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Effects of Olfactory Tract Section on the Immunohistochemical Distribution of Brain GnRH Fibers in the Female Goldfish, *Carassius auratus*

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ABSTRACT—The organization of the gonadotropin-releasing hormone (GnRH) system originating from the terminal nerve (TN) neuron in the olfactory bulb was examined immunohistochemically by tracing the changes in the distribution of salmon type (sGnRH) and chicken-II type (cGnRH-II) GnRH fibers after olfactory tract section (OTX) in the female goldfish. Following OTX, which blocks the axonal transport of GnRH from the TN to other brain regions, the density of sGnRH-ir fibers in various brain regions decreased from 7 days to 28 days, with the exception of rather restricted areas surrounding cell bodies in the ventral telencephalon, preoptic area, and hypothalamus. The density of cGnRH-II-ir fibers decreased only in the telencephalon from 7 days to 14 days. In spite of the decrease of GnRH-ir fibers in several brain areas, neither type of GnRH fibers showed marked changes in the pituitary gland during the experiment. These results indicate that the TN GnRH system project fibers to wide brain areas and that most of sGnRH and some of the cGnRH-II-ir fibers in the brain of goldfish are TN origin. Furthermore, the GnRH system that project fibers to the pituitary does not primarily originate from the TN-GnRH system.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide which stimulates the release of gonadotropins (GTH) from the pituitary gland. To date, twelve types of GnRH molecules have been isolated, and their primary structures have been determined (See reviews by Okuzawa and Kobayashi, 1999; Parhar, 1999). Recent studies have shown that in teleosts, more than one type of GnRH molecule exists even within the same species, and that GnRH neurons are found not only in the hypothalamic region but also in the extrahypothalamic regions of the brain (Amano *et al*., 1997; Okuzawa and Kobayashi, 1999; Parhar, 1999; Yamamoto *et al*., 1995). These results led us to consider whether all of the brain GnRHs function in the regulation of GTH release, and if not, which population and which type of GnRH regulates GTH release in teleosts.

In the goldfish, *Carassius auratus*, two types of GnRH, salmon (sGnRH) and chicken-II (cGnRH-II) have been identified in the brain and pituitary gland at peptide and mRNA levels by chromatography combined with radioimmunoassays (RIA) and molecular cloning, respectively (Yu *et al*., 1988; Kobayashi *et al*., 1992; Lin and Peter, 1996, 1997; Suetake *et al*., 2000). Our previous immunohistochemical study in the goldfish brain revealed that sGnRH-immunoreactive (ir) cell bodies are localized in the terminal nerve (TN) ganglion (TN GnRH system), the ventral telencephalon, the preoptic area (POA), and the hypothalamus (POA GnRH system) (Kim *et al*., 1995a). cGnRH-II-ir cell bodies occur in the same areas as those of sGnRH and also in the midbrain tegmentum (midbrain GnRH system). sGnRH and cGnRH-II fibers are widely distributed in the brain and the pituitary. The wide distribution of GnRH-ir fibers in the brain suggested that, in the goldfish, sGnRH and cGnRH-II regulate not only gonadotropin release from the pituitary gland, but also function as neuromodulators in the brain (Oka, 1997). In fact, changes in brain GnRH content does not always reflect levels of gonadal maturation in goldfish (Yu *et al*., 1987). Since the olfactory bulbs of the goldfish are of the pedunculated type with elongated olfactory tracts and the TN cell bodies are localized in the rostral part of the olfactory bulb, the axonal transport of GnRH from the TN to

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other brain regions can be easily blocked by sectioning the olfactory tracts. Our studies using RIA showed that olfactory tract section (OTX) causes remarkable decreases in brain sGnRH content without inhibiting gonadal maturation (Kim *et al.*, 1995b,c; Kobayashi *et al.*, 1992,1994,1997), suggesting that GTH secretion in the goldfish is primarily regulated by GnRH systems other than the TN-GnRH system. However, it is difficult to confirm by RIA whether the origin of the decreased sGnRH is solely the TN, or alternatively, whether the TN influenced GnRH production in other GnRH neurons.

In the present study, we examined the chronological changes in the distribution of sGnRH- and cGnRH-II-immunoreactive fibers after OTX in order to elucidate the anatomical organization of the TN GnRH system in the goldfish.

MATERIALS AND METHODS

Experiment 1

Sexually regressed female goldfish weighing 40 to 70 g (gonadosomatic index 0.5–1.5) were obtained from a commercial source in August and kept in stock tanks at 20°C.

Fish were olfactory tract-sectioned (N=35) or sham-operated (N=35) as described in the previous reports (Kim *et al.*, 1995b; Kobayashi *et al.*, 1992, 1994). For the olfactory tract section (OTX), a four-sided flap was cut in the skull cap using a disc saw. The first cut was made aligned with the center of the iris. The second cut, parallel to the first, was made just behind the nares. The third and fourth cuts, parallel to the longitudinal axis of the fish, was made to connect the first and second cuts. After removal of a square flap detached from the skull cap, fat and cranial fluid were gently removed. The olfactory tracts were bilaterally sectioned at two places with Wecker's scissors, and the resultant sections were removed to prevent possible regeneration (OTX fish). The cavity resulting from the operation was filled with gelatin sponge (Spongel, Yamauchi Pharmaceutical Co., Tokyo, Japan). Sham operations were performed in the same manner without cutting the olfactory tracts (sham-operated fish).

