Electron Microscopic Cytochemistry of Catecholaminergic Innervation of LHRH Neurons in the Medial Preoptic Area of the Rat*

Tsuneharu WATANABE and Yasumitsu NAKAI
Department of Anatomy (Prof. Y. NAKAI), Showa University School of Medicine, Tokyo, Japan

Received November 11, 1986

Summary. The synaptic interactions between catecholaminergic terminals and luteinizing hormone releasing hormone (LHRH)-containing neurons in the medial preoptic area of the rat was studied by electron microscopy using LHRH immunocytochemistry combined with 5-hydroxydopamine labeling or autoradiography after injection of 3H-dopamine or 3H-noradrenaline in the same tissue section.

Axon terminals labeled with 5-hydroxydopamine, 3H-dopamine or 3H-noradrenaline were found to make synapse-like contacts with LHRH-immunoreactive nerve cell bodies and fibers in the medial preoptic area and also 5-hydroxydopamine-labeled terminals made synaptic contacts with the same LHRH-immunoreactive nerve fibers with unlabeled terminals.

These findings suggest that catecholaminergic neurons may innervate LHRH-containing neurons to regulate LHRH secretion via synapses with other unknown neurons in the medial preoptic area of the rat.

Extensive studies have established that catecholamines are involved in the control of gonadotropin secretion. In vitro studies of the roles of catecholamines in controlling the secretion of luteinizing hormone releasing hormone (LHRH) have shown stimulatory effects of dopamine (BENNETT et al., 1975; NEGRO-VILAR et al., 1979) and noradrenaline (NEGRO-VILAR et al., 1979; HEAULME and DRAY, 1984) on LHRH release at the level of the median eminence. Furthermore, it has been suggested that in the catecholamine control of gonadotropin secretion at the level of the LHRH-producing area, noradrenaline stimulates LHRH release by acting in the medial preoptic area (HONMA and WUTTKE, 1980) and dopamine does so in the anteroventricular periventricular nucleus (SIMERLY et al., 1985).

On the other hand, morphological studies have shown using double immunostaining for LHRH and dopamine-β-hydroxylase (DBH) (JENNES et al., 1982) or tyrosine hydroxylase (TH) (JENNES et al., 1983) that in the rat brain DBH-positive (noradrenergic) fibers are in close contact with the LHRH perikarya in the septopreoptic region and TH-positive (dopaminergic) fibers are in close contact with the LHRH perikarya in the septopreoptic diagonal band complex. However, the catecholaminergic synaptic input to LHRH-containing neurons has not yet been demonstrated at the electron-microscopic level.

The present study therefore aims at the elucidation of the synaptic relationships

*This study was supported by a grant from the Ministry of Education, Science and Culture, Japan.
between the catecholaminergic terminals and the LHRH-containing neurons in the medial preoptic area by combining 5-hydroxydopamine labeling or autoradiography after injection of \(^3\)H-dopamine or \(^3\)H-noradrenaline with LHRH immunocytochemistry at the ultrastructural level.

**MATERIALS AND METHODS**

Twenty-four adult male Wistar rats (150–200 g) were used in this study. The animals were anesthetized with sodium pentobarbital, and 200 \(\mu\)g of colchicine dissolved in 25 \(\mu\)l of saline was injected into the lateral ventricle stereotaxically. Two days later eight animals were infused into the lateral ventricle through a microsyringe with 6–11 mg of 5-hydroxydopamine (Sigma, U. S. A.) dissolved in 25 \(\mu\)l of saline containing 0.02% ascorbic acid, and the other twelve animals were infused into the lateral ventricle through a microsyringe with 200–400 \(\mu\)Ci of \((7,8-\)\(^3\)H\) dopamine (sp. act. 46 Ci/mmole, Amersham, England, Code TRK. 582) or \((7-3\)H\) noradrenaline (sp. act. 10.6 Ci/mmole, Amersham, England, Code TRK. 174) in aliquots of 200–300 \(\mu\)l of saline containing 0.02% ascorbic acid. Some of the animals were injected intraperitoneally with 100 mg of the monoamine oxidase inhibitor (phenylhydrazine hydrochloride) per kg of body weight 2 hr prior to the injection of \(^3\)H-dopamine, \(^3\)H-noradrenaline or 5-hydroxydopamine. Five to 20 min after the 5-hydroxydopamine injection and 40–60 min after \(^3\)H-dopamine or \(^3\)H-noradrenaline injection, the animals were sacrificed by perfusion with an ice-cold mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4). The hypothalamus was excised and immersed in the same fixative for 2–3 hr at 4°C followed by 0.1 M phosphate-buffered saline (PBS, pH 7.2) containing 7.5% sucrose. The tissue blocks were sectioned at 30–40 \(\mu\)m on a Vibratome (Oxford Instrument, U. S. A.).

