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Catecholaminergic Innervation of GnRH Neurons in the Rat Median Eminence

Young Ki Lee*, Kyungjin Kim

Department of Anatomy, College of Dentistry, Kangnung National University, Kangnung 210-702, Department of Molecular Biology, and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742

The present study was carried out 1) to show the ontogenic development of CA- and GnRH-containing nerve fobres in the median eminence, 2) to simultaneously demonstrate the synaptic contact between these two nerve fibres in the rat median eminence at the ultrastructural level using light and electron microscopic double-label immunostainings. GnRH-and CA-nerve terminals were detectable in the median eminence at embryonic day 19.5. The CA-nerve terminals were observed in the entire gegion of the external layers, while GnRH-nerve terminals only in the lateral portion. At the 14th postnatal day, both types of nerve terminals showed a very similar distribution to those of adult one. In the median eminence of adult rats, a substantial overlap existed in the distribution of GnRH fibres with CA-containing nerve fibres. This overlap was most intense throughout the external palisade zone. Furthermore, an electron microscopic double label immunostaining showed that there was a close apposition of CA- and GnRH-nerve fobres. These axo-axonic contacts occurred frequently in the internal and palisade zones, i.e. at the level of the fobre preterminals. These morphological results suggest that the CA-mediated GnRH secretion may occur via axo-axonic interaction in the median eminence.

KEY WORDS: GnRH, Catecholamines, Electron microscopic immunohistochemistry, Median eminence, Synaptic contact

The median eminence with the adjacent hypophysial portal system provides the morpho-functional link between the hypothalamus and the anterior lobe of the pituitary (Moore and Johnston, 1982). Several hypothalamic releasing or inhibiting hormones and monoamines are transferred to their axonal terminals in the median eminence and released into the portal vessels. Abundant evidences indicate that a number of neurotransmitters and neuromodulators are involved in the regulation of gonadotropin releasing hormone (GnRH) secretion from the hypothalamus (Barraclough and Wise, 1982; Karla and Karla, 1983; Ramirez *et al.*, 1984, 1985; Jacobowitz, 1988). Among them, catecholamines (CA) are best studied. However, it is of importance to study the anatomical basis of the interactions between CA- and GnRH-containing neurons in the median eminence (McNeill and Sladek, 1978; Hoffman *et al.*, 1982; Jennes *et al.*, 1982, 1983; Wray and Hoffman, 1986; Leranth *et al.*, 1988). Only recently, these interactions between CA- and GnRH-containing neurons have been studied using immuno-histochemistry with a double-labeling technique in ewe (Kuljis and Advis, 1989), newt (Corio *et al.*, 1990) and rat (Ugrumov *et al.*, 1989). Neverthless, there still remains to be elucidated the precise synaptic specialization at the ultrastructural level. It is more important to examine when these

relationships occur during development. To this end, electron microscopic double-label immunostaining method as well as light microscopic immunohistochemistry were employed to examine this issue.

Materials and Methods

Animals

In this study, pregnant Sprague-Dawley rats (Seoul National University Animal Breeding Center) were used. The rats were mated overnight and examined for the presence of semen in the vagina on the next morning, and this time was designated embryonic day 0.5 (E 0.5). All rats were housed with lights on 06:00-20:00 hr, food and water were available ad libitum. The pregnant rats were caged individually after insemination and the pups were separated from the mothers on the postnatal day (P) 21. The prenatal series were obtained from fetuses at E15.5, E17.5 and E19.5 and the postnatal series from animals at ages P1, P4, P14, P21, P28 and adult. Each series contained four litter mates from at least two different litters.

Tissue Preparations

Pregnant female rats were anaesthetized with ethyl ether in the morning (10:00- 11:00h). Subsequently, fetal rats removed from the uterus were decapitated, and their whole brains were obtained and immersed in 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). At all other stages of development the animals were perfused through the left cardiac ventricle. Perfusion began with a rinse of saline, followed by the same fixative as the fetal brain. The fixed brains were taken out from the skull and postfixed for another 4 hr at 4°C. The fixed brains were rinsed overnight in PB containing 30% sucrose and frontal serial sections in the region of median eminence, 8 μm thick, were made in cryostat (American Optics) at −20°C. Each sections were then mounted on gelatin coated slides and stored at −70°C until use.

