Immunohistochemical Demonstration of Opioids and Tachykinins in Human Pineal Gland

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The human pineal gland secretes melatonin in a circadian rhythm manner. The rhythm of melatonin synthesis is primarily controlled by the noradrenergic sympathetic system originating from the superior cervical ganglion. Several neurotranmitters/neuropeptides have been reported to influence the production of melatonin in the pineal glands of many mammalian species. Both opioid peptide, a pain suppressing peptide and substance P, a pain inducing peptide were also reported to be present in the pineal gland of several kinds of mammals. However, few studies have been demonstrated in humans. Therefore, in the present study, the immunohistochemical investigation was performed in the human pineal gland by using antisera raised against leu-enkephalin, met-enkephalin and beta-endorphin to demonstrate an opioidergic system; and antisera raised against substance P, neurokinin A, and neurokinin B to study a tachykinin system. A high amount of leu- and met-enkephalin immunoreactivities were observed in intrapineal neuronal-like cells while very few were presented in nerve fibers. This result suggests a local regulatory function or paracrine opioidergic control in human pineal. Substance P- and neurokinin A-immunoreactivities, but not neurokinin B were observed in the human pineal gland. They are located mostly in nerve fibers but a few in neuronal-like cells. The tachykininergic control of human pineal is mainly from the nerve fibers which have their perikaryal origin outside the gland. Some of the nerve fibers might originate from neurons in the brain and/or from a peripheral ganglion.

Keywords: *Opioid peptides, Tachykinins, Human pineal, Immunohistochemistry*

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The pineal gland is a neuroendocrine gland, which secretes the hormone melatonin[1]. It has been reported to involve in many physiological controls, for example, reproductive hormone and organs, thyroid hormone levels, aging, immune system. The secretion of melatonin exhibits a circadian rhythm with maximum during the night. This circadian secretory activity is generated in the suprachiasmatic nucleus of the brain and modified by the length of the daily photoperiod[2]. The melatonin synthesis is primarily regulated by the noradrenergic sympathetic system from the superior cervical ganglion[3]. However, numerous peptidergic nerve fibers, not belonging to the sympathetic nervous system, have anatomically been shown to innervate the mammalian pineal gland[4,5]. Opioid peptide, a pain inhibitory suppressing peptide and substance P, a pain inducing peptide were also reported to be present in the pineal gland of several mammalian species[6].

Opioid peptides, a group of neuropeptides, act as endogenous mediators at morphine receptor sites[7]. They constitute four peptide families, a)

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56-65.pmd 56 25/5/2548, 16:10

pro-opiomelanocortin, b) proenkephalin, c) prodynorphin and d) pronociceptin, which are translated from separate genes and give rise to beta-endorphin, enkephalins, dynorphins and nociceptin, respectively. A number of previous studies reported the presence of opioid peptides in the pineal gland and their influence on the synthesis of melatonin[8]. For example, the subcutaneous injection of des-tyrosine-γ-endorphin increased melatonin level[9] while morphine stimulated the release of melatonin from a rat pineal gland[10,11]. Furthermore, the presence of opioidergic innervation has been reported in the pineal gland of guinea pigs[12], cows[13], European hamsters[14], and tree shrews[15]. Moreover, opioid receptors have been characterized in the bovine pineal[16]. These receptors were demonstrated to be mu- and delta- opioid receptors subtypes and located on the pinealocytes[17,18].

