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 and human 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.3 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.3

The presence of a high concentration of Glu in the mammalian pineal gland has been repeatedly reported.8 Later, Glu binding sites were pharmacologically characterized in the pineal glands of cow and rat.9 Furthermore, L-Glu was shown to inhibit NE-stimulated NAT activity9 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 immunoperoxidase 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% H2O2 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% H2O2 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
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 number 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 addition, 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 perivascular 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.

**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 and its receptor sites 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 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. Intrapineal neurons have been previously demonstrated in various mammalian species including humans. Furthermore, by immunohistochemical study, these human intrapineal neurons were shown to contain different kinds of neurotransmitters and neuropeptides, e.g., opioid peptides and substance P. 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. 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 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. Furthermore, GABA transporter proteins have been demonstrated in pinealocytes, as well as in interstitial glial cells. 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.
more, the presence of GABA-IR and Glu-IR pinealocytes supports the previous reports of the heterogeneity 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 glutamatergic, 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.

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**References**