For an electron microscopic double-label immunostaining, adult female rats (250-270g) were decapitated and the middle portion of the hypothalamus was rapidly dissected and fixed in the 4% paraformaldehyde plus 0.5% glutaraldehyde fixative for 24 hr at 4°C. Serial frontal sections, 50 μm thick, were cut with a vibratome (Dosaka EM Co.) and post-fixed with 0. 1% osmium tetroxide for 20 min. Subsequently the area containing median eminence is trimmed and dehydrated in a graded series of ethanols and embedded in Epon mixture. Serial ultrathin sections (70 nm) were obtained with a ultramicrotome (Sorvall) and placed on 200 mesh nickel grids.

Light Microscopic Immunohistochemistry

For the study of developmental relationships between TH- and GnRH-immunoreactive fibres, a peroxidase-anti-peroxidase (PAP) method was used (Sternberg *et al.*, 1970) and alternate serial sections were processed for localization of TH and GnRH. Tissue sections on slides were air dried and rinsed several times in 20mM phosphate buffered saline (PBS, pH 7.4). Tissue sections were then incubated with 40 μl of normal goat serum, diluted 1:20, for 30 min to exclude the nonspecific binding before the primary antibody incubation. Slides were applied with 50 μl of diluted primary antibody, rabbit derived anti-GnRH or anti-TH at 4°C for 16-24 hr. Anti-TH (generous gift from Joh D.H., Cornell University) and anti-GnRH (Chen-Ramirez GnRH antiserum, CRR-11-B-72) were used at a dilution of 1:2000 and 1:1000, respectively in PBS containing 0.4% to 1.0% Triton X-100. The sections were then rinsed in PBS containing 0.02% Triton X-100. Approximately five such rinses for 10 min each were performed. The sections were then incubated with 0.05% periodic acid for 30 min to exclude the endogeneous peroxidase activity and applied with goat anti-rabbit Ig G (Sigma) diluted 1:100 in PBS containing 0.02% Triton X-100 for 1 hr at room temperature. Following rinsed with the same buffer for 15 min twice, the sections were incubated for 1 hr in peroxidase-anti-peroxidase complex (Sigma), diluted 1:500 in PBS-0.02% Triton X-100. After the last four times 15-min washes with PBS, the sections were incubated with 0.5% 3,3'-diaminobenzidine (DAB)

in 0.01% hydrogen peroxide in PBS for 5 min, washed, dehydrated, mounted with synthetic mounting medium, and observed in a light microscope. The specificity of immunostaining was confirmed by the substitution of normal rabbit serum for GnRH or TH antiserum.

Electron Microscopic Double-label Immunostaining

Silver-to-gold sections of median eminence, affixed to nickel grids, were carefully blotted on filter paper with section side-up and allowed to dry. The sections were then etched by placing the grids on a drop of saturated sodium metaperiodate for 1 hr, then jet-washed with PBS for 2 min and placed on a drop of PBS containing 0.5% bovine serum albumin (Sigma) and 0.1% gelatin (Sigma, PBG) for 30 min prior to use for double-label immunohistochemistry. The first immunostaining employed protein A-gold method as previously described (Roth *et al.*, 1978). Briefly, the etched grids were incubated on a large drop of anti-GnRH, diluted 1:200 in PBG, for 2 hr at room temperature. Following three times rinses for 15min and brief jet-washes with PBG, the grids were placed on a drop of protein A-gold (15nm, Janssen Pharmaceutics), diluted 1:200 in PBS, for 1 hr at room temperature and washed with PBS. In order to verify the proper labeling of GnRH antibody and protein A-gold in the GnRH nerve fibres of median eminence, some grids were washed with distilled water and contrasted with uranyl acetate and lead citrate before observation with electron microscope.

In the second immunostaining for the localization of TH, PAP method was used according to Sternberg *et al.* (1970). After the completion of the first immunostaining, the grids were incubated with section side down on a drop of anti-TH, diluted 1:500 in PBG, for 2 hr at room temperature and washed with PBG for 15 min three times. The grids were then placed on a drop of goat anti-rabbit IgG diluted 1:100 for 1 hr, washed again with PBG, incubated for 1 hr with a 1:100 dilution of PAP, and finally washed in PBS. Subsequently, the grids were incubated on drop of 0.5% DAB containing 0.3% H_2O_2 for 5 min in the dark, jet-washed with tap-water for 1 min, incubated for 10 min in 2.5% glutaraldehyde and washed again with tap-water. The grids were contrasted with uranyl acetate for 1 min, then lead citrate for 5 min, and observed with electron microscope (Zeol CX-II).

