Glu-IR intrapineal neuronal-like cells (5-
7 mm in diameter) were also observed occasionally
(Figs 4A, 4C).

A small number of Glu-IR nerve fibers were found
within the gland. They were located both in the perivas-
cular space and the intraparenchyma of the gland (Fig
4D). Immunostaining of the antiserum against Glu was
completely abolished by liquid phase absorption of the
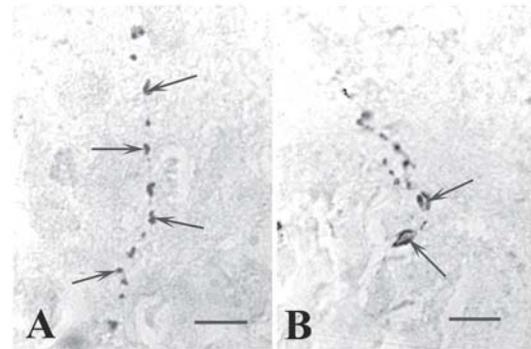antiserum to Glu.

Fig 3. GABA immunoreactive (IR) nerve fibers in the human
pineal gland. A, Thin GABA-IR nerve fiber with a dot-like
varicosities (arrows). B, IR nerve fiber fiber with big
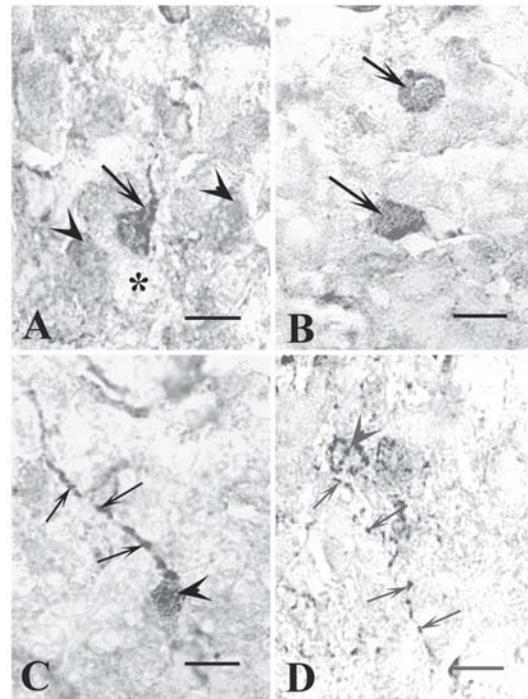boutons *en passage* (arrows). Bar, 10 μm (A, B).

Fig 4. Glutamate immunoreactivity in the human pineal gland.
A) A deeply-stained Glu-IR cell (arrow), a weakly-stained
cell (arrowheads) and an unstained cell (star). B) Two
deeply-stained Glu-IR cells (arrows). C) Glu-IR neuronal-
like cell (arrowhead) with a long process (arrows). D)
Thin Glu-IR nerve fiber (arrows) with dot-like varicosities
and Glu-IR cell (arrowhead). Bar, 10 μm (A, B, C, D).

DISCUSSION

The presence of GABA-IR cells and nerve fibers in
the human pineal gland indicates GABAergic
innervation of the gland and supports previous studies
that detected GABA[12] and its receptor sites[3] in human
pineal. As for classification of the GABA-IR cells in the
human pineal, most of them could be pinealocytes,

because they correspond to the pinealocytes in adjacent sections stained by cresyl violet. These cells have spherical nuclei containing a conspicuous, centrally located nucleolus surrounded by a large quantity of heterochromatin, and lacking Nissl substance. A previous immunohistochemical study in bovine pineal gland[4] had also demonstrated that GABA-positive cells exhibited the morphological characteristics of pinealocytes. The presence of 2% of GABA-IR pinealocytes supports the concept of the heterogeneity of pinealocytes from the previous study. By using morphological criteria, the other kinds of GABA-IR cells in the present study are probably positive neuronal-like cells, since they are larger than the pinealocytes and send their long processes from their soma. These cells were also reported in the cat pineal.[13] Intrapineal neurons have been previously demonstrated in various mammalian species including humans.[14] Furthermore, by immunohistochemical study, these human intrapineal neurons were shown to contain different kinds of neurotransmitters and neuropeptides, e.g, opioid peptides[15] and substance P.[16] The presence of GABA-IR nerve fibers located in the parenchyma of the human pineal gland implies that these fibers might originate either from the intrapineal neuronal-like cells or from the perikarya outside the gland. However, those fibers at the base of the gland which connect to the human pineal stalk found in the present study indicate a central innervation from the brain in humans. The habenular complex is of interest as possibly being the perikarya origin of GABAergic innervation. Autoradiography of [³H]-GABA accumulation and immunocytochemistry of glutamic acid decarboxylase (GAD) showed a very heavy innervation of the habenular complex.[17] In addition, paracrine regulation may be another possible explanation for GABA existing in the human pineal because of the appearance of GABA-IR pinealocytes in the present study. These GABA-containing pinealocytes can synthesize, uptake, accumulate and secrete GABA[1] which has a physiological effect on pineal function. Moreover, the role of endogenous GABA in the modulation of human melatonin production has also been demonstrated. After administration of sodium valproate, a GABAergic drug, to healthy humans during the evening, a significant suppression of nocturnal plasma melatonin levels was observed.[18] Furthermore, GABA transporter proteins have been demonstrated in pinealocytes, as well as in interstitial glial cells.[19] These previous studies, together with the present results showing the presence of GABA-containing pinealocytes and neuronal-like cells in the human pineal, support the idea that GABA within the pineal itself may participate in the modulation of the activity of the gland as a paracrine regulator.