The sections were washed overnight in PBS containing 7.5% sucrose and then immunostained according to the peroxidase-antiperoxidase (PAP) technique (STERNBERGER et al., 1970) as follows. The sections were incubated with 1) LHRH antiserum \((1 : 100–1 : 1,000, Ferring Co., F. R. G. and Immuno Nuclear Corporation, U. S. A.) for 48–72 hr at 4°C, 2) goat anti-rabbit IgG \((1 : 100, Polyscience, U. S. A.) for 3 hr at 20°C, and 3) PAP \((1 : 100, Cappel Lab., U. S. A.) for 3 hr at 20°C. Each incubation interval was followed by washing in PBS for 1–2 hr. Finally they were incubated for 10 min at 20°C in a medium consisting of 3,3'-diaminobenzidine tetrahydrochloride, 0.05 ml of 5% hydrogen peroxide, and 50 ml of 0.05 M Tris buffer (pH 7.6).

As a control, the sections were incubated with the LHRH antiserum preabsorbed with synthetic LHRH \((25 \mu\)g/ml diluted LHRH antiserum) for 24 hr.

After incubation, the tissue sections were postfixed in 1% OsO\(_4\) in 0.1 M phosphate buffer (pH 7.4) for 1 hr at 4°C, dehydrated in a graded series of ethanol and embedded in a mixture of Epon-Araldite. Ultrathin sections cut on an LKB ultratome and a Porter Blum microtome were stained with uranyl acetate and lead citrate.

The ultrathin sections were processed for electron microscopic autoradiography as described elsewhere (NAKAI et al., 1983). Following exposure for 30–50 days at 4°C, the autoradiograms were developed in Microdol X and fixed with sodium thiosulfate. All the sections were examined at 75 and 100 kV with Hitachi HS-9 and H-800 electron microscopes.
RESULTS

The nerve cell bodies containing LHRH-immunoreactivity were localized in the medial preoptic area of the rat hypothalamus. Nerve fibers with the immunoreactivity were distributed in and around the medial preoptic area. Dense reaction products were found in the secretory granules (about 100–130 nm in diameter) in the perikarya (Fig. 1a) and fibers; they were distributed throughout the cytoplasmic matrix. Immunonegative terminals making synaptic contacts with LHRH-immunoreactive perikarya bodies (Fig. 1a) and fibers (Fig. 1b) were found in the medial preoptic area. The presynaptic terminals contained numerous clear spherical vesicles either exclusively (about 40 nm in diameter) or intermingled with a few larger granular vesicles (80–100 nm in diameter) (Fig. 1a, b). Most of the synapses were asymmetrical types as seen in Figures 1a, b, but a few symmetrical types could also be found.

The combined method of LHRH immunocytochemistry and 5-hydroxydopamine labeling demonstrated, 5-hydroxydopamine-labeled terminals containing numerous small vesicles with dense cores and a few larger granular vesicles with increasing electron density, making synaptic contacts with the LHRH-immunoreactive perikarya and fibers in the medial preoptic area (Fig. 2a-c). Some LHRH-immunoreactive nerve cell bodies and fibers received synaptic inputs from both 5-hydroxydopamine-labeled and non-labeled terminals (Fig. 2c).