Result

Light Microscopic Immunohistochemical Experiment

GnRH-containing nerve fibres were detectable as small brown dots on E19.5 in the lateral portion of the external layer (Fig. 1A), while CA-containing nerve fibres were found in the whole external layer of the rostro-caudal median eminence at the same stage (Fig. 1B). At this stage, however, the internal layer was completely devoid of both types of immunoreactive nerve fibres, and CA-containing nerve fibres were more intensively labeled and widely distributed.

At P1, nerve fibres immunostained for GnRH were accumlated in the external layer of the median eminence medially in the rostral or postinfundibular portion and laterally in the pre-infundibular portion, showing a similar distribution pattern to that of adults. The CA-containing nerve fibres exhibited a similar distribution to that of GnRH immunoreactive fibres, but a few fibres could be seen in the internal layer. From this stage, GnRH and CA nerve fibres in the median eminence displayed a very similar distribution to the previous stage (P1), with some difference in their reaction intensity. However, at P4 stage, GnRH nerve fibres parallel to tanycyte were seen and some of CA-containing nerve fibres were found in the subependymal region under the floor of third ventricle. Moreover, at P14 stage, GnRH- and CA-containing nerve fibres penetrated the ependymal layer and situated very close to the ventricular lumen (data not shown).

In the adult median eminence, GnRH and CA nerve fibres were localized with a substantial overlap in the ventrolateral portion and a partial overlap existed in the remaining medial part (Fig. 1C and 1D). In the rostral median eminence, both GnRH and TH nerve fibres were localized medially, but in the central and caudal median