Substance P (SP) was detected by Von Euler and Gaddum in 1931[19]. SP was extracted from brain and gastrointestinal tract. In the early 1970s, SP was identified as an undecapeptide[20]. SP is a member of the tachykinin family of peptides, which all contain the carboxyl terminal sequence of -Phe-X-Gly-Leu-Met-NH_2[21]. The tachykinin family consists, in addition to SP, of neurokinin A, and neurokinin B. SP and neurokinin A are encoded from the preprotachykinin I gene, which through alternative splicing express four different forms of mRNA (the alpha-, beta-, gamma-, and delta-forms). Neurokinin B synthesis is encoded from a second gene, preprotachykinin II gene. In the nervous system, numerous SP-immuno-reactive perikarya are located in the dorsal root ganglia as well as in the trigeminal ganglion[22]. The SP-immunoreactive perikarya in the trigeminal ganglion extend processes to the brain vasculature and release of SP increases the blood flow in the dura[23]. The mammalian pineal gland is highly vascularized with arterioles and capillaries, which are connected with the pial blood vessels[24]. In addition, by the use of immunohistochemistry, SPergic nerve fibers have been reported in the pineal gland of several mammalian species, in rodents: rat[25,26], gerbil[27], golden hamster[28], cotton rat[29], in non rodents: hedgehog[30], cow[31], tree shrew[32], pig[33] and macaque monkey[34]. However, few studies have been performed in the human pineal gland.

An interaction among melatonin, opioids analgesia has been suspected for many years. Several reports have shown that melatonin has a potent and long-lasting antinociceptive effect[35-37]. In studying this phenomenon further, it is of interest to demonstrate that these two pain modulating peptides, the opioidergic and tackykinergic pathways innervated in the human pineal gland and their precise location were studied by immunohistochemical technique.

Material and Method

Tissue preparation

Seven human pineals were obtained from the Institute of Forensic Medicine, Police Hospital, Bangkok, Thailand. The use of them was approved by the Ethics Committee of the Faculty of Medicine, Srinakharinwirot University. After dissecting from the cadavers, the pineals were immediately fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 and transported to the laboratory. In the laboratory, a pial capsule with the blood vessels was removed from the tissue specimens. They were postfixed in 4% paraformaldehyde in 0.1 M PB, pH 7.4 at 4°C for three weeks. Some of the specimens were then cryoprotected in 20% sucrose in phosphate buffered saline (PBS) overnight for the case of cryostat sections (sagittal sections of 20 μm). Part of the specimens was embedded in paraffin and sectioned on a sliding microtome (sagittal sections of 7 μm).

Immunohistochemistry

The immunostaining was performed on glass slides by the use of the ABC streptavidin-biotin complex technique with the following primary antisera: mouse anti-met-enkephalin (Sera Lab, England); mouse anti-leu-enkephalin (Sera Lab, England); mouse anti-β-endorphin (Sera Lab, England); rabbit anti-substance P (Euro-Diagnostica AB, Sweden); rabbit anti-neurokinin A (Euro-Diagnostic AB, Sweden); and rabbit anti-neurokinin B (gift of Dr. K.Tateishi, Department of Biochemistry, School of Medicine, Fukuoka University, Japan[38]. The tissue sections were, first, rinsed for 2 x 5 min in PBS-A (PB with 0.25% bovine serum albumin and 0.1% Triton X-100). They were then pretreated in 1% H_2O_2 in PBS for 10 min to reduce endogenous peroxidase activity and followed by the incubation in a solution of 5% normal swine serum (Dakopatts, Denmark) in PBS-B (PB with 1% bovine serum albumine and 0.3% Triton X-100) for 20 min. The sections were then incubated in the specific antisera (as described above, diluted 1:1000 in PBS-B) for 48 h at 4°C. After washing for 3x10 min in PBS-A, they were then incubated with biotinylated secondary antisera directed against the host of the primary

56-65.pmd 57 25/5/2548, 16:10

antisera diluted 1:200 in PBS-A for 1 h at room temperature and were followed by washing in PBS-A for 3x10 min. The sections were then incubated at room temperature in the ABC-streptavidin biotin-horseradish peroxidase complex (Vector, USA, diluted 1:250 in PBS-A) for 1 h. After washing of the sections in PBS-A and followed by 0.05 M Tris-HCl buffer, pH 7.6 for 10 min, the peroxidase activity visualized by incubation in a solution of 0.025% diaminobenzidine (Sigma, USA) and 0.001% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6 for 15 min. After rinsing in distilled water for 2 x 5 min, the sections were dried and cover slipped with Permount®. Results were examined with a light microscope (BX-2 Olympus) with conventional illumination.