In the combined LHRH immunocytochemistry and ^3^H-dopamine or ^3^H-noradrenaline autoradiography, axosomatic and axodendritic and/or axoaxonic synapses

![Fig. 1](image.jpg)

**Fig. 1.** Two immunonegative axon terminals making synapses with an LHRH-immunoreactive cell body (note the immunostained secretory granules indicated by small arrows) (a) and with an LHRH-immunoreactive nerve fiber (LHRH) (b). Rat medial preoptic area. a: \( \times 15,000 \), b: \( \times 22,000 \)
Fig. 2. 5-hydroxydopamine-labeled terminals (black stars) making synapses (arrows) with LHRH-immunoreactive neurons in the medial preoptic area. a. A 5-hydroxydopamine-labeled terminal making a symmetrical synapse with an LHRH-immunoreactive cell body. ×25,000. b. A 5-hydroxydopamine-labeled terminal making synaptic contact with an LHRH-immunoreactive fiber. ×39,000. c. A 5-hydroxydopamine-labeled and three unlabeled terminals (white stars) making synaptic contacts with an LHRH-immunoreactive fiber. ×18,000. Inset shows a higher magnification of a 5-hydroxydopamine-labeled terminal. ×28,000
Fig. 3. Axon terminals (black stars) labeled with silver grains after $^3$H-dopamine injection are shown making synapse-like contacts (large arrows) with the LHRH-immunoreactive neurons. a. An axon terminal showing selective uptake of $^3$H-dopamine making synapse-like contact with an LHRH-immunoreactive cell body. N nucleus. $\times 24,000$. b. $^3$H-dopamine labeled terminal making synapse-like contact with an LHRH-immunoreactive fiber. $\times 21,000$

Fig. 4. Axon terminals (black stars) labeled with silver grains after $^3$H-noradrenaline injection making synapse-like contacts (large arrows) with an LHRH-immunoreactive cell body (LHRH) (a) and with an LHRH-immunoreactive nerve fiber (LHRH) (b) in the medial preoptic area. LHRH-like immunoreaction is seen in the secretory granules (small arrows) in the cytoplasm. It is unclear whether the terminal labeled with an open star is labeled with $^3$H-noradrenaline or unlabeled. a: $\times 19,000$, b: $\times 35,000$
were found between axon terminals labeled with \(^3\)H-dopamine (Fig. 3a, b) or \(^3\)H-noradrenaline (Fig. 4a, b) and LHRH-immunoreactive nerve cell bodies and fibers in the medial preoptic area. However we could not determine whether the postsynaptic LHRH-immunoreactive processes were axons or dendrites. Silver grains in the axon terminals occurred commonly in the serial sections. A majority of the silver grains occurred on the axon terminals containing numerous small clear vesicles and a few large granular vesicles such as in the 5-hydroxydopamine-labeled ones in the medial preoptic area.

In the combination method, synapses between catecholaminergic terminals labeled with 5-hydroxydopamine, \(^3\)H-dopamine or \(^3\)H-noradrenaline and LHRH-immunoreactive neurons were mostly asymmetrical types (Fig. 2b, 4b), though symmetrical types also occurred relatively often (Fig. 2a). In the latter, both pre- and post-synaptic membranes appeared to be thickening by the accumulation of a dense substance in the cytoplasm beneath both synaptic membranes. However, it was often difficult to distinguish the dense material in the postsynaptic structure of asymmetrical synapses from dense reaction products.

In the control sections incubated with LHRH antisera preabsorbed with synthetic LHRH, the immunoreactive LHRH neurons were completely abolished.

DISCUSSION

The distribution of LHRH neurons and their processes in the rat brain has been extensively studied immunocytochemically by light and electron microscopy. It has been generally accepted that LHRH neurons located in the preoptic area project their processes to the median eminence (IBATA et al., 1979a, b, 1983; KAWANO and DAIKOKU, 1981; HOFFMAN and GIBBS, 1982; KING et al., 1982; MERCENTHALER et al., 1984). On the other hand, the distribution of dopaminergic and noradrenergic terminals in the rat preoptic area has been revealed by immunocytochemistry using antisera against the catecholamine synthesizing enzymes such as TH and DBH (HÖKFELT et al., 1978).