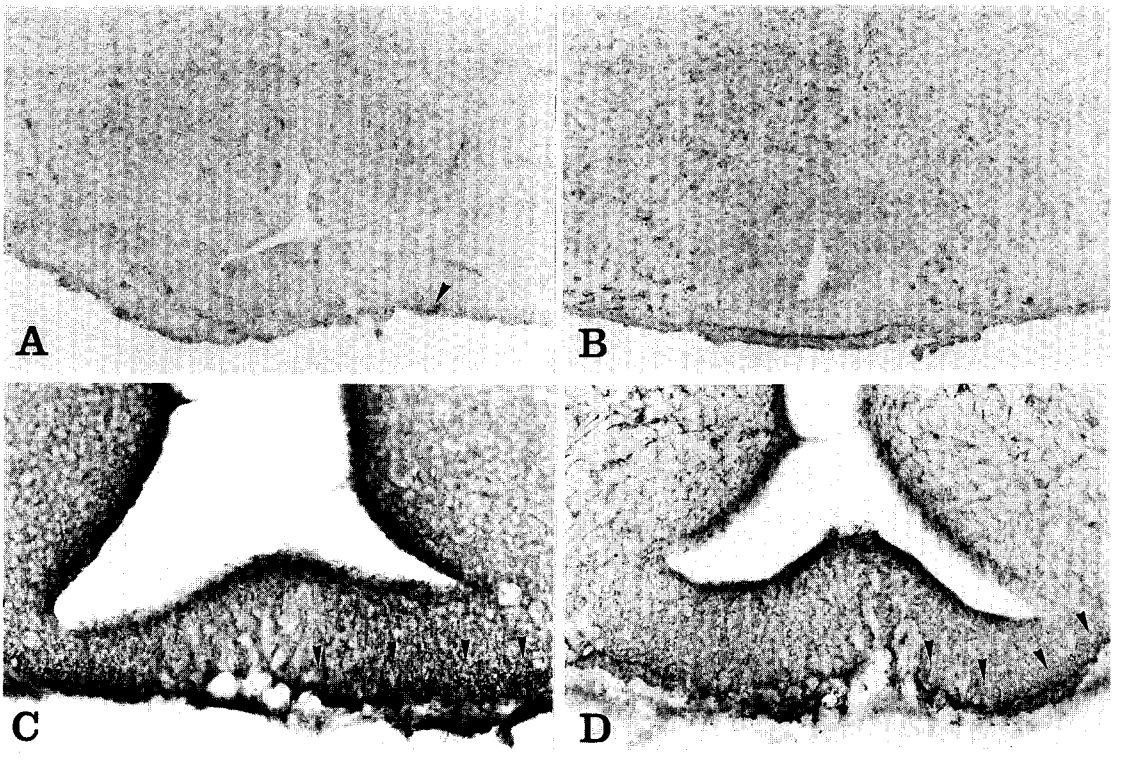

Fig. 1. Immunohistochemical staining for GnRH (A and C) and TH (B and D) in the median eminence at E 19.5 (A and B) and adult (C and D). At E 19.5 stage, GnRH-immunoreactive fibres are seen in the lateral portion of the external layer. In case of adult rat, a substantial overlap between two nerve fibres is seen in the external layer of lateral median eminence (arrowheads) × 100.

eminence, these overlaps were mainly confined to the lateral portion of the external layer.

Electron Microscopic Double-Label Experiment

The double-label immunohistochemical procedure at the ultrastructural level produced the consistent and reproducible results. Critical factors in producing clean sections without DAB or gold artifact included 1) immunostaining of one section side (section side up) for GnRH and the opposite section side (section side down) for TH, 2) optimal dilution of each antibody and 3) jet-washing of the grids between each steps.

In this postembedding electron microscopic double-label method, the difference in the reaction products was very easily discernible at the ultrastructural level. GnRH nerve fibres were labeled as gold particles (Fig. 2A), whereas CA nerve fibres as covering the neurosecretory granules with electron-dense flocculent DAB reaction product (Fig. 2B and 2C). Furthermore, the incubation in a second set of immunological reagents did not result in an artifactual labeling of GnRH nerve fibres, and similarly no DAB reaction products were present in tissues in which normal rabbit serum was substituted for the TH antiserum (Fig. 2D). These findings serve as an internal control for the doubl-label method and permit to use two rabbit-derived anti-sera to detect two substances without the confounding effects of nonspecific binding of the second set of immunological reagents.

In the palisade layer of the median eminence, GnRH and CA nerve fibres were scattered among the non-reactive nerve fibres and tanycyte processes. Some GnRH and CA nerve fibres were found to terminate near the basal lamina (Fig. 2A

