Immunohistochemical demonstration of dopamine receptor D2R in the primary cilia of the mouse pituitary gland

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ABSTRACT
Dopamine regulates the synthesis and secretion of prolactin and α-MSH/β-endorphin in lactotrophs and melanotrophs, respectively. While a predominant dopamine receptor, D2R, is known to be expressed in both the anterior and intermediate lobes of the pituitary gland, no previous immunohistochemical studies have shown the existence of D2R in the plasma membrane of pituitary endocrine cells. The present study clearly demonstrated a selective localization of the D2R immunoreactivity in primary cilia of lactotrophs and melanotrophs in the mouse adenohypophysis. Another immunoreactivity of D2R was found along the plasma membrane of melanotrophs. The intensity of immunoreactivity for D2R in the primary cilia of lactotrophs changed during the estrous cycle and with genital conditions in contrast to a consistent immunolabeling in the melanotrophs. Since there is accumulating evidence that the primary cilium functions as a sensory device at a cellular level, the D2R-expressing primary cilia in the pituitary gland may be involved in the sensation of dopamine and dopaminergic compounds—though their involvement differs between the anterior and intermediate lobes.

Dopamine is well established as a primary inhibitor of both prolactin secretion (6) and the proliferation of lactotrophs (1, 13). Dopamine is released at the median eminence and conveyed by the hypothalamic portal system to regulate the activities of pituitary lactotrophs mainly via the type 2 dopamine receptor (D2R). Accordingly, D2R knockout mice display a phenotype characterized by chronic hyper-prolactinemia and the hyperplasia of lactotrophs (16, 25). Although dopamine must exert its actions by binding to specific membrane receptors on pituitary endocrine cells, previous immunohistochemical studies of normal and tumoral lactotrophs have detected D2R immunoreactivities in the cytoplasm but not in the plasma membrane (18, 41, 48). This is an inconsistent finding concerning the subcellular localization of D2R—though we sometimes experience such a discrepancy with the immunostaining of membrane-bound receptors.

The pituitary intermediate lobe is also under the control of dopamine (10–12, 17) and expresses D2R at a high density, unlike the anterior lobe (3). In situ hybridization analyses for D2R mRNA and radioligand binding assays in rats have demonstrated a condensed expression of D2R or dopamine binding sites in the intermediate lobe (28, 32, 35). The intermediate lobe morphologically differs from the anterior lobe by having rich innervation and poor vascularization (4, 26, 27) and being composed of essentially one type of endocrine cell, melanotrophs. The melanotrophs receive dopamine input from tuberohypophyseal neurons that actually terminate within the intermediate lobe (4). Dopamine has been experimentally shown to decrease the capacity of the intermediate lobe to synthesize pro-opiomelanocortin

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(POMC) and release its products: α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (12, 14, 43, 44). It is known that dopaminergic compounds differentially control the D2R mRNA levels and activities of target endocrine cells in two lobes of the rat pituitary gland (3, 10). However, the difference in the regulatory mechanism via D2R remains to be elucidated.

The primary cilium is immotile and occurs extensively as a more fundamental structure as compared with the secondary, motile cilium (46). Increasing evidence suggests that the primary cilium functions as a sensory apparatus in neurons, sensory cells, and several types of epithelial cells which face liquid flow, such as pancreatic ducts (2, 9) and urinary tubules (36). Neuronal primary cilia in the brain frequently express G protein-coupled receptors (GPCRs), including somatostatin receptor 3 (SSTR3) (21), 5-HT₄ serotonin receptor (20), and melanin-concentrating hormone receptor 1 (MCHR1) (7), indicating an important role for these cilia in the extra-synaptic signaling of neurons (47). Endocrine cells also possess primary cilia in the adenohypophysis, pancreatic islets, and adrenal gland. Electron microscopic studies of these endocrine cells have demonstrated the existence of single cilia which display a structural organization unlike that of the motile cilium (5, 45, 49). Our recent immunohistochemical study revealed an abundance of SSTR3-expressing solitary cilia in growth hormone (GH)-secreting cells and insulin-secreting β-cells of the mouse (24). Further application of immunostaining for various receptors in the nervous and endocrine tissues holds promise for establishing the primary cilium as universal sensory devices.