After the operation, fish were kept at 20°C and fed commercial trout pellets once a day. Fish were sampled at 3, 7, 14, 21, and 28 days after OTX for immunohistochemical analysis. At each sampling time, two or three fish were sampled. Intact fish were also sampled as initial controls.

Experiment 2

Sexually immature female goldfish weighing 8.0 to 22 g (gonadosomatic index 1.5–4.2) were obtained from a commercial source in September and kept at 20°C. Fish were olfactory tract-sectioned (N=20) or sham-operated (N=20). After the operation, fish were kept at 20°C and fed commercial trout pellets once a day. Four fish were sampled before and 14 and 28 days after the operation to trace changes in the density of GnRH-ir fibers. In this experiment, changes in the density of GnRH fibers were quantified after the operation. Representative parasagittal sections containing the whole brain regions except the olfactory bulb and the tract were selected from each of the sGnRH and cGnRH-II staining. The density of GnRH-ir fibers were calculated as total length of fiber (mm) / brain areas (mm^2) in representative sections (48 μm thickness) using KIMA image analyzing system (Ecosolution Co., Seoul, Korea). The difference of the means among groups was analyzed by ANOVA and Duncan's test with raw or log-transformed data.

Experiment 3

Sexually maturing or mature female goldfish weighing 20 to 30 g (gonadosomatic index 5.0–8.5) were obtained from a commercial source in May and kept at 18°C, which is favorable for maintianing the maturity of the fish. Fish were olfactory tract-sectioned (N=23) or sham-operated (N=23). After the operation, fish were kept at 18°C and fed commercial trout pellets once a day. Fish were sampled for immunohistochemical analysis 28 days after the operation.

Immunohistochemistry

Fish were deeply anesthetized by immersion in 0.02% tricane methanesulfonate. They were then perfused through the heart with 0.75% NaCl, followed by a fixative that had been freshly prepared by mixing 40 ml of 10% paraformaldehyde, 50 ml of 0.2 M phosphate buffer (pH 7.4), 7 ml of saturated picric acid, and 3 ml of distilled water. The head was post-fixed in the same fixative for 24 hr at 4°C. Next, the brain and the pituitary were dissected out of the head and fixed again for 24 hr at 4°C. Tissues were embedded in 15% gelatin dissolved in a 0.1 M phosphate buffer (pH 7.4) and were quickly frozen by immersing them in powdered dry ice. Forty-eight micrometer sagittal and frontal sections were cut on a cryostat, and the subsequent procedures were done on floating sections. Alternate sections were used for sGnRH and cGnRH-II staining. We used antisera raised against sGnRH (lot No. 2) and cGnRH-II (lot R-A) (Okuzawa *et al.*, 1990; Hasegawa *et al.*, 1986). These antisera were diluted 10,000- and 20,000-fold respectively, with 0.1 M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100 (PBST). For immunohistochemical reactions, we used the streptavidin method with a commercial kit (Histofine; Nichirei Co., Tokyo, Japan). Immunoreactivity of each GnRH neuron was judged to be positive when the labeling was denser than that of the background. Meanwhile, there was no difference of immunoreactivity in each individual. Representative parasagittal sections that are considered to be the best areas to show the localization of GnRH neurons (Kim *et al.*, 1995a), were selected for the camera lucida drawings (Figs. 1, 2). Localization of cell bodies shown in Figs. 1, 2 were determined from the observation of several sections. The specificity of anti-sGnRH and cGnRH-II have been demonstrated by RIA (Kobayashi *et al.*, 1992; Okuzawa *et al.*, 1990) and by immunocytochemistry (Amano *et al.*, 1991; Kim *et al.*, 1995a). To test the specificity of the immunoreactions, the diluted sGnRH and cGnRH-II antisera were each incubated overnight at 4°C with an excess amount of synthetic sGnRH and cGnRH-II (1 μg GnRH in 1 ml of diluted antiserum) respectively, or conversely, sGnRH and cGnRH-II antisera with synthetic cGnRH-II and sGnRH, respectively. The subsequent histological procedures were performed in the same manner as in the experimental sections. No immunoreactive fibers were observed when the antisera were preincubated with respective GnRHs. On the contrary, immunoreactive fibers were observed when the antisera were preincubated with different GnRHs. When the sGnRH and cGnRH-II antisera were incubated with seabream GnRH (1 μg GnRH in 1ml of diluted antiserum) (Powell *et al.*, 1994), they were not absorbed, and immunoreactive fibers were observed with both the sGnRH and cGnRH-II antisera. Synthetic seabream GnRH was obtained from Fujiya Bioscience Co. (Hadano, Kanagawa, Japan). Moreover, we tested the specificity of the antisera by immunoreaction on nitrocellulose membranes. Both synthetic sGnRH and cGnRH-II of different amount, 1 ng, 100 ng, 1 μg, 10 μg, 20 μg, and 30 μg in 100 μl of 0.1 M PBST were spotted on nitrocellulose membrane. The membrane was treated in the same fixative as for the brain tissues. The subsequent immunoreaction procedures were done in the same manner as in the experimental sections. When the antisera were incubated with heterologous antigens of 10 μg or more, only faint staining reactions were observed, while strong reactions were observed between the antisera and the homologous antigens at these doses.

We used the terminology of brain structures or subdivisions according to Northcutt and Davis (1983) for the telencephalon, Braford and Northcutt (1983) for the diencephalon and pretectum, and Nieuwenhuys and Pouwels (1983) for the brainstem.