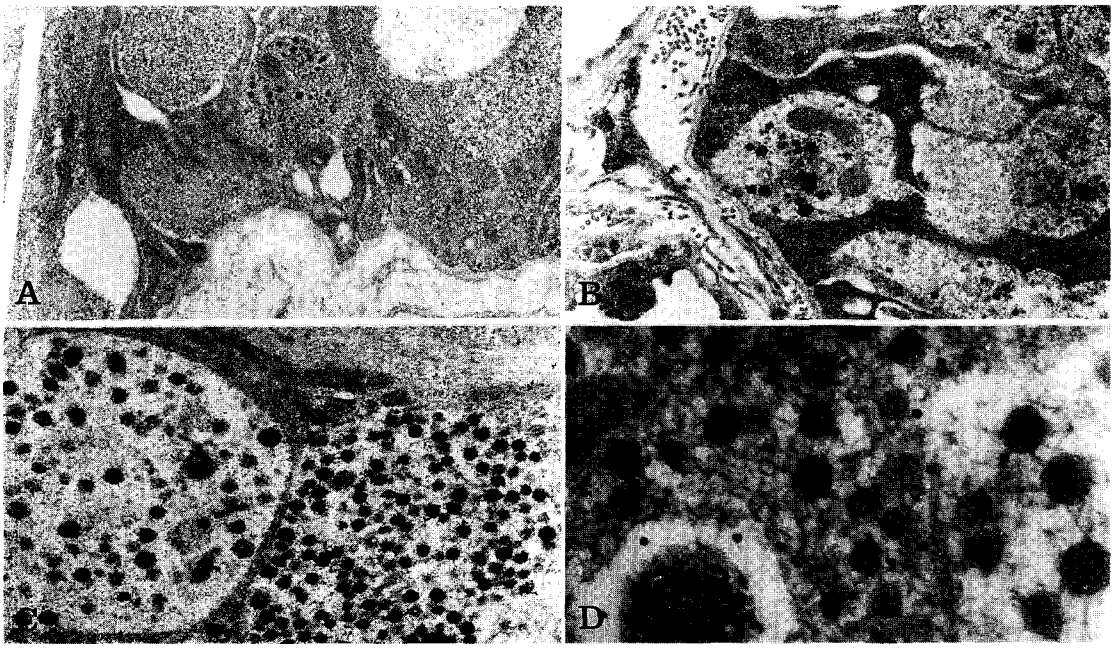

Fig. 2. Demonstration of GnRH in the rat median eminence by protein A-gold method (A). Note the discrete localization of gold-particles on the secreting granule of GnRH nerve fibre. ×55,000. Localization of TH-containing nerve fibre in the rat median eminence by electron microscopic PAP method (B and C). Note the electron dense flocculent DAB reaction product over the secretory granules of one fibre and an adjacent non-immunoreactive fibre. × 51,000. Control of electron microscopic double-label immunohistochemistry (D) ×201,000.

and 2B). In the subependymal layers of the median eminece, GnRH nerve fibres were detectd among the non-immunoreactive ependymal elements (Fig. 3A and 3B), and in the ependymal layer, gold particle-labeled GnRH axons were found in juxtaposition with the ependymal cell bodies (Fig. 3C). Some GnRH and TH fibres extended almost as far as the third ventricle. However, in the present electron microscopic immunohistochemical study, neither GnRH nor TH fibres were observed protruding into the ventricle.

Since the present light microscopic immunostaining revealed that there is a significant overlapping in the distribution of GnRH and CA nerve fibres in the lateral palisade zone of the median eminence (see above), an electron microscopic double-label immunostaining was carried out mainly in this selected area. The gold particle-labeled GnRH nerve fibres were found frequently in close apposition to CA nerve fibres whose secretory granules were covered with electron dense DAB reaction product (Fig. 4).

These relationships, however, could not be seen in the external layer in the proximity of the portal vessels, but in the relatively internal layer of the lateral palisade zone.

Discussion

The first appearance and subsequent development of GnRH- and CA-immunoreactive nerve fibres and their anatomical relationship during development are studied by a light microscopic immunohistochemistry in the rat median eminence. Furthermore, to elucidate the topographical relationship between these two nerve fibres at the ultrastructural level, an electron microscopy in combination with immunogold and peroxidase- anti- peroxidase procedure is applied in the same ultra-thin sections of the median eminene of adult female rats.

CA-containing nerve fibres are found in the

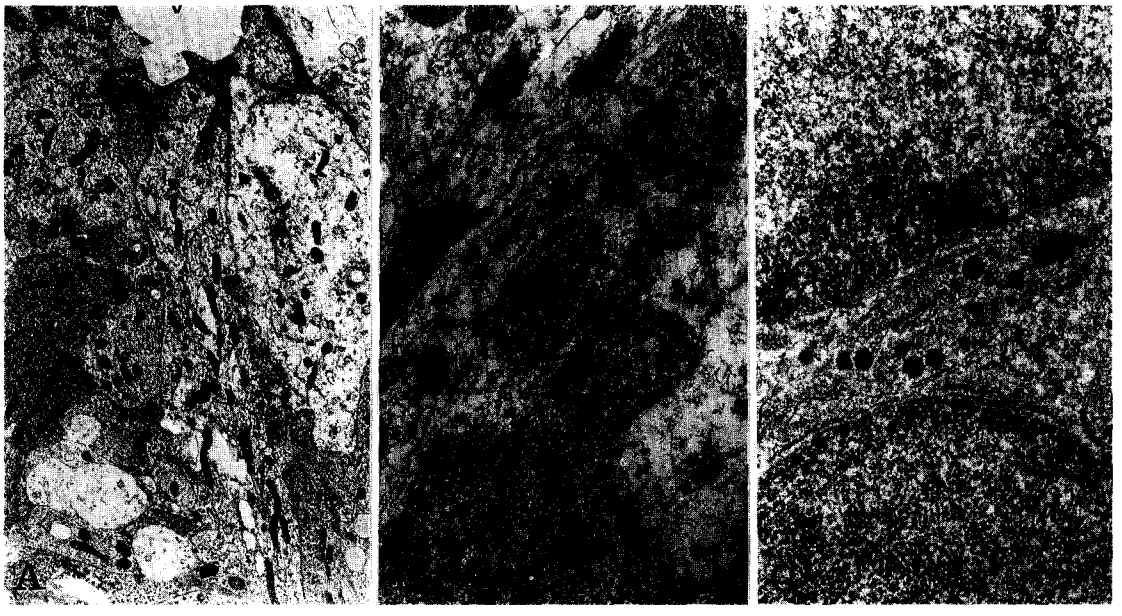

Fig. 3. Electron micrograph of GnRH nerve fibres in the subependymal region. The gap juctions (GJ) between columnar ependymal cells (A) ×12,000. A higher magnification of arrow-indicated region in A (B) ×55,000. GnRH nerve fibre in juxtapposition with the ependymal cell bodies (C) ×57,000 v, third ventricle.