The present immunohistochemical study reports the expression of D2R in the primary cilia of the mouse adenohypophysis, suggesting the involvement of these cilia in the special sensation of dopamine and regulation of the secretion of prolactin and α-MSH/β-endorphin.

MATERIALS AND METHODS

Animals. Twelve female mice and five male mice of the ddY strain (8-week-old, weighing about 30 g) were used for basic observations. The mice were housed under freely ranging circumstances in our animal facilities with controlled temperatures (23 ± 1°C), relative humidity (30–60%) and lighting (lights on 08:00–20:00). They were allowed ad libitum access to normal rodent chow and water. The estrous cycles of females were checked by observation of vaginal smears and tissue sections from the vagina obtained after sacrifice. In addition, groups of three to five pregnant mice (14.5 gestational day), lactating mice (2 and 5 days after parturition), and ovariectomized mice (seven days after surgery) were used for the immunohistochemistry of D2R. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

In situ hybridization. For in situ hybridization, the hypophysis was removed and directly embedded into a freezing medium (OCT compound; Sakura Finetech Co. Ltd., Tokyo, Japan). The embedded tissues were quickly frozen in liquid nitrogen and stored at −80°C until use. Three non-overlapping antisense oligonucleotide probes (45 mer in length) were designed to be complementary to the 461–505, 741–785, and 1021–1065 of mouse D2R mRNA (accession number: NM_010077). The probes were labeled with 33P-dATP using terminal deoxy- nucleotidyl transferase (Invitrogen, Carlsbad, CA). Fresh frozen sections, 14-μm-thick, were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 10 h with a hybridization buffer containing 10,000 cpm/μL 33P-labeled oligonucleotide probes. Glass slides were rinsed at room temperature for 30 min in 2 × SSC (1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate) containing 0.1% N-lauroylsarcosine sodium, then rinsed twice at 55°C for 40 min in 0.1 × SSC containing 0.1% N-lauroylsarcosine sodium, dehydrated through a graded series of ethanol, and air-dried. Sections were dipped in an autoradiographic emulsion (NTB-2, Kodak) at 4°C for 8 weeks. The hybridized sections used for autoradiography were counterstained with hematoxylin after development.

In situ hybridization using the three non-overlapping antisense probes for D2R mRNA exhibited identical labeling in all the tissues examined. The specificity of the hybridization was also confirmed by the disappearance of the signals upon the addition of an excess of an unlabeled antisense probe serving as a negative control.

Immunohistochemistry. For immunohistochemistry, mice were perfused with a physiological saline through the heart, followed by 4% paraformaldehyde plus 0.2% picric acid in 0.1 M phosphate buf-
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The hypophysis was dissected out and immersed in the same fixative for an additional 8 h. The fixed tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound, and quickly frozen in liquid nitrogen. Frozen sections of a 10-μm thickness were mounted on poly-L-lysine-coated glass slides. After immersion in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton-X 100, the sections were preincubated with a normal donkey serum. For double immunofluorescence, the sections were first incubated at a concentration of 1 μg/mL with guinea pig antibody raised against C-terminal 45 amino acids of mouse D2R (NM010076) (Frontier Institute Co. Ltd, Hokkaido, Japan) (33) and then incubated with one of the following antibodies: rabbit anti-mouse prolactin (1 : 800 in dilution; AbD Serotec—MorphoSys UL Ltd, Oxford, UK), rabbit anti-α-MSH (1 : 800; Peninsula Laboratories, San Carlos, CA), rabbit anti-rat ACTH (RY55, 1 : 800; Yanaihara Institute, Shizuoka, Japan), rabbit anti-human GH (0.4 μg/mL; Zymed Laboratories, South San Francisco, CA), rabbit anti-mouse/rat SSTR3 (1 : 5,000 in dilution; Gramsch Laboratories, Schwabhausen, Germany), or rabbit anti-adenylate cyclase type 3 (AC3) (0.2 μg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Dopaminergic neurons were detected by use of a rabbit antiserum (1 : 900 in dilution) against bovine tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. The sites of antigen-antibody reactions were detected by incubation with a combination of Cy3-labeled anti-guinea pig IgG and FITC-labeled anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan). Some of immunostained sections were counterstained with SyTO 13 (Invitrogen) before observation.