For control of the specificity of the reactions, human pineal sections were incubated with the specific primary antisera, which had been preabsorbed for 24 h with the peptide fragments against which the antisera had been raised (50 µg peptide fragment/ml diluted antiserum). In addition, some pineal sections were stained with 0.1% cresyl violet for Nissl staining.

Results

Anatomy of human pineal gland

In the sagittal section, a human pineal gland is cone-shaped with its base pointing toward the pineal recess and the tip extending in dorso-caudal direction (Fig. 1A). The base of the gland extends between the habenular commissure rostrally, and the posterior commissure caudally. On the surface, a pial capsule covers the gland, which penetrates into the gland as the interlobular septa. The septum separates a parenchyma into small lobules which exhibits a classical follicular pattern (Fig. 1B). The follicles are between 0.2-0.8 mm in diameter and are surrounded by the fairly thick interlobular septum. In several specimens, brain sands are found in the gland (Fig. 1A).

Immunoreactivity of opioid peptides

In all investigated human pineals, a high number of leu- and met-enkephalin but a low number of b-endorphin immunoreactivities were observed. Most of the leu- and met-enkephalin immunoreactivities were presented in the cells which were distributed in the basal and apical part of the gland (Fig. 1C, 2A). However, the number of met-enkephalin immunoreactive (IR) cells were higher than leu-enkephalin IR cells. The IR cells were often located in the periphery of the lobules and their processes penetrated into the surrounding interstitial pial septum (Fig. 2B). These cells frequently exhibited a neuron-like appearance with a cell body from which one (Fig. 2C) or two processes emerged (Fig. 2F). Most of the cellular processes were endowed with varicosities (Fig. 2C, 2D). However, some thick smooth processes were also observed (Fig. 2B). At the periphery of the gland, some IR nerve fibers were found penetrating into the gland (Fig. 2D). Within the pineal stalk, the IR nerve fibers were also observed (Fig. 2E) and these nerve fibers could be followed in the direction to the base of the gland. In addition, very little β-endorphin immunoreactivity was detected in the small nerve fibers located at the pial septum (Fig. 2G).

After incubation of the sections with specific antisera which had been absorbed with 50 µg of leu- or met-enkephalin, or β-endorphin/ml diluted antisera, neither IR cell nor nerve fiber was observed in any section.

Immunoreactivity of tachykinin

By using the polyclonal antiserum against substance P, a moderate number of IR fibers were found throughout the gland (Fig. 1D). The IR fibers predominantly located in the interlobular septa but penetrated often into the parenchyma of the gland between the pinealocytes (Fig. 3A). The IR nerve fibers were endowed with boutons en passage with medium or large boutons en passage (Fig. 3B). A number of substance P-IR nerve fibers were seen in the rostral and caudal part of the pineal stalk and could be followed to the base of the pineal gland (Fig. 1D). Within the gland, few substance P-IR neuron-like cells were present (Fig. 1D, 3C).

A number of neurokinin A-IR nerve fibers were also observed throughout the human pineal gland. Some intraparenchymal fibers also created a diffuse network among pinealocytes (Fig. 3D). The distribution and morphology of the neurokinin A-IR nerve fibers (Fig. 3E) did not differ from those obtained with the antiserum against substance P. However, the number of neurokinin A-IR fibers was less than that of the substance P-IR fibers. Some neurokinin A-IR neuron-like cells were found within the gland (Fig. 3F).

Neither neurokinin B-IR nerve fiber nor cell was found in the human pineal gland.