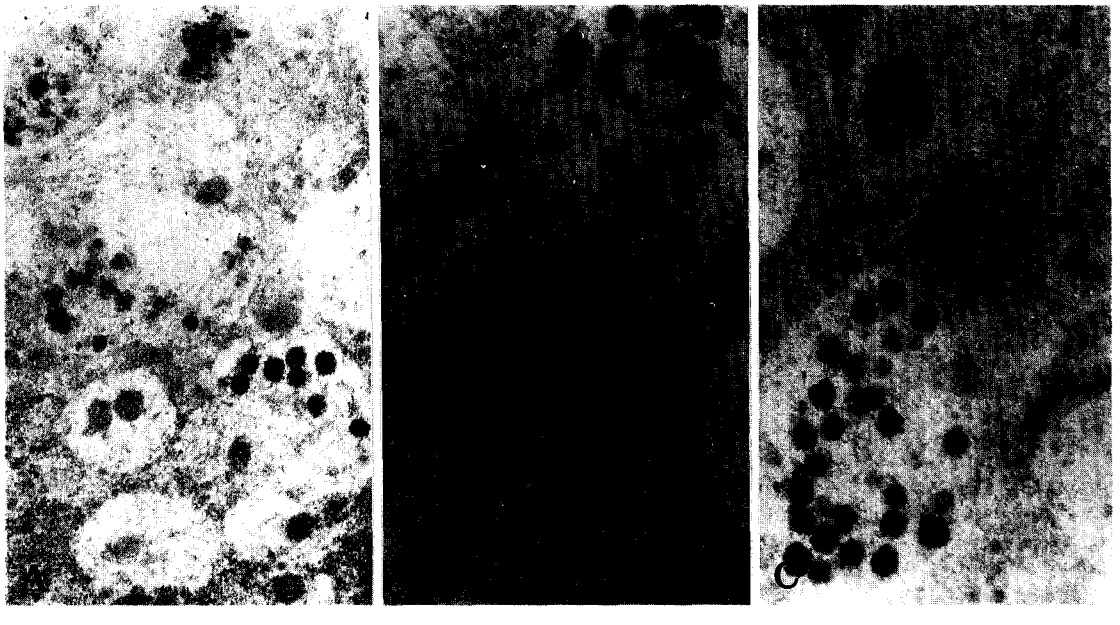

Fig. 4. Electron microscopic double-label immunostaining for simultaneous visualization of GnRH and TH. Note that there is the close apposition between the gold particles-labeled GnRH nerve fibres and TH-containing nerve fibres with electron dense DAB reaction product. ×56,000.

entire external layer and GnRH-containing nerve fibres appeared in the lateral portion of external layer of median eminence at E19.5. At P1 stage, immunostainings for GnRH and TH show some overlapping in the ventrolateral portion of the external layer of the median eminence. However, considering the fact that the portal vessels become functional at P5 following penetration of the primary plexus into the median eminence (Glydon, 1954), it is resonable to suggest that the functional relationship between CA and GnRH may be established at more later stage. In fact, at P14, both types of nerve fibres show a very similar distribution, with substantial overlap, to those of adult one.

Using an electron microscopic double-label immunostaining method, the present study clearly demonstrates that CA-containing nerve fibres are closely juxtapposed to GnRH-containing nerve fibres in the ventrolateral portion of the median eminence, somewhat distal to the primary portal plexus. This implies that the axo-axonic contacts, a possible functional contact between CA- and GnRH-nerve fibres, mainly occurs at the presynaptic level, and CA may directly modulate GnRH secretion. Although the present study does not reveal the direct synaptic connection between two nerve fibres, it is now believed that CA can establish not only synaptic, but also non-synaptic neurosecretory effect in the CNS (Swanson *et al.*, 1978; Buijs *et al.*, 1984; Leranth *et al.*, 1988). The present study is the first demonstration that there is a direct contact between GnRH- and CA-nerve fibres at the ultrastructural level in the female rats.

