ONTOGENETIC STUDY ON THE MONOAMINE AND PEPTIDE
CONTAINING CELLS IN THE PITUITARY AND HYPOTH-
ALAMUS OF THE BULLFROG *RANA CATESBEIANA*
BY IMMUNOHISTOCHEMISTRY

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By the immunofluorescence method, development of the cells containing catecholamine-synthesizing enzymes [tyrosine hydroxylase (TH), dopamine- β -hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT)], serotonin (5-HT), calcitonin (Cal), somatostatin (Som), β -endorphin (End) and adrenocorticotropin (ACTH) was examined in the pituitary and tubero-infundibulum of *Rana catesbeiana* tadpoles, and the localization was compared with that of adult bullfrogs.

A large number of End- and ACTH-like immunoreactive cells were observed in the rostral part of the pars distalis of the tadpole pituitary before metamorphosis (stage 31 by Gosner). At this time, 5-HT-positive cells were also observed throughout the pars distalis, though far less than those of the adult frog. TH-positive cells first appeared at Gosner's stage 40 together with the appearance of hindlimbs, but these cells were DBH-negative. The cells reacting with antiserum against DBH appeared after the resorption of the tail.

TH-positive, DBH-negative dopaminergic fibers were observed around the capillary sinusoid of the pars distalis at Gosner's stage 31, but at stage 40 these fibers disappeared.

TH-, 5-HT-, Cal-, Som- and End-immunoreactive neurons in the hypothalamus, and their fibers which may be derived from this brain area, in the neural lobe and median eminence, were already observed in the tadpole before metamorphosis (stage 31), as those in the adult frog.

The authors recently found many tyrosine hydroxylase (TH)- and dopamine- β -hydroxylase (DBH)-positive but phenylethanolamine-N-methyltransferase (PNMT)-negative noradrenaline cells and serotonin (5-HT) cells in the pars distalis of the adult frog pituitary together with adrenocorticotrophic hormone (ACTH)-positive cells by immunohistochemical method (10). In the pituitary of *Xenopus laevis* tadpoles, the time of appearance of various hormone-secreting cells have been studied by some investigators. According to the developmental stage of the *Xenopus laevis* tadpole, the first sign of melanocyte stimulating hormone (MSH)-like immunoreactive cells was observed in the pituitary at Nieuwkoop and Faber (18)'s stage 38 (19). ACTH-cells appeared at stage 39 (12), lactotrophic hormone (LTH)-, thyroid stimulating hormone (TSH)- and luteinizing hormone (LH)-cells

at stage 42 (11, 12), and somatotropin hormone (STH)-cells at stage 44 (11). The hindlimb bud was still not visible at stage 44 of *Xenopus laevis* tadpole before metamorphosis. In the present study, the time of appearance of the catecholamine- and serotonin-containing cells was investigated in the pars distalis of *Rana catesbeiana* tadpole pituitary with ACTH- and End-like immunoreactive cells, and was compared with the appearance of monoaminergic neurons in the hypothalamus. Preliminary reports were published elsewhere (9, 10).

MATERIALS AND METHODS

Ten frogs (300–600 g body weight) and 15 tadpoles (Gosner's stage 31–42) of *Rana catesbeiana* were used. Frogs were anesthetized with ether and perfused via the arterial trunk with the Zamboni's fixative (2% paraformaldehyde–0.2% picric acid in 0.1 M phosphate buffer, pH 7.2). Brains attached with the pituitary glands of adult frogs and tadpoles were dissected out and postfixed in the same fixative for 6–18 hr in a cold room at 4°C. Subsequently they were washed, rinsed in the phosphate buffer containing 10% sucrose, and frozen on dry ice. Frozen sections 10 μ m in thickness were cut on a cryostat and processed for the indirect immunofluorescence technique (15). After immunofluorescent staining, the sections were mounted in buffered glycerol (0.5 M carbonate, pH 8.6: glycerol, 1 : 2) and examined by a Leitz Orthoplan fluorescence microscope, equipped with a Ploem vertical illuminator (activation filter KP490, red suppression filter BG38 and barrier filter K530 or K510) with a mercury vapor lamp as the light source (13).

For the present study, specific antiserum against TH, DBH or PNMT from bovine adrenal glands was raised in rabbits and the specificity was tested as previously described (13–16). 5-HT-, calcitonin (Cal)-, somatostatin (Som)-, β -endorphin (End)- and porcine pituitary ACTH^{1–39}-antisera were purchased from Immuno Nuclear Corporation, Stillwater, Minn., USA. Rabbit antiserum against 5-HT was also produced in our laboratory using 5-HT coupled to bovine serum albumin (BSA) as antigen, according to the method of Steinbusch *et al.* (20, 21). The two kinds of rabbit antiserum against 5-HT produced specific 5-HT staining in brain sections and similar results were obtained in dilution of about 1 : 3000. Pre-immune rabbit serum and antiserum against BSA were used for controls.