The D2R antibody used selectively stained the striatum in the mouse brain (33) but never stained any structures in the adrenal gland or pancreatic islets. SSTR3 and AC3 antibodies used specifically labeled the primary cilia on neurons in several regions of the mouse brain, in agreement with previous studies (24). The specificity of immunoreactions with the anti-D2R antibody was confirmed by disappearance of the immunoreactivity when the antibody was preincubated with a peptide antigen (Frontier Institute Co.Ltd).

Silver-intensified immunogold method for electron microscopy. Frozen sections of a 15-μm thickness were mounted on poly-L-lysine-coated glass slides, incubated with the guinea pig anti-D2R antibody (1 μg/mL in concentration), and subsequently reacted with goat anti-guinea pig IgG covalently linked with 1.4-nm gold particles (1 : 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon (Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

RESULTS

In situ hybridization of D2R mRNA

An in situ hybridization analysis demonstrated expression sites of D2R mRNA in the hypophysis of female adult mice under a light microscope (Fig. 1). A condensed expression of D2R mRNA was found throughout the intermediate lobe. The anterior lobe displayed a dispersed distribution of silver grains for

Fig. 1 In situ hybridization analysis for D2R mRNA in the mouse pituitary gland. The same area is shown in bright- (a) and dark-field images (b). Silver grains are condensed in the intermediate lobe (I). A: anterior lobe, P: posterior lobe Bar 50 μm
D2R mRNA, which appeared to correspond to lactotrophs.

**Immunohistochemistry for D2R**

To confirm the distribution of dopaminergic neurons in the mouse hypophysis, immunohistochemistry for tyrosine hydroxylase (TH) was applied to sections from female mice. Varicose immunoreactive fibers accumulated in the intermediate lobe, but were sparse in the anterior lobe (Fig. 2a). Only a small number of TH-immunoreactive beaded fibers were found in restricted areas of the posterior lobe adjacent to the intermediate lobe. When tissue sections from female mice at diestrus were stained with the anti-D2R antibody, many rod-shaped structures in the anterior lobe were immunoreactive—though the immunoreaction in the cell bodies was faint or undetectable (Fig. 2b–d). These rod-like structures were identified at the electron microscopic level to be primary cilia extending from endocrine cells, as described below. Double staining for D2R and prolactin revealed an intimate relation between D2R-

![Fig. 2](image)

**Fig. 2** Immunohistochemistry in the mouse pituitary gland. Tyrosine hydroxylase (TH)-immunoreactive nerve fibers gather at the intermediate lobe (I in a). The antibody against D2R labels the plasma membrane and primary cilia in the intermediate lobe (I) and primary cilia in the anterior lobe (A) (b). In double staining, most of the D2R-immunoreactive cilia (red) have contact with the Golgi area of prolactin cells (green) (c). However, it is hard to correlate the cilia with many ACTH cells in the double staining of D2R and ACTH (d). Cells of the intermediate lobe possess the D2R immunoreactivity in the plasma membrane and rod-shaped primary cilia (e). All cells of the intermediate lobe are immunoreactive for both D2R (red) and α-MSH (green) (f). In a, b, and e, nuclei are stained green with a nucleic acid-sensitive dye. Bars 20 μm (a, b), 10 μm (c–f)
immunoreactive cilia and prolactin-containing cells, namely lactotrophs (Fig. 2c). The prolactin immunoreactivity in lactotrophs was more intense in the Golgi apparatus than other parts of the cell body. The cilia appeared to be extended from the intensely immunolabeled Golgi area of lactotrophs. Non-lactotrophs such as GH cells did not possess any D2R-immunoreactive cilia (data not shown). ACTH cells were highly unlikely to be associated with the D2R-positive cilia (Fig. 2d) although it was difficult to deny the existence of the D2R-immunoreactive cilia in some ACTH cells.