A large number of studies indicate that CA is involved in the control of GnRH and gonadotropin secretion. The role of dopamine in the regulation of GnRH release is still under debate, but the effect of this monoamine, particularly in restoration of age-related gonadal dysfunction (Vijayan and McCann, 1978), appears to be facilatory. On the other hand, the association of low rates of dopamine turnover with high gonadotropin secretion led to the hypothesis that dopamine may be inhibitory to GnRH secretion (Krulich, 1979; Ramirez *et al.*, 1984). These findings are supported by more direct pharmacological evidence (Beck *et al.*, 1978; Sarkar and Fink, 1981; Karla and Karla, 1983). Noradrenaline is known to evoke initially the accumulation of GnRH in the MBH and subsequently its release into the hypophysial portal system (Clifton and Sawyer, 1980; Rance *et al.*, 1981; Karla, 1985). It is of interest to know whether the present CA-nerve fibres juxtapposed to GnRH-nerve fibres are dopaminergic or noradrenergic. Daikoku *et al.* (1986) confirmed that the presumptive dopaminergic neurons of the arcuate nucleus, A12 group, send their axons to the lateral median eminence, whereas its medial portion is thought to be innervated by the dopaminergic neurons of the anterior periventricular nucleus, A14 group. However, it may be premature to characterize the CA-nerve fibres reported here as strictly dopaminergic, since several lines of evidence have shown that the median eminence, to a less extent, is also innervated by the adrenergic and noradrenergic fibres originating outside the hypothalamus (Hokfelt *et al.*, 1978; Palkovits, 1981; Jennes *et al.*, 1982).

It is now well documented that gonadal steroids act at both the level of hypothalamus where they probably lead to changes in GnRH release, and directly at the level of anterior pitutary where they markedly change the responsiveness of gonadotrophs to GnRH. The TH-immunoreactive neurons contain estrogen or progesterone receptors (Heritage *et al.*, 1980; Sar, 1984, 1988; Blaustein and Turcotte, 1989), while GnRH neurons contain neither estrogen nor progesterone receptors (Shivers *et al.*, 1983; Fox *et al.*, 1986). Moreover, estrogen and progesterone treatments are known to influence CA levels (Barraclough and Wise, 1982; Ramirez *et al.*, 1984) and the activity of TH (Luine *et al.*, 1977). Thus, taken together with the previous results, the present study indicates that gonadal steroids may initially exerts its effect on the catecholaminergic neurons leading to altered CA synthesis or release at the various level, which, in turn, modulate GnRH secretion from the median eminence.

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흰쥐 시상하부의 정중융기에서 카테콜아민 신경세포와 GnRH 신경세포와의 연접에 관한 연구

이영기* · 김경진(*강릉대학교 치과대학 해부학교실, 서울대학교 자연대학 분자생물학과)

본 연구는 광학현미경 및 전자현미경적 이중면역 염색방법을 이용하여, 카테콜아민 신경세포와 GnRH 신경세포의 신경종말이 뇌의 초기 발생과정에서 어느 시기에 시상하부의 정중융기에 분포되는 가를 밝히고, 성숙한 흰쥐에서 두 신경세포 사이에서의 신경연접 여부를 밝히고자 하였다. 광학현미경적 면역염색에서 카테콜아민 신경섬유와 GnRH 신경섬유는 임신 19.5일부터 각각 시상하부의 전체 바깥 층과 바깥 층의 외측 부분에서 나타나기 시작하여 출생 14일 부터는 성숙한 흰쥐와 비슷한 분포를 보여 주었다. 성숙한 흰쥐의 정중융기에서 이들 두 신경세포의 신경섬유는 매우 유사한 분포양상을 보여 주었는데 이러한 양상은 특히 정중융기의 외측 울타리 부위(lateral palisade region)에서 가장 뚜렷하였다. 또한 이 부위에 대한 전자현미경적 이중면역 염색을 시행한 결과, 카테콜아민 신경세포와 GnRH 신경세포의 신경섬유는 서로 연접을 이루고 있는 것이 밝혀졌다. 이러한 결과로 미루어볼 때, 카테콜아민에 의한 GnRH의 분비는 이들 두 신경세포의 신경섬유가 시상하부의 정중융기에서 직접적으로 연접을 이룸으로써 조절되는 것으로 사료된다.