RESULTS

Pituitary glands of *Rana catesbeiana* tadpoles were studied between stage 31 and 42 by Gosner (8), which correspond to the stage 50 and 62 by Nieuwkoop and Faber (18). At Gosner's stage 31 (Fig. 2c), tadpoles had the paddle-shaped hindlimbs, and subsequent stages through stage 37 witness the appearance of individual toes. Proportional changes in the length of individual toes and in the appearance of the metatarsal and subarticular tubercles occurred in stages 38–40. The latter appeared as light patches in stage 39 and as actual tubercles in stage 40. In stage 40 (Fig. 3c), the hindlimb was almost completed, and in stage 42 both the hindlimb and forelimb appeared and the resorption of the tail began (8).

1) The stage of appearance of TH-, DBH-, or 5-HT-immunoreactive cells and fibers

Figs. 2a-c. At stage 31 of tadpoles (c). TH-immunofluorescent micrographs in the pituitary.

Sagittal sections. The right side is rostral. a, b. TH-positive varicose fibers are observed along the sinusoid in the pars distalis (D), around the cells of the pars intermedia (I), and near the pars intermedia in the neural lobe (N). No TH-positive cells in the pars distalis are found. a $\times 75$, b $\times 215$

Cells: In the pars distalis, TH-positive cells began to appear simultaneously with hindlimbs at Gosner's stage 40 (Figs. 3a, b), but showed DBH-negative reaction. These cells were dopamine-containing cells. DBH-positive cells appeared after resorption of the tail (Table 1). A large number of TH- and DBH-positive but PNMT-negative noradrenaline cells were widely distributed throughout the pars distalis of adult frog pituitary (9, 10).

At stage 31, 5-HT-positive cells (Fig. 1b) were found a few in number, but TH-positive cells (Fig. 1a) were not observed in the pars distalis. 5-HT-immunoreactive cells increased gradually in the pars distalis of adult frog.

In the pars intermedia, no TH-positive cells were found in any stage of

Figs. 1a-f. At Gosner's stage 31, immunofluorescent micrographs of the tadpoles (*Rana catesbeiana*) pituitary in sagittal sections. The right side is rostral. a-f. Serial sections. a. TH-positive fibers and terminals along the sinusoid in the pars distalis (D), around cells in the pars intermedia (I), near the pars intermedia in the neural lobe (N) and in the tuberoinfundibular tract. But, no TH-positive cells were observed in the pars distalis. b. A few 5-HT-positive cells in the pars distalis. c. Only Cal-like immunoreactive fibers and terminals in the infundibulum, in the tuberoinfundibular tract and near the pars intermedia in the neural lobe. d, e. ACTH- (d) and β -End- (e) like immunoreactive cells in the rostral region of the pars distalis and all cells of the pars intermedia. f. Som-like immunoreactive fibers and terminals in the infundibulum, tuberoinfundibular tract and neural lobe. a-f $\times 145$

Figs. 3a–c. At stage 40 of tadpoles (c). Immunofluorescent micrographs of TH in the pituitary. Horizontal sections. The upside is rostral. a. TH-positive cells (arrow) in the pars distalis (D) begin to appear together with hindlimbs. Also, TH-positive varicose fibers are observed around the cells of the pars intermedia (I), near the pars intermedia in the neural lobe (N) and in the median eminence (M), whereas TH-positive varicose fibers disappear from the pars distalis. b. TH-positive cells in the pars distalis. a $\times 85$, b $\times 215$

development. In contrast, 5-HT-immunoreactive cells with fine processes were scarcely detected among cells of the pars intermedia of adult frog pituitary, as already reported (10).

In the neural lobe, no monoamine-containing cells were found.

Fibers: In the pars distalis, TH-positive DBH-negative dopaminergic varicose fibers, were observed along the sinusoid at Gosner's stage 31 (Figs. 1a, 2a, b), but disappeared at stage 40 (Fig. 3a).

In the pars intermedia, TH-positive, dopaminergic fibers were already distributed around the cells at stage 31 (Figs. 1a, 2a, b), as in the case of the adult frog (Fig. 4c).

In the neural lobe, TH-immunoreactive fibers were found near the pars intermedia at stage 31 (Figs. 1a, 2a, b, 3a), as in the adult frog (Figs. 4c, e).