In the intermediate lobe, the D2R antibody immunostained the plasma membrane of all endocrine cells (melanotrophs) linearly along their entire length (Fig. 2b, e). These endocrine cells were heavily immunolabeled with the antibody against α-MSH (Fig. 2f). The melanotrophs possessed D2R-immunoreactive cilia which were more intense in reaction than the plasma membrane. The cilia of melanotrophs tended to be longer and thicker than those in the D2R-immunoreactive cilia of lactotrophs.

Since neurons and some endocrine cell types express adenylate cyclase 3 (AC3) in the primary cilia (8, 24), double staining for D2R and AC3 was done in the hypophysis. As shown in Fig. 3a–c, most of the primary cilia in the anterior lobe were immunoreactive for both D2R and AC3. In contrast, the primary cilia of melanotrophs in the intermediate lobe were free of any immunoreactivity for AC3 (data not shown).

Changes of D2R immunoreactivity in the anterior lobe
The immunoreactivity of D2R in the primary cilia of the anterior lobe changed during the estrous cycle: the immunoreactivity was intense at met-estrus and di-estrus but decreased in the intensity from pro-estrus to estrus (Fig. 4a, b). Especially, the decrease was remarkable when the vaginal epithelium developed a thick keratinized layer on the surface. In contrast, the D2R immunoreactivity of melanotrophs was essentially unchanged during the estrous cycle in both the primary cilia and plasma membrane.

![Fig. 3](image)

**Fig. 3** Double staining for D2R and adenylate cyclase 3 (AC3) in the anterior lobe. D2R-immunoreactive cilia of lactotrophs largely correspond to the AC3-immunolabeled cilia. Fig. 3c is a merged image. Bar 10 μm

**Fig. 4** Comparison of D2R immunoreactivities in the adenohypophysis between the di-estrus (a) and pro-estrus (b). D2R-immunoreactive cilia are numerous in the anterior lobe at the di-estrus (a) but undetectable at the pro-estrus (b). I: intermediate lobe. Nuclei are stained green. Bar 20 μm
More intense degrees of labeling were found in the plasma membrane of primary cilia (Fig. 6b). The D2R-immunolabeled cilia tended to be longer and run more straightly than those in the anterior lobe. Such cilia were embedded in their own cytoplasm of endocrine cells and enveloped by deep invaginations of the plasma membrane (Fig. 7b). Some cilia reached an intercellular gap and were further inserted into the cell body of adjacent endocrine cells.

**DISCUSSION**

It is generally believed that D2R is a predominant dopamine receptor that is expressed on the plasma membrane of lactotrophs and melanotrophs, where it regulates the hormone secretion and proliferation of target cells. The present immunohistochemical study is the first to demonstrate the localization of D2R in the plasma membrane of melanotrophs as well as the condensed localization in the primary cilia in both melanotrophs and lactotrophs. Only in the cilia of lactotrophs did the D2R immunoreactivity change during the estrous cycle and under various genital conditions, in contrast to the constant stainability in the intermediate lobe. The expression pattern of D2R as well as the contrasting modes of dopamine input—hormonal vs. neuronal—suggest that regulatory mechanisms by hypothalamic dopamine differ between lactotrophs and melanotrophs, as has been indicated by many physiological and pharmacologi-