Concerning the control of LHRH secretion by various materials, neurotransmitters such as dopamine (BENNET et al., 1975; NEGRO-VILAR et al., 1979; WILKES et al., 1979), noradrenaline (HEAULME and DRAY, 1984), adrenalin, serotonin (WEINER and GANONG, 1978), neuropeptides such as opioid (ADLER and CROWY, 1984; KALRA and KALRA, 1984) and neurotensin (FERRIS et al., 1984) have been shown to be involved in the regulation of LHRH release.

However, little is known morphologically about the regulatory mechanisms of these materials in LHRH secretion.

It has been demonstrated that LHRH immunoreactive perikarya and their processes receive synaptic input from unknown immunonegative terminals in the preoptic area of the rat (HISANO et al., 1981; WITKIN and SILVERMAN, 1985) and guinea pig (SILVERMAN and WITKIN, 1985) and in the preoptic area and the diagonal band of Broca of the rat (JENNES et al., 1985). HISANO et al. (1981) reported that the immunonegative presynaptic terminals possess a large number of clear spherical vesicles (54 nm) intermingling with several cored vesicles (100 nm) in the preoptic area of early postnatal rats of both sexes. On the other hand, JENNES et al. (1985) reported that in female rats 40 to 50 days of age, most presynaptic terminals contained clear vesicles only, although occasionally presynaptic terminals with both clear and dense-core vesicles also occurred. In the present study, both immunonegative presynaptic termi-
nals containing only clear vesicles and terminals containing both these vesicles and a few larger granular ones were found. Recently, Leranth et al. (1985) demonstrated using a double-label electron microscopic immunostaining procedure that terminals containing the immunoreactivity for glutamic acid decarboxylase, the enzyme responsible for GABA biosynthesis, made symmetrical synapses on the LHRH immunoreactive perikarya and their processes in the rat medial preoptic area.

Concerning the interactions between the catecholamine- and LHRH-containing neurons, studies with light microscopic immunohistochemistry suggest that dopamine may control the LHRH release via an axoaxonic interaction in the median eminence, for it is there that the dopamine and LHRH nerve endings overlap each other (Hökfelt et al., 1978; Ajika, 1979; Hoffman et al., 1982; Jennens et al., 1983). Using electron microscopic double immunostaining, Ajika (1979) demonstrated an axoaxonic contact of dopamine and LHRH terminals in close proximity to portal vessels in the rat median eminence and suggested a synaptic influence of dopamine on the LHRH release into the portal vessels.

Recently, synaptic contacts between 3H-5-hydroxytryptamine-labeled terminals and LHRH-immunoreactive dendrites have been demonstrated in the rat medial preoptic area, using a combination of immunocytchemistry and high resolution autoradiography (Kiss and Halász, 1985). Using the same combined technique, the present study demonstrated axon terminals labeled with 3H-dopamine, 3H-noradrenaline or 5-hydroxydopamine making synaptic contacts with both LHRH cell bodies and processes in the rat medial preoptic area. It has been demonstrated in the central and peripheral nervous system that 5-hydroxydopamine is selectively accumulated in catecholaminergic terminals and causes an increased electron density of the granular vesicles as well as the appearance of a dense core in the small clear vesicles in them (Richards and Tranzer, 1970; Thoenen and Tranzer, 1971; Ajika and Hökfelt, 1973; Ibata et al., 1974; Mazzuca and Poulain, 1974). Furthermore, it has been reported that 3H-noradrenaline is not retained in dopaminergic (Descarries et al., 1980) or serotonergic terminals (Rothman et al., 1976), and that metabolites of noradrenaline are not retained through the autoradiographic technique (Descarries and Dupin, 1974). The presynaptic terminals labeled with 3H-noradrenaline found in this study are probably considered to be noradrenergic, but those labeled with 3H-dopamine are unclear whether they contain dopamine or some other amine. These findings suggest that in the rat, at least some catecholaminergic neurons innervate LHRH neurons to regulate LHRH secretion via synapses with other unidentified neurons in the rat medial preoptic area.

REFERENCES