No immunostaining was detected in the human pineal gland after absorption of the specific antisera with the peptide against which they had

56-65.pmd 58 25/5/2548, 16:10

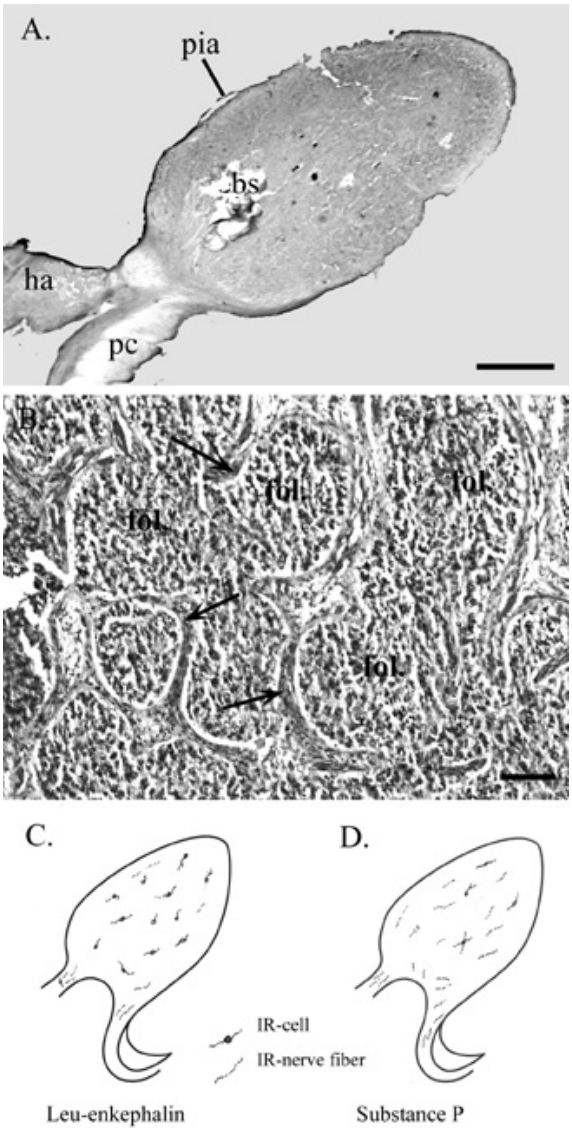

Fig. 1 (A)and (B), Photomicrographs of a human pineal gland stained with cresyl violet. (A), A sagittal section of the whole human pineal gland that covered by pia mater (pia) extending from habenular area (ha) and posterior commissure (pc). Some brain sands (bs) were found within the gland. (B), A micrograph at medium magnification showed that a human pineal was seperated by pial septae (arrows) into a follicular-like structure (fol.). Scale bars, 1mm (A), 50 μm (B). (C)and (D), Drawings of a sagittal section of human pineal gland showing the density and distribution of leu-enkephalin and substance P immunoreactive nerve fibers and cells, respectively.

been raised. The antiserum against substance P has previously been characterized and shown in radioimmunoassay not to cross-react with neurokinin A and neurokinin B[39].

Discussion

Opioidergic innervation in human pineal gland

In the present study of the Thai human pineal gland, leu- and met-enkephalin immunoreactivities were observed in intrapineal neuronal-like cells much more than in nerve fibers. This result agrees with the previous study in the pineal gland of human[40] and European hamster[14]. Contrarily, opioid peptides were immunolocalized in a few cells but in moderate amounts of nerve fibers in the pineal gland of guinea pig[12], cow[4], and tree shrew[15]. Thus, an inter species variation might exist in the opioidergic innervation.

Ultrastructural analysis of the enkephalin-immunoreactive cells in the European hamster[14] showed that they were pinealocytes. Some of them made contact with other pinealocytes. This might be an anatomical indicator of a local regulatory function of these positive cells on other pinealocytes[41]. The opioidergic cells may affect other nearby pinealocytes through presynaptic contacts or by release of peptides to the interstitial space from where molecules may diffuse to receptors on other pinealocytes. Furthermore, delta and mu opioid receptors have been demonstrated to express in the human pineal gland by using reverse transcriptase polymerase chain reaction (RT-PCR) technique[42]. Therefore, the paracrine control should be the main opioidergic regulation in the human pineal gland. The stimulatory effect of opioid peptides on melatonin synthesis has been previously reported[8]. Recently, this stimulatory effect has been demonstrated to mediate via delta and mu opioid receptors with the activation of adenylate cyclase system[43]. In addition, the analgesic effect of melatonin and opioid on modulation of nociception were reported by using a tailflick and formalin tests (the authorsí unpublished data).