RESULTS

Experiment 1

The distribution of sGnRH-ir fibers 3 days after OTX showed no changes in various brain regions and in the pituitary gland in comparison with those of the initial controls (Fig. 1). At Day 7, sGnRH-ir fibers in various brain areas started to diminish. In particular, the decrease in the density of ir fibers in the telencephalon, the optic tectum, and the thalamus was more remarkable than in the hypothalamus, cerebellum, and medulla oblongata. At Day 14, further decrease of sGnRH-ir fibers was observed in the brain areas except in the ventral telencephalon, the preoptic area, and the hypothalamus where ir cell bodies were located. Thereafter, there were no further changes in the distribution of ir fibers in all brain areas.

cGnRH-II-ir fibers decreased mainly in the telencephalon from Day 7 to Day 14 in comparison with that of the initial control (Fig. 2). Thereafter, there were no further changes in the distribution of cGnRH-II-ir fibers in all brain areas.

On the other hand, both types of GnRH fibers showed no marked changes in the pituitary gland during the experiment (Figs. 1, 2). The ovaries of the OTX and sham-operated fish remained regressed.

Experiment 2

Distribution of GnRH fibers in initial control fish, OTX fish, and sham-operated fish was similar to that in equivalent fish in Experiment 1 (data not shown). When the density of GnRH fibers was calculated by summing the length of all GnRH-ir fibers observed in the representative parasagittal brain section (olfactory bulb and tract are not included) and normalized by the brain area, the density of sGnRH fibers showed a significant decrease after OTX compared to initial control fish (day 14, $p<0.01$; day 28, $p<0.01$) and sham-operated fish of the same sampling days (day 14, $p<0.01$; day 28, $p<0.01$) (Fig. 3). There were no significant differences in the density of sGnRH-ir fibers between initial control fish and sham-operated fish.

The density of cGnRH-II-ir fibers in the brain showed a significant decrease after OTX compared to initial control fish