2) *The stage of appearance of Cal-, Som-, β -End- or ACTH-like immunoreactive cells and fibers*

Cells: β -End- and ACTH-like immunoreactive cells were already observed in the rostral region of the pars distalis and in the pars intermedia at Gosner's stage 31 (Figs. 1d, e) as seen in the adult frog (Fig. 4a). Distribution pattern of these End/ACTH-like immunoreactive cells were different from that of noradrenaline and serotonin cells in the pars distalis.

No Cal- or Som-like immunoreactive cells were found in adult frog and tadpole

FIGS. 4a-h. Immunofluorescent micrographs of the adult bullfrog (*Rana catesbeiana*) pituitary. a-d. Sagittal sections. The right side is rostral. a. β -End-like immunoreactive cells in the rostral region of the pars distalis (D) and all cells of the pars intermedia (I). b. 5-HT-positive cells in the pars distalis and 5-HT-positive fibers and terminals in the median eminence (M). c, d. A large number of TH- (c) and DBH- (d) positive cells in the pars distalis. TH-positive (c) but DBH-negative (d) fibers and terminals in the median eminence and around cells of the pars intermedia. e-h. TH- (e), 5-HT- (f), Cal- (g) and Som- (h) like immunoreactive fibers and/or terminals in the neural lobe (N). a-d $\times 25$, e-f $\times 190$

		Tadpoles															Adult Frog				
		Stage 31*					Stage 40*					Stage 42*									
		PD	PI	PN	ME	Inf	PD	PI	PN	ME	Inf	PD	PI	PN	ME	Inf	PD	PI	PN	ME	Inf
TH	cell body					NID ●●●	●				NID ●●●	●				NID ●●●	●●●				NID ●●●
	fiber	●	●●●	●	●●●	●●		●●●	●	●●●	●●		●●●	●	●●●	●●		●●●	●	●●●	●●
DBH	cell body																●●●				
	fiber																				
5-HT	cell body	●				PVO ●●●	●				PVO ●●●	●				PVO ●●●	●●●	●			PVO ●●●
	fiber				●●	●●				●●	●●				●●	●●			●	●●	●●
ACTH	cell body	●●	●●●●				●●	●●●●				●●	●●●●				●	●●●●			
	fiber																				
β-End	cell body	●●	●●●●			NIV ●	●●	●●●●			NIV ●	●●	●●●●			NIV ●	●●	●●●●			NIV ●
	fiber																				
Cal	cell body					NIV ●●					NIV ●●					NIV ●●					NIV ●●●
	fiber				●●●	●●				●●●	●●				●●●	●●			●	●●●	●●
Som	cell body					NIV ●●					NIV ●●					NIV ●●					NIV ●●
	fiber			●●	●●●	●●			●●	●●●	●●			●●	●●●	●●			●●	●●●	●●

*Stage 31, 40 and 42: Kenneth L. Gosner (1960) Herpetologica 16, 183-190.

TABLE 1. The time of appearance of the immunoreactive cells and fibers in the pituitary and related nuclei of bullfrog (*Rana catesbeiana*)

PD: pars distalis, PI: pars intermedia, PN: neural lobe,
ME: median eminence, Inf: infundibulum, NID: nucleus infundibularis dorsalis,
PVO: paraventricular organ, NIV: nucleus infundibularis ventralis.

pituitary glands (Table 1).

In the neural lobe, cells positive to any antisera used were not found (Figs. 1a-f, 4a-h).

Fibers: A small number of Cal-like immunoreactive fibers were seen at boundary of the pars intermedia and neural lobe (Fig. 4g). A large number of Som-like immunoreactive varicose fibers were observed throughout the neural lobe (Fig. 4h). However, End- and ACTH-like immunoreactive fibers were not seen in the whole pituitary.

DISCUSSION

The stage of appearance of TH-, DBH-, 5-HT-, ACTH- and End-containing cells in the pituitary

Based on the results obtained from the present immunofluorescent study, the time of the appearance of the TH-, DBH-, 5-HT-, ACTH-like-, End-like-, Cal-like- and Som-like-immunoreactivity in the pituitary and infundibular region of tadpoles and adult bullfrog (*Rana catesbeiana*) is given in Table 1.