**Electron microscopy**

Immunohistochemistry for D2R at the electron microscopic level detected many immunolabeled primary cilia in the anterior lobe of female mice at the di-estrus (Figs. 5, 7a). Endocrine cells with primary cilia contained irregularly shaped electron-dense secretory granules which characterized the lactotrophs. Gold particles showing the existence of D2R immunoreactivity were localized densely along the plasma membrane of cilia (Fig. 7a). The cilia were projected from the Golgi area into an intercellular space among the endocrine cells and did not face the perivascular space (Fig. 5). The primary cilium was an immotile one which lacked a central pair of microtubules and possessed two centrioles, the proximal and the distal centrioles, as observed in primary cilia of other cell types (46, 47).

In the intermediate lobe, gold particles for D2R immunoreactivity appeared at the plasma membrane along the entire length of melanotrophs (Fig. 6a).
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The present findings could also serve as a representative case for the regulation of endocrine functions by the primary cilia.

**Anterior lobe**

Dopamine and dopaminergic agonists diminish both prolactin synthesis at the mRNA level and prolactin secretion in lactotrophs (29). A radioligand binding assay using dispersed bovine anterior pituitary cells has indicated that dopamine receptors are restricted to the outer plasma membrane and not internalized during the receptor assay (38). In contrast to the established findings for the dopaminergic regulation of lactotrophs, previous immunohistochemical studies failed to demonstrate a membrane-bound localization of D2R in the pituitary gland, with the exception of an immunohistochemical study by Hozumi et al. (22). Immunostaining of the human pituitary gland for D2R labeled only the cytoplasm and nuclei of endocrine cells (41, 48). These findings are in accordance with studies performed on other cell types, where the D2 receptor was found intracellularly upon overexpression (37, 42), or was localized in secretory granules in vivo (40). The present study clearly showed a membrane-associated localization of D2R in the primary cilia of lactotrophs without

![Fig. 6](image_url) Electron microscopic pictures showing the D2R immunoreactivity in the intermediate lobe. The immunoreactivity is found linearly and evenly along the plasma membrane (a). More condensed labeling for D2R localizes at two primary cilia which appear to be embedded in the cytoplasm of melanotrophs (b). N: nucleus Bars 1 μm
any significant immunolabeling in the cytoplasm and nucleus. The frequent occurrence of primary cilia and their ultrastructure in the anterior lobe of mouse hypophysis have been documented in detail by Barnes (5). In agreement with her data, our electron-microscopic observations confirmed that the D2R-expressing primary cilia in lactotrophs extended from the Golgi area and showed features unique to the immotile and solitary cilia. The primary cilia of lactotrophs protruded into intercellular spaces among endocrine cells and did not face the perivascular spaces. This morphology resembles the SSTR3-expressing primary cilia of GH cells and pancreatic β-cells that we documented in our recent study (24). The best characterized signal transduction pathway of D2R is the inhibition of adenylate cyclase, which results in the decrease of intracellular cAMP levels (30, 31). The immunohistochemical colocalization of adenylate cyclase type 3 in the primary cilia supports the functional significance of D2R being selectively localized in the primary cilia.