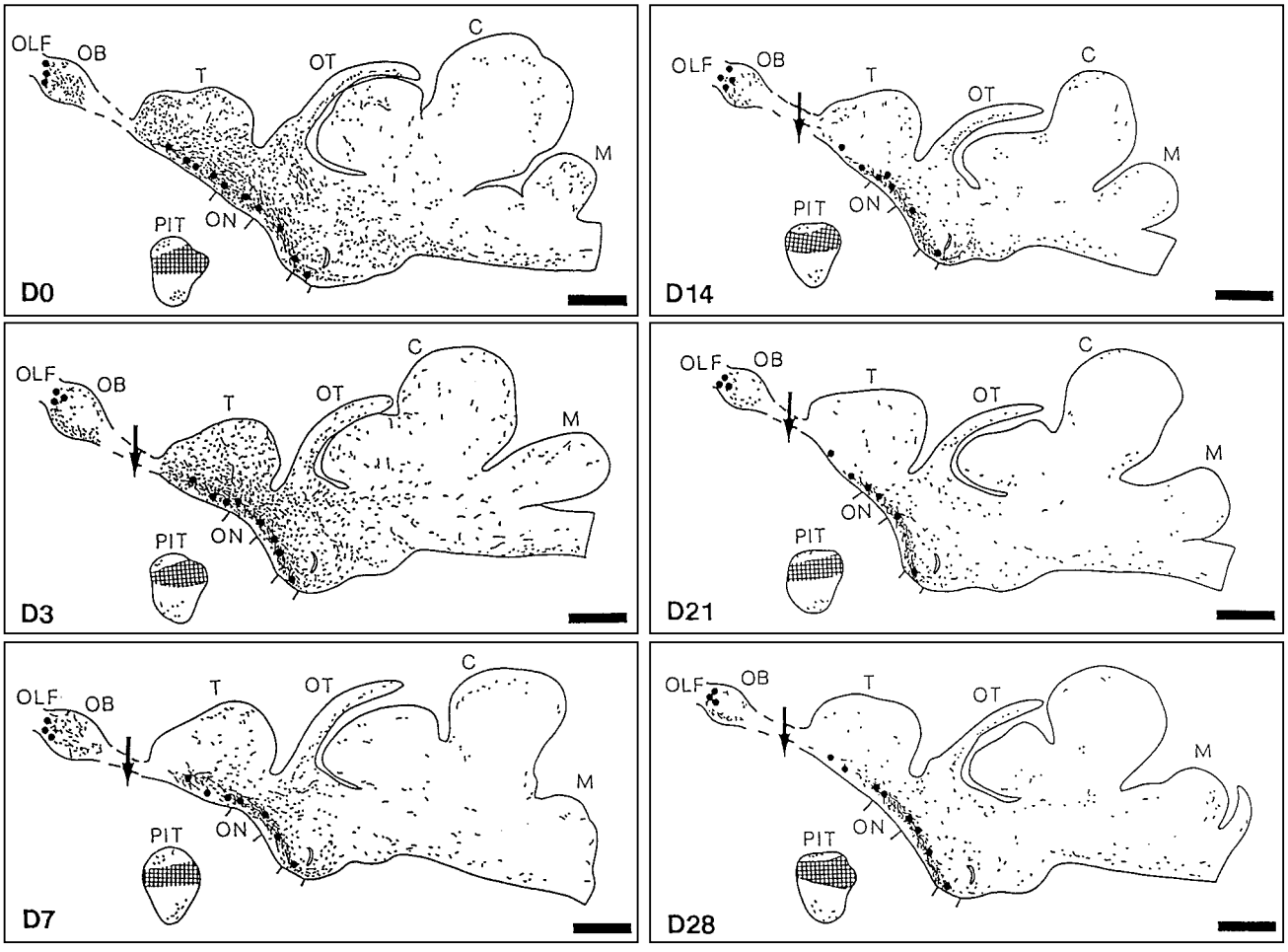

Fig. 1. Semidiagramatic illustrations of salmon gonadotropin-releasing hormone-immunoreactive (sGnRH-ir) cell bodies (solid circles) and fibers (lines) in a parasagittal section of the immature goldfish brain (Exp. 1). Shaded areas (dense) and lines (scarce) in the pituitary indicate sGnRH-ir fibers. D0, initial; D3, 3 days after olfactory tract section (OTX); D7, 7 days after OTX; D14, 14 days after OTX; D21, 21 days after OTX; D28, 28 days after OTX. Arrows indicate location of OTX. C, cerebellum; M, medulla oblongata; OB, olfactory bulb; OLF, olfactory nerve; ON, optic nerve; OT, optic tectum; PIT, pituitary; T, telencephalon. Scale bar, 1 mm.

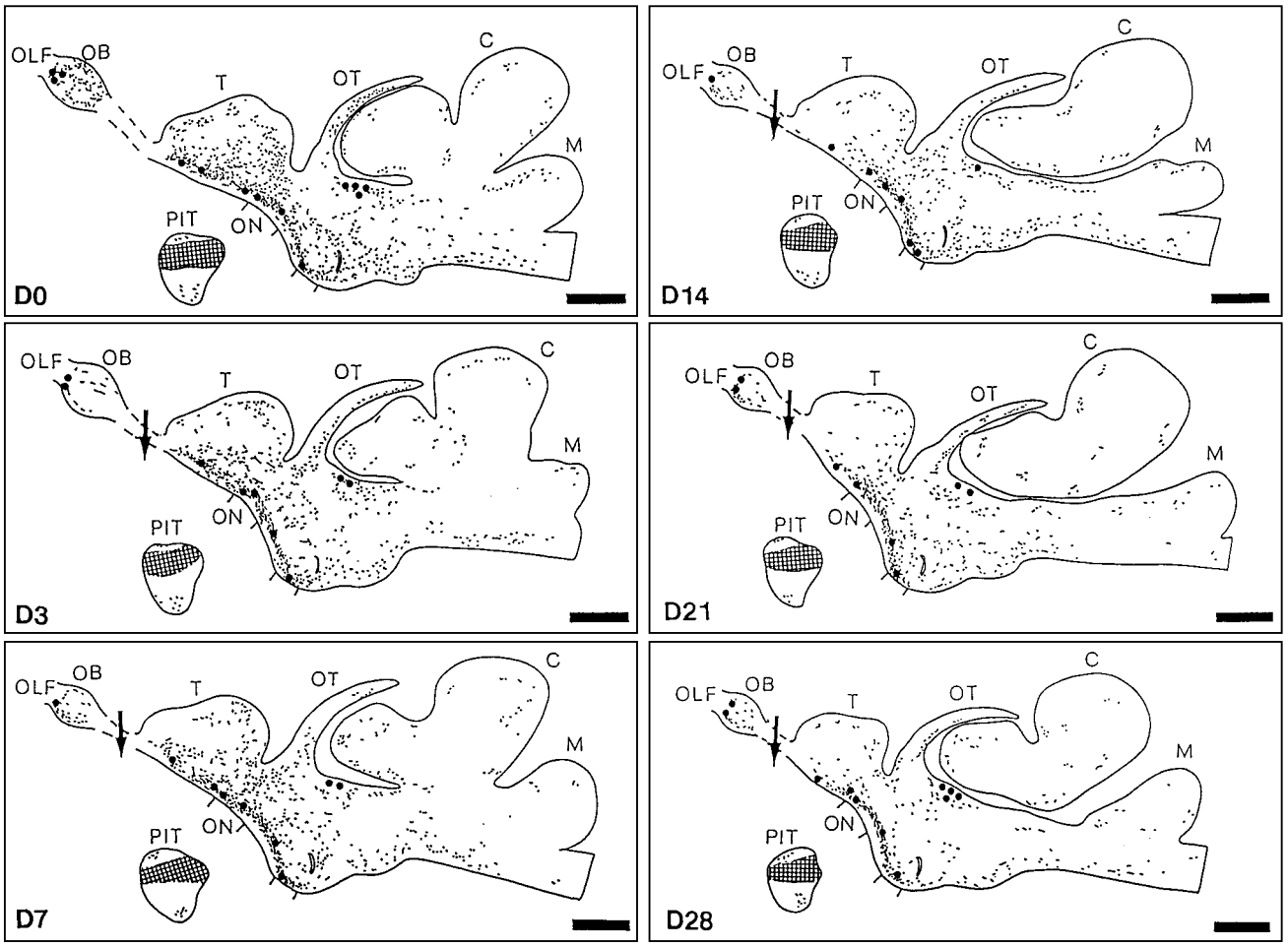

Fig. 2. Semidiagramatic illustrations of chicken gonadotropin-releasing hormone-II-immunoreactive (cGnRH-II-ir) cell bodies (solid circles) and fibers (lines) in a parasagittal section of the immature goldfish brain (Exp. 1). Shaded areas (dense) and lines (scarce) in the pituitary indicate cGnRH-II-ir fibers. D0, initial; D3, 3 days after olfactory tract section (OTX); D7, 7 days after OTX; D14, 14 days after OTX; D21, 21 days after OTX; D28, 28 days after OTX. Arrows indicate location of OTX. Abbreviations for brain areas are as in Fig. 1. Scale bar, 1 mm.