Moreover, the presence of opioidergic nerve fibers in the pineal stalk and the pial capsule of human pineal gland indicates that these opioidergic nerve fibers have their perikaryal origin outside pineal glands. A previous study[4] suggested that the origin of these fibers may not be the peripheral ganglion, but rather the central origin. The intergeniculate leaflet of the lateral geniculate nucleus may be the origin of this central innervation[4].

56-65.pmd 59 25/5/2548, 16:10

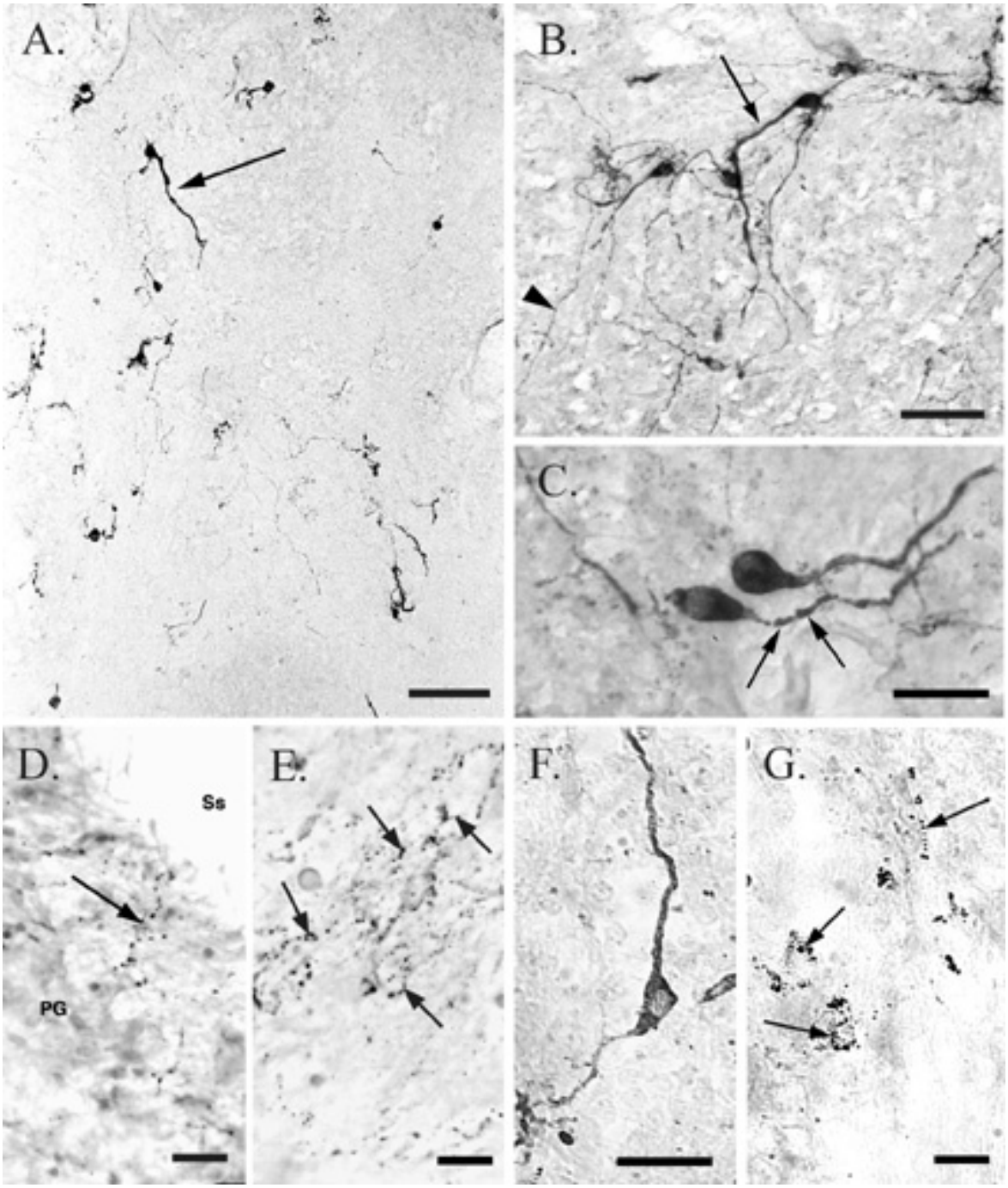

Fig. 2 Photomicrographs showing opioid peptides immunoreactivity in the human pineal gland. (A), A survey micrograph of a sagittal section showing the distribution of many neuronal-like immunoreactive cells throughout the gland (arrow). (B), A thick long smooth process (arrow) from one of the cell bodies and a dense network of leu-enkephalin-immunoreactive, varicose, nerve fibers (arrow head). (C), Two leu-enkephalin immunoreactive unipolar neuronal-like cells and their processss endowed with large varicosities (arrows). (D), A thin leu-enkephalin immunoreactive nerve fiber (arrow) penetrated from the pial capsule into the gland (PG). (E), Thin leu-enkephalin immunoreactive nerve fibers (arrows) in the pineal stalk. (F), A met-enkephalin immnureactive bipolar neuronal-like cell. (G), Short β-endorphin immunoreactive terminal-like dots (arrows) in the pial septum, Ss = subarachnoid space, Scale bars, 100 μm (A), 50 μm (C-G)