Prolactin secretion is regulated by dopamine levels in the portal plasma and also by the number of dopamine binding sites, but not by changes in the affinity to legands (22, 34). Estrogen and the estrous cycle affect the number of binding sites for dopamine in the anterior lobe (22, 34, 35), although there is a discrepant finding concerning the exact time of change during the estrous cycle. Estrogen reverses the inhibitory effect of dopamine on prolactin secretion at the pituitary level (19, 34, 39) while progesterone reverses the stimulatory effect of estrogen (15). The number of dopamine receptors was reported by Pasqualini et al. (34) and Pazos et al. (35) to be constantly high at the di-estrus and decreased in the pro-estrus. In accordance with these reports, the present study showed a pattern of change for D2R in the primary cilia of lactotrophs: the immunoreactivity significantly decreased in the pro-estrus to estrus as compared with the met-estrus to di-estrus. The stainability of D2R in the cilia also varied under sexual and genital conditions in contrast to the consistent staining for D2R in the melanotrophs. The less intense immunoreactivity or disappearance of D2R in primary cilia was found in male mice as well as ovariectomized, pregnant, and lactating female mice. This decreased expression of dopamine receptors is in agreement with the general idea that lactotrophs are hypertrophied and/or hyperplastic in conditions of hyper-prolactinemia such as pregnancy and lactation. Although the exact regulatory mechanism by the ovarian steroid hormones has yet to be determined, it is worth noting that the expression of D2R on the primary cilia of lactotrophs either appeared or disappeared during the estrous cycle and by hormonal conditions. This strongly suggests that the primary cilia play a key role in the dopaminergic regulation of prolactin.

**Intermediate lobe**

Dopaminergic neurons densely innervate the intermediate lobe to exert tonic inhibitory actions on the growth and function of melanotrophs. The present immunohistochemical staining for TH confirmed the rich existence of dopaminergic nerve terminals in the intermediate lobe of the mouse pituitary gland, as reported in the rat (4). Several *in situ* binding studies using dopaminergic agents demonstrated their
intense binding in the intermediate lobe of rats (28, 35). Under the light microscope, our in situ hybridization analysis in the mouse detected a dense expression of D2R mRNA in the intermediate lobe that was equivalent to the rat pituitary gland (3, 32). Immunohistochemically, melanotrophs expressed D2R linearly along the entire length of their plasma membrane, reconfirming that most of the D2R-expressing sites are not involved in synaptic formation which usually aggregate as spots on the cell surface. Electron microscopic studies of the rat intermediate lobe have found two types of nerve terminals: 1) neurosecretory axons—possibly a major type in the intermediate lobe—and 2) fine nerve fibers which contain small, so-called synaptic vesicles and make an occasional synaptic contact with endocrine cells (26, 27). Although it is not known which types of nerves contain dopamine, it is generally accepted that dopamine released from the nerve terminals inhibits the activities of melanotrophs in a paracrine manner.

Melanotrophs expressed D2R in the primary cilia at a higher density than that in the plasma membrane of cell bodies. Unlike the primary cilia of lactotrophs, the D2R-expressing cilia of melanotrophs appeared to be embedded in their own cell body or the cytoplasm of adjacent cells. This feature should be attributed to the construction of the intermediate lobe where parenchymal cells are densely packed without forming any sufficient intercellular space. Based on the subcellular localization of D2R, it is safe to say that melanotrophs can receive dopamine signals released from adjacent nerve terminals at both the primary cilia and the plasma membrane of the cell body. The dissimilar expression patterns of D2R between two pituitary lobes may be related to different regulations by dopamine/D2R between melanotrophs and lactotrophs. In fact, dopaminergic compounds differentially control D2R mRNA levels between the intermediate and anterior lobes of rats, with no significant changes in the anterior lobe (3). Another important difference between the two pituitary lobes is the change in dopamine receptors during the estrous cycle and by hormonal states. Some binding studies have demonstrated that dopaminergic receptors in the intermediate lobe did not fluctuate in number during the estrous cycle, in contrast to the anterior lobe (35). In accordance with this finding, the stainability of D2R in melanotrophs here was constant during the estrous cycle and in various genital states—sex, pregnancy, and lactating.

Our recent study demonstrated the selective localization of SSTR3 in the primary cilia of pituitary GH cells. It is interesting that both receptors for inhibitory signals—dopamine and somatostatin—are localized to the primary cilia of pituitary endocrine cells. The regulation of prolactin secretion involves a complex interaction of ovarian steroids with hypothalamic releasing and inhibitory factors. The switching on or off of D2R on primary cilia in the lactotrophs must be involved in this regulatory mechanism.

REFERENCES