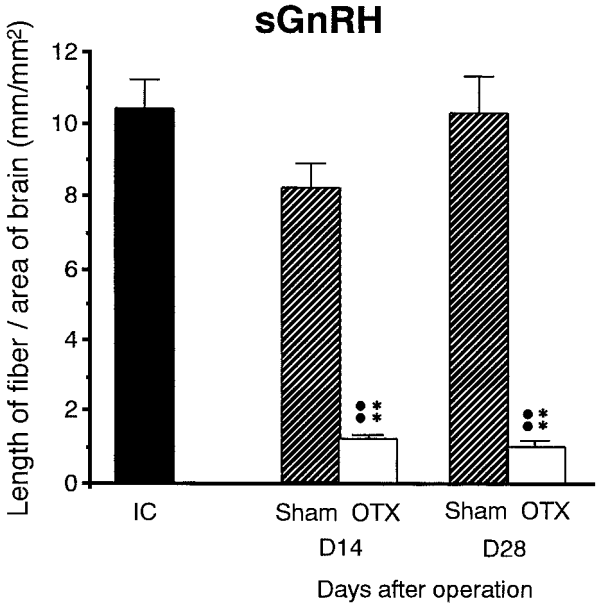

Fig. 3. Changes in the density of sGnRH-ir fibers in the brain after olfactory tract section in goldfish. The density was calculated by summing the length of sGnRH-ir fibers observed in the representative parasagittal brain section (the olfactory bulb and tract are not included) and normalized by brain area. IC, Initial control; Sham, sham-operated fish; OTX, olfactory tract-sectioned fish. D14, 14 days after operation; D28, 28 days after operation. Each column represents the mean and SEM (N=4). ●●, Significant difference compared to initial control fish ($p<0.01$). **, Significant difference compared to sham-operated fish ($p<0.01$).

(day 14, $p<0.001$; day 28, $p<0.001$) and sham-operated fish of the same sampling days (day 14, $p<0.05$; day 28, $p<0.001$) (Fig.4). Sham-operated fish showed a decrease in cGnRH-II-ir fibers at day 14 after operation compared to that in initial control fish ($p<0.01$).

Experiment 3

Distribution of GnRH fibers in OTX fish, and sham-operated fish was similar to that in equivalent fish in Experiments 1 and 2. Most of sGnRH-ir fibers and some cGnRH-II-ir fibers disappeared in various brain areas with the exception of those in the pituitary gland, the ventral telencephalon, the preoptic area, and the hypothalamus where immunoreactive cell bod-

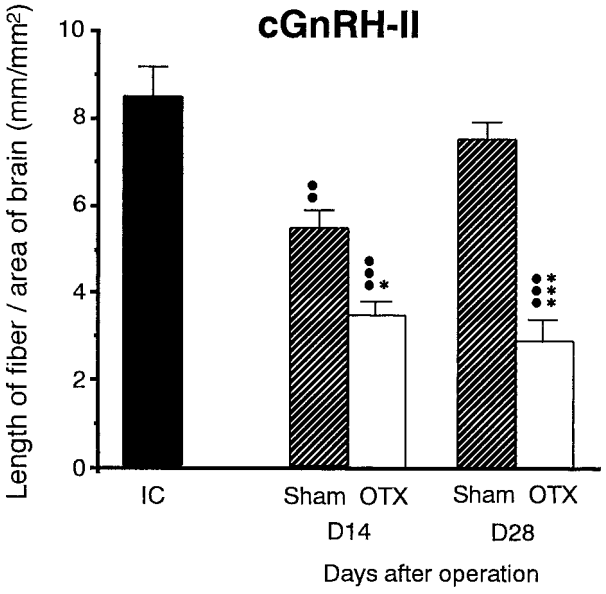

Fig. 4. Changes in the density of cGnRH-II-ir fibers in the brain after olfactory tract section in goldfish. The density was calculated by summing the length of cGnRH-II-ir fibers observed in the representative parasagittal brain section (the olfactory bulb and tract are not included) and normalized by brain area. IC, Initial control; Sham, sham-operated fish; OTX, olfactory tract-sectioned fish. D14, 14 days after operation; D28, 28 days after operation. Each column represents the mean and SEM (N=4). ●, Significant difference compared to initial control fish. *, Significant difference compared to sham-operated fish. Levels of significance: one symbol, $p<0.05$; two symbols, $p<0.01$; three symbols, $p<0.001$.

ies were located (Figs. 5–16). Despite the disappearance of most of the sGnRH-ir fibers and some of the cGnRH-II-ir fibers, the fish maintained mature ovaries exhibiting vitellogenic oocytes and showed no difference from those of sham-operated fish.