The demonstration of Glu-IR pinealocytes in human pineal, in the present study, is in good agreement with previous reports in other mammalian pineal. In addition, the human Glu-IR pinealocytes were arranged into clusters intermingled with unstained pinealocytes. Each cluster contained deeply-stained, weakly-stained and unstained Glu-IR pinealocytes. This result suggests a paracrine control within the cluster. It is also supported by a previous study wherein Glu immunoreactivity was observed in the gerbil pinealocytes.[11] Furthermore, synaptic-like micro-vesicles were demonstrated as the storage and release sites of Glu within pinealocytes, and the release of Glu from them acted as a paracrine signal on other pinealocytes.[20] In the present study, a small amount of Glu-IR intrapineal neuron-like cells and Glu-IR nerve fibers were demonstrated in the human pineal. Glu has also been found to accumulate in the large secondary neurons of other mammalian pineal.[21] Therefore, Glu-IR fibers in this study may be the nerve processes from either intrapineal neurons or neurons outside the gland. Numerous pharmacological and functional investigations concerning the effects of Glu on the pineal gland have been conducted. The α-amino-3-hydroxy-5-methyl-4-iso-xazolepropionic acid (AMPA) type of Glu receptor has been reported in the bovine pineal gland.[7] A high-affinity kainate receptor subunit transcript (KA-2) has also been localized in the rat pineal gland. The affinity of [³H] Glu binding sites in rat pineal gland were found to increase following superior cervical ganglionectomy.[8] Furthermore, the expression of Glu receptor subunits has been demonstrated by *in situ* hybridization studies in rat and macaque monkey[22] pineal glands. Recently, several types of Glu receptors have been reported in the pineal, i.e., metabotropic Glu receptor type 5 (mGluR5) in rat pinealocytes,[23] and mGluR2/3 and mGluR5 in the interstitial glial cells of gerbil pineal.[24] As well, it has been showed that Glu activated pinealocytes via these metabotrophic Glu receptors and inhibited NE-stimulated melatonin secretion.[24,25] The activation of mGluR5 in rat pinealo-cytes could trigger a calcium efflux from intracellular storage and then inhibit cAMP production, thus resulting in a reduction of melatonin synthesis.[23]

From an anatomical point of view, the density and distribution of both the GABAergic and glutamatergic systems are compared in the present study. The GABA-IR cells are more scattered, while most of the Glu-IR cells are arranged in clusters. This finding may indicate different patterns for their roles in controlling pineal function. The glutaminergic system may have a feed-back paracrine control among each glutaminergic cell within the cluster, while the GABAergic cells exert a paracrine control on the other pinealocytes. Further-

more, the presence of GABA-IR and Glu-IR pinealo-
cytes supports the previous reports of the hetero-
geneity of the pinealocyte population. Only GABA-IR
and Glu-IR pinealocytes can synthesize these two kinds
of neurotransmitters and then have a paracrine
control on the other pinealocytes in melatonin
synthesis. In conclusion, the results from the present
study indicate that both amino acid neurotransmitter
systems, GABAergic and glutama-tergic, are involved
in the control of human pineal function. Although
glutamate is known as an excitatory neurotransmitter,
it exerts an inhibitory control of melatonin synthesis
like GABA, an inhibitory transmitter. Such systems have
direct and/or indirect inhibitory effects on melatonin
synthesis by mainly paracrine control, but also partly
by neuronal control.

ACKNOWLEDGEMENTS

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