56-65.pmd 60 25/5/2548, 16:10

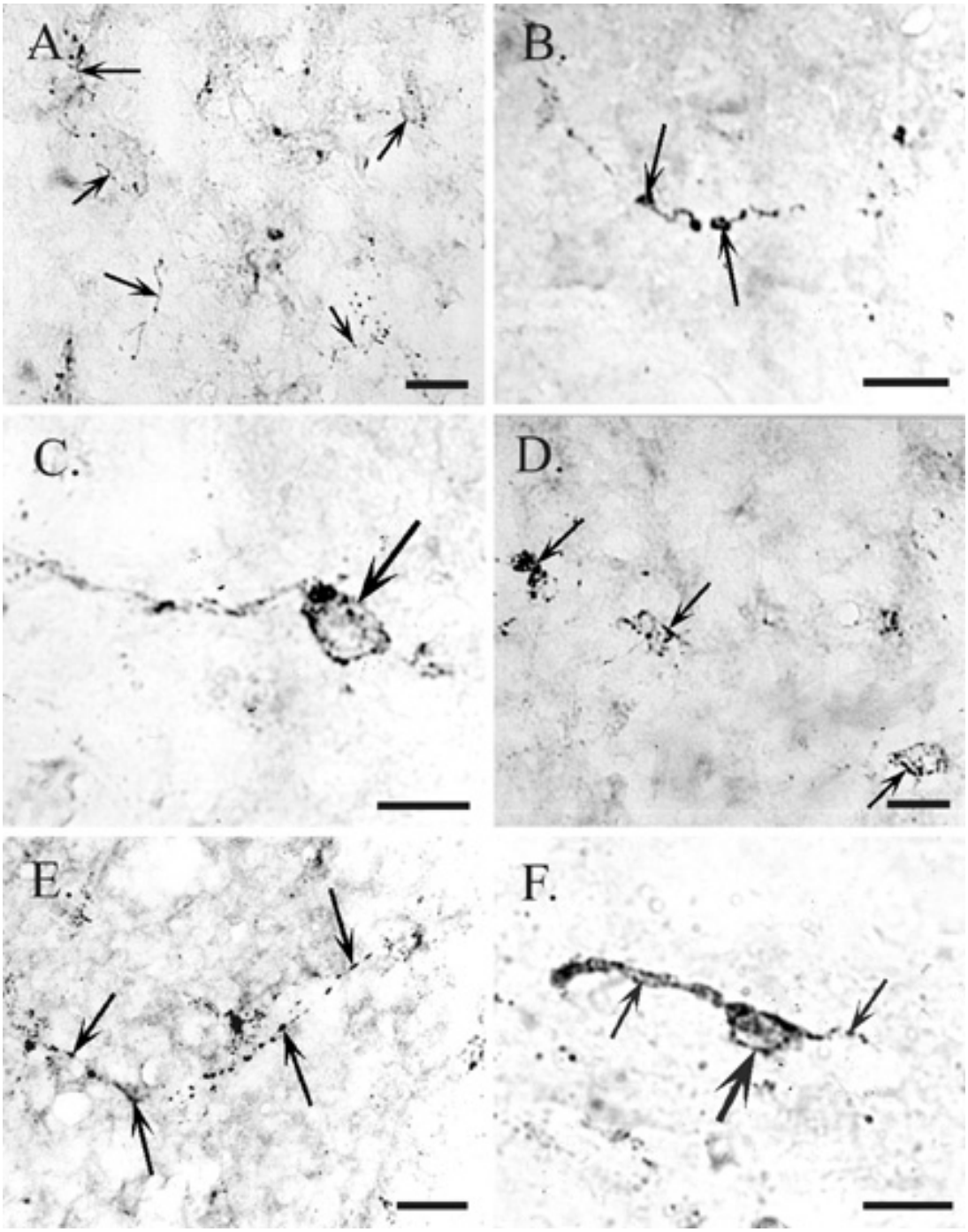

Fig. 3 Photomicrographs showing tachykinin immunoreactivity in the human pineal gland. (A), short substance P immunoreactive nerve fibers (arrows) located at the parenchyma of the gland. (B), A substance P immunoreactive nerve fiber with large- and medium-sized boutons en passage (arrows). (C), A substance P immunorective neuronal-like cell (arrow). (D), Diffuse networks of neurokinin A immunoreactive nerve fibers organized among pinealocytes (arrows). (E), A fine neurokinin A immunoreactive nerve fiber with boutons en passage (arrows). (F), A cell body (large arrow) with two processes (small arrows) of a neurokinin A immunoreactive neuronal-like cell. Scale bars, 30 μm (A, C), 15 μm (B, D, E, F)

56-65.pmd 61 25/5/2548, 16:10

Tachykininergic innervation in human pineal gland

The authorsí immunohistochemical demonstration of substance P immunoreactive nerve fibers and cell bodies in the human pineal gland corresponds well to the previous detection of high amounts of substance P in the human pineal by radioimmunoassay[44]. The results of neurokinin A immunoreactive nerve fibers and intrapineal neuronal-like cells in human pineal are demonstrated for the first time in the mammalian pineal gland. They are in accord with a 1998 study in detecting the neurokinin A in the rat pineal gland[45].

The origin of the tachykininergic innervation in the human pineal is enigmatic. Many substance P-containing neurons were found in the medial habenular nucleus of most species[46]. In the present study, some substance P immunoreactive nerve fibers have been also demonstrated in the pineal stalk. Therefore, some of the substance P containing nerve fibers in the human pineal might originate from the median habenular nuclei[31,34.25] and to be a part of the central innervation of the pineal gland. The fibers observed in the caudal part of the pineal stalk might also originate from the substance P-containing neurons in periaqueductal gray[47]. However, some substance P immunoreactive nerve fibers in the human pineal were observed in the capsule and many of these fibers could be followed into the gland. This indicates that some of tachykinin immunoreactive nerve fibers originate from a peripheral ganglion. Several studies reported that bilateral superior cervical ganglionectomy did not alter the density of the substance P- and neurokinin A-containing nerve fibers in the pineal gland[28,29,32,45]. This verified that the superior cervical ganglion was not the origin of tachykininergic nerve fibers. Recent neuroanatomical tracing studies have shown that the substance P-containing nerve fibers of the mammalian pineal gland, in several species, originate from a peripheral ganglion[5] and sensory trigeminal ganglion[26,27]. In humans, the trigeminal ganglion contains substance P-immunoreactive perikarya[48].