DISCUSSION

The localization of two types of GnRH cell bodies and fibers in initial controls and sham-operated goldfish showed a pattern similar to that in goldfish reported in our previous study (Kim *et al*., 1995a). Both sGnRH- and cGnRH-II-ir cell bodies were localized in the TN (TN GnRH system), the ventral telencephalon, the preoptic area, and the anterior hypothalamus (POA GnRH system). In addition, cGnRH-II-ir but sGnRH-negative cell bodies were also observed in the midbrain tegmentum (midbrain GnRH system). Both sGnRH- and cGnRH-II-ir fibers were detected in diverse brain areas and in the pituitary. In OTX fish, there was a marked decrease in the GnRH-ir fibers in the brain. The density of sGnRH- and cGnRH-II-ir fibers started to decrease 7 days after OTX, and then Day 14, the density of sGnRH-ir fibers continued to decrease in various brain regions except for the rather restricted areas surrounding cell bodies in the ventral telencephalon, the preoptic area, and the hypothalamus. At 21 and 28 days, the density of fibers immunoreactive for both molecular types showed no further decrease. The results of present immunohistochemical study support those of our RIA study which showed that brain sGnRH contents starts to decrease at 7 days after OTX and reach stable levels at 14 days (Kim *et al*., 1995b) as well as immunohistochemical studies which showed the disappearance of TN GnRH-derived fibers in the dwarf gourami, *Colisa lalia* two weeks after TN lesion (Yamamoto *et al*., 1995;1997).

Chicken GnRH-II-ir fibers decreased in the telencephalon after OTX. However, in the RIA study (Kim *et al*., 1995b), no decrease in the content of cGnRH-II was observed in all brain areas after OTX. This difference between RIA and immunocytochemistry studies is most likely due to the difference in sensitivities between the two different methods. Another possibility is that the GnRH content was measured by RIA using the whole telencephalon, whereas the number of GnRH-ir fibers was counted only in the restricted areas including mainly parasagittal sections.

The present immunohistochemical study indicates that the TN-GnRH neuronal system projects fibers to wide brain areas and that most of sGnRH and some of cGnRH-II in the brain originate from the TN GnRH system. Therefore, the decrease in the brain GnRH content after OTX as shown by RIA studies (Kim *et al*., 1995b,c; Kobayashi *et al*., 1992, 1994) is mostly attributable to the blockade of axonal transport of GnRH from the TN GnRH system rather than to the decrease in GnRH production in other GnRH systems. The projection of GnRH fibers to wide brain areas has also been reported in the dwarf gourami by TN lesion study (Yamamoto *et al*., 1995, 1997).

Despite the decrease in GnRH-ir fibers after OTX in the various brain regions, GnRH-ir fibers and cell bodies of both molecular types in the ventral telencephalon, the preoptic area, the hypothalamus, and the midbrain tegmentum remained almost unchanged. Therefore, it is considered that remaining GnRH-ir fibers originate from the GnRH-ir cell bodies in these brain regions. Brain GnRH in OTX fish measured by RIA is considered to be GnRH produced in these remaining GnRH neurons (Kim *et al*., 1995a,b,c; Kobayashi *et al*., 1992, 1994). GnRH-ir fibers of both types in the pituitary showed no marked changes in immunohistochemical sections, although we did not conduct quantitative analysis because of the difficulty in tracing individual fibers in the pituitary. Meanwhile, RIA studies showed no marked changes in the pituitary GnRH contents after OTX, suggesting that GnRH fibers in the pituitary do not primarily originate from the TN system (Kim *et al*., 1995b; Kobayashi *et al*., 1992, 1994).

Our previous studies showed that OTX fish underwent normal gonadal development as did sham-operated fish (Kim *et al*., 1995c; Kobayashi *et al*., 1992, 1994). Taken together, the results of the present study strongly indicates that GnRH of TN origin is not a prerequisite for gonadal development in the goldfish. Recent studies in teleost GnRH neuronal systems showed that GnRH fibers in the pituitary gland mainly originate from the POA GnRH system and suggested that these GnRH neurons play hypophysiotropic function (Kah *et*