At Gosner's stage 31, ACTH- and End-like immunoreactive cells in the pituitary were already seen in the rostral portion of the pars distalis and throughout the pars intermedia (Figs. 1d, e), as the adult frog (Fig. 4a). This agrees with the

finding on α -, β -End-like cells in *Rana temporaria* (L) (7). Recently, Doerr-Schott *et al.* have reported that the first signs of MSH- and ACTH-like immunoreactivity were found at stages 38 and 39 in the pituitary of *Xenopus laevis* tadpoles, respectively (19, 12). The stage 39 of *Xenopus laevis* used by them corresponds to stage 21 of Gosner's. Therefore, it is thought that ACTH- and MSH-cells in the pituitary have already appeared before this time. Moreover, Moriceau-Hay *et al.* (11, 12) have observed other pituitary hormone-secreting cells in early stages of *Xenopus laevis*: stage 42 for LTH, LH and TSH, and stage 44 for STH. Varicose TH-positive fibers in the pars intermedia of the pituitary and TH-, 5-HT-, Cal- and Som-like immunoreactive fibers in the neural lobe were also found at Gosner's stage 31, besides these kinds of fibers in the median eminence (Figs. 1a-f).

In contrast, the time of appearance of monoamine containing cells in the pars distalis of the tadpole pituitary was very late. Especially TH-positive catecholamine cells were not found until this time (Figs. 1a, 2a, b), though a few 5-HT-positive cells were observed at stage 31 (Fig. 1b). TH-immunoreactive cells first appeared at stage 40 (Figs. 3a, b) simultaneously with the appearance of hindlimbs, but these cells were identified as dopamine cells because of their negative reaction to DBH at stages 40 and 42 (Table 1), in contrast to noradrenaline cells in the adult bullfrog (Fig. 4d) (9, 10). Terlouw and Ploemacher (22) observed histofluorescent cells in the pars distalis of *Xenopus laevis* tadpoles at Nieuwkoop and Faber's stage 57 by the formaldehyde-induced histofluorescence method, but they could not identify whether this fluorophore was specific or not. The stage 57 of *Xenopus laevis* used by them corresponds to Gosner's stage 42 used by us because of the presence of both fore- and hindlimbs, and of beginning of tail atrophy. At this stage, we were able to recognize dopamine cells in the pars distalis.

Although the function of these monoamine cells is not clear, the following facts on the pituitary of the frog and tadpoles should be noted: (1) the cells which show immunoreactivity to catecholamine-synthesizing enzymes appeared considerably later than those in the brain, (2) 5-HT-cells were found earlier than catecholamine cells, and (3) DBH-immunoreactivity appeared further later than TH-immunoreactivity, and was present only in the cells of the pituitary of adult bullfrog.

The existence of TH-positive fibers in the pars distalis of the pituitary at early stage

At stage 31, TH-positive fibers and terminals were observed along the sinusoid ventrally in the pars distalis (Figs. 1a, 2a, b), but at stage 40 these fibers disappeared (Fig. 3a). This finding shows that dopaminergic innervation in the pars distalis occurs only transiently in prometamorphic stage. These dopaminergic fibers probably derive from hypothalamohypophyseal tract which originates in the brain and reaches into the pars intermedia and median eminence. Similar finding was noted in the tadpoles *Rana temporaria* at Gosner's stage 36-39 by the formaldehyde-induced histofluorescence technique (1). Aronsson and Enemar suggested that these monoamine fibers were remnants of an aminergic innervation as a functional link between brain and pituitary in the fish-like ancestor of amphibians (2) and were not of the vasomotor type.

Peptide-like immunoreactive cells and fibers in the pituitary

More recently, Defetos *et al.* (4, 5) reported the occurrence of the cells containing Cal throughout the intermediate lobe and in the anterior lobe of the rat pituitary. Cooper *et al.* (3) reported that 2 of 15 anti-human Cal sera examined showed

immunoreactive scattered cells in the rat and human anterior lobe using an immunoperoxidase method. These 2 antisera to human Cal showed Cal-positive reaction towards entire cells in the rat intermediate lobe. As already reported, antiserum to Cal used by us showed positive reaction to C-cells of the dog thyroid gland (17) and to the ultimobranchial bodies of grass parakeets and quail (24), but not to the pituitary gland of adult frog and tadpole (*Rana catesbeiana*) (Fig. 1c, Table 1). Similar negative finding was obtained in the bullfrog pituitary by Yui (27).

In the neural lobe, no immunoreactive cells to any antisera used were found, but a large number of varicose fibers and terminals showing Som-like immunoreactivity were observed throughout the neural lobe (Figs. 1f, 4h), as shown by Vandesande and Dierickx (23) and Dierickx *et al.* (6) in *Rana temporaria*. In addition, TH-, 5-HT- and Cal-immunoreactive varicose fibers were rarely seen at boundary of the pars intermedia and neural lobe (Figs. 4e, f, g).

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