The diffuse network of substance P-containing nerve fibers among pinealocytes indicates a direct effect of tachykinin on the pinealocyte. This is supported by the pharmacological demonstration of substance P-receptor binding sites in the bovine pineal gland[49,50]. However, the functional role of tachykinins in the pineal gland is still obscure. Substance P has been reported to stimulate adenylate cyclase in the post-mortem human pineal[44], however, the lack of effect of substance P on melatonin secretion has also been reported in perifused rat pineal organs[51] as well as in cultures pinealocytes[52].

Conclusion

The present study demonstrates the presence of opioidergic and tachykininergic innervation in the pineal gland of Thai humans. A large amount of enkephalin-immunoreactive intrapineal cells indicates a paracrine opioidergic control in human pineal. Moderate amounts of substance P and neurokinin A, but not neurokinin B are present in nerve fibers and in a few neuronal-like cells of the human pineal gland. It indicates that the tachykininergic innervation is mainly from the nerve fibers which have their perikaryal origin outside the gland. Some of the nerve fibers might originate from neurons in the brain and from a peripheral ganglion, possibly the trigeminal ganglion.

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56-65.pmd 62 25/5/2548, 16:10

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56-65.pmd 64 25/5/2548, 16:10

การแสดงถึงระบบประสาทชนิด opioid และ tachykinin ในต่อมไพเนียลของคนโดยวิธี immunohistochemistry

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ต่อมไพเนียลของคนสร้างฮอร์โมนเมลาโทนิน โดยมีระดับขึ้นลงตามเวลากลางคืนและกลางวัน การสร้างเมลาโทนินถูกควบคุมด้วยระบบประสาทอัตโนมัติซิมพาเทติกที่หลั่งสารสื่อประสาทชนิดนอร์อะดรีนาลินจาก superior cervical ganglion เป็นหลัก นอกจากนี้มีรายงานว่ามีระบบประสาทและสารสื่อประสาทชนิดอื่น ๆ มาควบคุมด้วย สารสื่อประสาทชนิด opioid peptide ซึ่งลดความเจ็บปวด และ ชนิด substance P ซึ่งกระตุ้นการเจ็บปวด ก็มีรายงานว่าพบในต่อมไพเนียลเช่นกัน ในการศึกษาครั้งนี้ได้ใช้วิธี immunohistochemistry เพื่อแสดงถึงระบบประสาท opioid และ substance P ในต่อมไพเนียลของคน โดยใช้ แอนติบอดีต่อ leu-enkephalin, met-enkephalin และ beta-endorphin เพื่อแสดงระบบประสาท opioid และแอนติบอดีต่อ substance P, neurokinin A และ neurokinin B เพื่อแสดงระบบประสาท tachykinin ผลการศึกษาพบ leu-enkephalin และ met-enkephalin ในเซลล์ที่คล้ายเซลล์ประสาทจำนวนมาก แต่พบในเส้นประสาทเล็กน้อย ซึ่งแสดงว่าระบบประสาทชนิด opioid ในคนนั้นมีการควบคุมการสร้างเมลาโทนินเป็นแบบ paracrine ส่วนระบบ tachykinin พบเฉพาะ substance P และ neurokinin A เท่านั้น และพบมากในเส้นประสาท พบในเซลล์คล้ายเซลล์ประสาทเล็กน้อย ซึ่งแสดงว่าระบบประสาท tachykinin ควบคุมการทำงานของต่อมไพเนียลของคน โดยส่งเส้นประสาทส่วนใหญ่มาจากเซลล์ประสาทที่มีต้นกำเนิดจากภายนอกต่อมไพเนียล

56-65.pmd 65 25/5/2548, 16:10