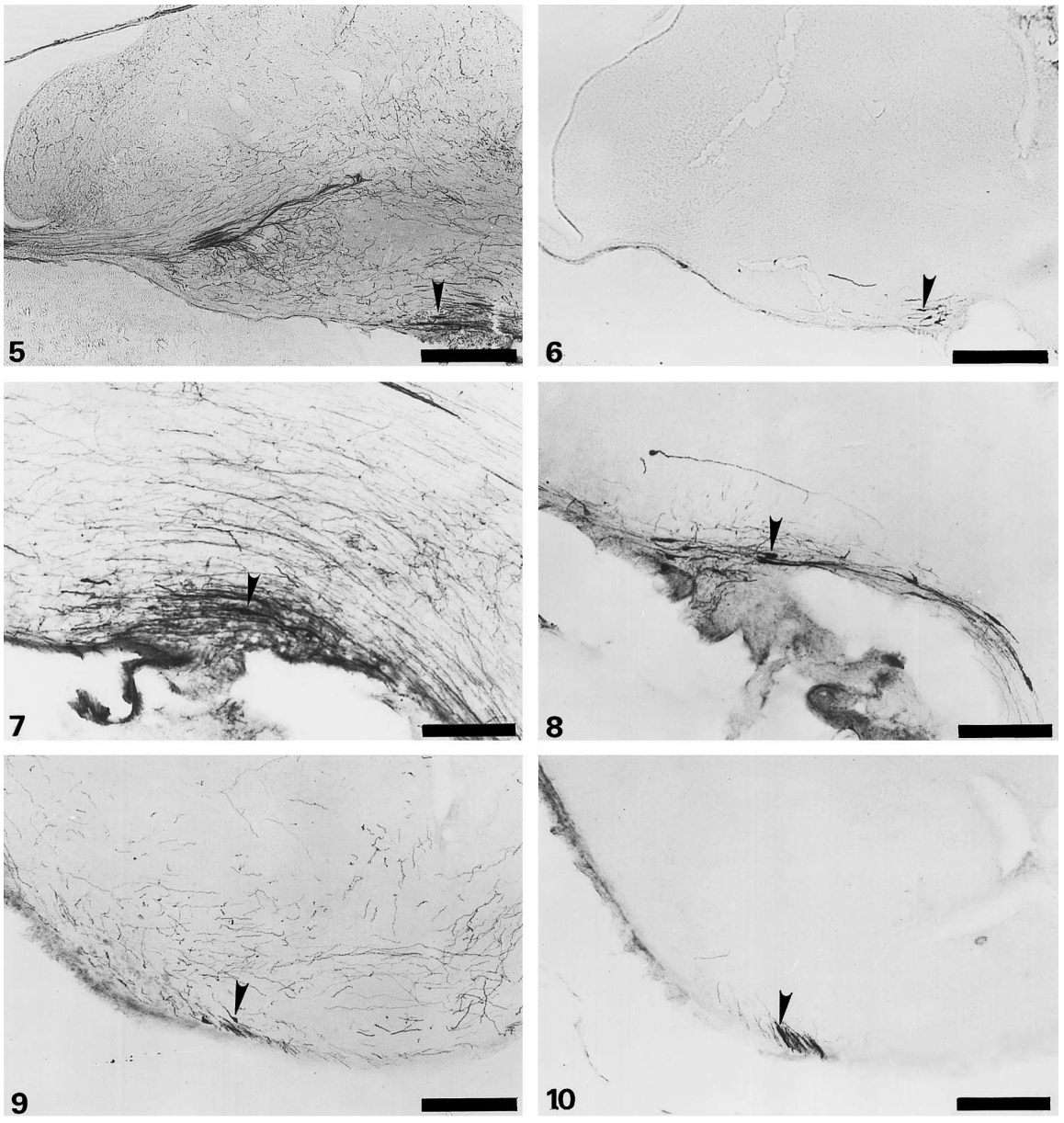

Fig. 5. Sagittal section through the telencephalon which shows sGnRH-ir cell bodies and fibers in sham-operated goldfish 28 days after olfactory tract section. Arrowhead indicates a sGnRH-ir cell body of POA GnRH system. Scale bar, 400 μm.
Fig. 6. Sagittal section through the telencephalon similar to Fig. 5 in olfactory tract-sectioned goldfish 28 days after olfactory tract section. Arrowhead indicates a sGnRH-ir cell body of POA GnRH system. Scale bar, 400 μm.
Fig. 7. Sagittal section through the preoptic area which shows sGnRH-ir cell bodies and fibers in sham-operated goldfish 28 days after olfactory tract section. Arrowhead indicates a sGnRH-ir cell body of POA GnRH system. Scale bar, 200 μm.
Fig. 8. Sagittal section through the preoptic area similar to Fig. 7 in olfactory tract-sectioned goldfish 28 days after olfactory tract section. Arrowhead indicates a sGnRH-ir cell body of POA GnRH system. Scale bar, 200 μm.
Fig. 9. Sagittal section through the hypothalamus which shows sGnRH-ir cell bodies and fibers in sham-operated goldfish 28 days after olfactory tract section. Arrowhead indicates a sGnRH-ir cell body of POA GnRH system. Scale bar, 200 μm.
Fig. 10. Sagittal section through the hypothalamus similar to Fig. 9 in olfactory tract-sectioned goldfish 28 days after olfactory tract section. Arrowhead indicates a sGnRH-ir cell body of POA GnRH system. Scale bar, 200 μm.

al., 1986; Yamamoto *et al*., 1995, 1998; Okuzawa and Kobayashi, 1999; Parhar, 1999). sGnRH and cGnRH-II neurons comprising the POA GnRH system is presumed to be the major GnRH population regulating gonadotropin release in the pituitary of the goldfish.

Physiological functions of the TN-GnRH and the midbrain GnRH systems in the goldfish remain unknown. Neuromodulatory function is proposed as one of the most probable physiological functions of TN GnRH in teleosts (Oka, 1997), and the involvement of midbrain GnRH in sexual behavior is suggested in the musk shrew, *Suncus murinus* (Dellovade *et al*., 1995) and in the regulation of the caudal neurosecretory

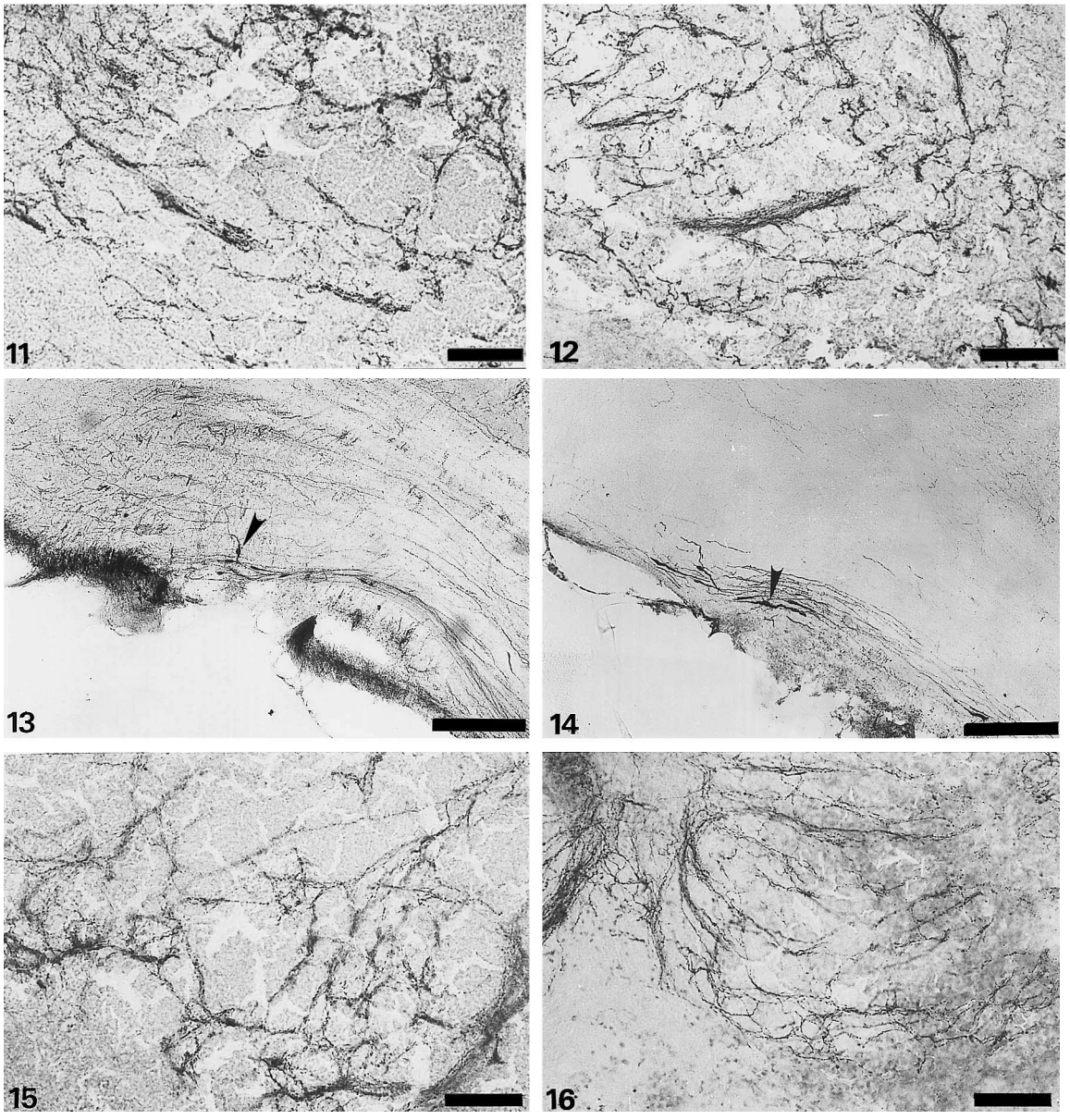

Fig. 11. Sagittal section through the pituitary (proximal pars distalis) in sham-operated goldfish showing sGnRH-ir fibers 28 days after olfactory tract section. Scale bar, 100 μm.
Fig. 12. Sagittal section through the pituitary similar to Fig.11 in olfactory tract-sectioned goldfish showing sGnRH-ir fibers 28 days after olfactory tract section. Scale bar, 100 μm.
Fig. 13. Sagittal section through the preoptic area which shows cGnRH-II cell bodies and fibers in sham-operated goldfish 28 days after olfactory tract section. Arrowhead indicates a cGnRH-II-ir cell body of POA GnRH system. Scale bar: 200 μm.
Fig. 14. Sagittal section through the preoptic area similar to Fig. 13 in olfactory tract-sectioned goldfish 28 days after olfactory tract section. Arrowhead indicates a cGnRH-II-ir cell body of POA GnRH system. Scale bar, 200 μm.
Fig. 15. Sagittal section through the pituitary (proximal pars distalis) in sham-operated goldfish showing cGnRH-II-ir fibers 28 days after olfactory tract section. Scale bar, 100 μm.
Fig. 16. Sagittal section through the pituitary similar to Fig. 15 in olfactory tract-sectioned goldfish showing cGnRH-II-ir fibers 28 days after olfactory tract section. Scale bar, 100 μm.

neurons in poecilid fishes (Miller and Kriebel, 1986). Recently, a stimulatory effect of GnRH on prostaglandin-induced female spawning behavior was shown in the goldfish by GnRH administration (Volkoff and Peter, 1999). GnRH from the TN GnRH and the midbrain GnRH neuronal systems may be involved in the modulation of spawning behavior in the goldfish (Stacey and Kyle, 1983).

In summary, the present immunohistochemical study together with our previous studies (Kim *et al*., 1995a, b, c; Kobayashi *et al*., 1992, 1994) indicate that in the goldfish the TN GnRH system projects fibers to wide brain areas and that most of sGnRH and a part of the cGnRH-II in the brain are TN

origin. However, the TN GnRH system is not a prerequisite for gonadal maturation in the goldfish, and the GnRH system that projects fibers to the pituitary does not primarily originate from the TN GnRH system.

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