Central Connection of the Optic, Oculomotor, Trochlear and Abducens Nerves in Medaka, Oryzias latipes

Authors: Deguchi, Tomonori, Suwa, Hiroshi, Yoshimoto, Masami, Kondoh, Hisato, and Yamamoto, Naoyuki

Source: Zoological Science, 22(3) : 321-332

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.22.321

ZOOLOGICAL SCIENCE **22**: 321–332 (2005)

Central Connection of the Optic, Oculomotor, Trochlear and Abducens Nerves in Medaka, *Oryzias latipes*

Tomonori Deguchi[1], Hiroshi Suwa[2], Masami Yoshimoto[3], Hisato Kondoh[1,2]* and Naoyuki Yamamoto[3]

[1]*Graduate School of Frontier Biosciences, Osaka University, Yamadaoka 1-3, Suita, Osaka 565-0871, Japan*
[2]*Japan Science and Technology Agency, ERATO Kondoh Differentiation Signaling Project/SORST Kondoh Research Team, Kinki-chihou Hatsumei Center Building, Yoshida-Kawaracho 14, Sakyo-ku, Kyoto 606-8305, Japan*
[3]*Department of Anatomy and Laboratory for Comparative Neuromorphology, Nippon Medical School, Sendagi 1-1-5, Bunkyo-ku, Tokyo 113-8602, Japan*

ABSTRACT—Medaka (*Oryzias latipes*) is one of the few vertebrate experimental animals in which inbred lines have been established. It is also a species that has advanced in genetic studies in a manner comparable to zebrafish. This fish is therefore a good model for studying functional organization of the nervous system, but anatomical analysis of its nervous system has been limited to embryonic stages. In the present study, we investigated anatomy of cranial nerves in adult fish focusing on the visual function, using an inbred strain of medaka. Cranial nerves of medaka were labeled using biocytin, revealing a central distribution of retinofugal terminals, retinopetal neurons, and oculomotor, trochlear and abducens motor neurons. The optic nerve of the adult medaka was of a complete decussation type. Retinofugal terminals were located in 8 brain nuclei, the suprachiasmatic nucleus, nucleus pretectalis superficialis, nucleus dorsolateralis thalami, area pretectalis pars dorsalis (APd), area pretectalis pars ventralis (APv), nucleus of the posterior commissure (NPC), accessory optic nucleus, and the tectum opticum. Retinopetal neurons were identified in 6 brain nuclei, the ganglion of the terminal nerve, preoptic retinopetal nucleus, nucleus dorsolateralis thalami, APd, APv, and NPC. The oculomotor neurons were mostly labeled ipsilaterally and were located dorsomedially, abutting the fasciculus longitudinalis medialis in the mesencephalon. The trochlear nucleus was located contralaterally and dorsolaterally adjacent to the fasciculus longitudinalis medialis in the mesencephalon. The abducens nucleus was located ipsilaterally in a ventrolateral part of the rhombencephalic reticular formation. These results, generally similar to those in other teleosts, provide the basis for future behavioral and genetic studies in medaka.

Key words: retinal projection, retinopetal neuron, extraocular motor neuron, brain, teleost fish, medaka

INTRODUCTION

Medaka (*Oryzias latipes*) has several advantages as an experimental animal model for genetic and developmental studies (Ishikawa, 2000; Wittbrodt *et al*., 2002). In recent years, large-scale mutagenesis screening of mutations affecting neurogenesis or organogenesis have been successfully performed using medaka (Furutani-Seiki *et al*., 2004). Many mutants displaying defects in histogenesis of the brain and cranial nerves have been identified (Ishikawa 2000; Kitagaka *et al*., 2004; Yoda *et al*., 2004; Yasuoka *et al*., 2004). Determination of the entire genome sequence is currently underway (http://dolphin.lab.nig.ac.jp/medaka/).

The brain of teleost fish shares the same basic organization with other vertebrates, consisting of the telencephalon, diencephalon, mesencephalon and rhombencephalon in the anterior to posterior order (Ito and Yoshimoto, 1991). Small fish have markedly fewer neuron numbers compared to other vertebrate species but exhibit common basic behaviors (e.g. feeding, escape and reproduction) as well as more sophisticated social behaviors exemplified by schooling (Yamamoto, 1975; Egami *et al*., 1990; Iwamatsu, 1997). This indicates their potential use as an experimental model

* Corresponding author. Phone: +81-6-6879-7963;
Fax : +81-6-6877-1738;
E-mail: j61056@hpc.cmc.osaka-u.ac.jp

for the analysis of various levels of behavioral responses. To take advantage of the biological characteristics of medaka in behavioral studies and to establish links between genes and behavioral functions, anatomical analysis of the medaka brain is an essential first step.

Neuroanatomical studies of teleost fish have been performed using various species, revealing a diversity of morphological features (Davis and Northcutt, 1983; Ito and Yoshimoto, 1991; Uematsu *et al*., 2002) partly ascribed to the variety of sensory inputs in the life of individual fish species (Ito and Yoshimoto, 1991; Ito, 2002). A gross brain atlas of the medaka has been published by Anken and Bourrat (1998) and Ishikawa *et al*. (1999). Previous analyses of the medaka brain, however, have stopped short of identifying neural circuits.

To provide fundamentals to the study of the medaka nervous system, we initiated an investigation into the cranial nerve connections and compared the results to other fish species. Medaka belongs to smegmamorphs, which is considered to be closely related to percomorphs, such as the archer fish and filefish, and holocentrids; all belong to neoteleostei (a newly-evolved branch of euteleostei) (Nelson, 1994). Medaka is very distantly related to another group of teleost fish frequently employed for experimentation, such as zebrafish, carp and goldfish that belongs to cyprinids (an old group of euteleostei). There are several teleost groups with well-developed eyes (e.g. holocentrids, some percomorphs), which presumably reflect the large dependence on vision in their ecological niche. Medaka also has large eyes, as implicated by its name (me=eye, daka=high in Japanese). The origin of the name also relates to the known feeding habits of medaka that includes the dependence on the visual sense to identify food while swimming close to the water surface. We therefore began our study with an analysis of the cranial nerves involved in visual function. In the present study, the central distribution of retinofugal terminals, retinopetal neurons, and oculomotor, trochlear and abducens motor neurons was analyzed using biocytin and tract-tracing experiments.

ABBREVIATIONS

AON, accessory optic nucleus; APd, area pretectalis pars dorsalis; APv, area pretectalis pars ventralis; CC, corpus cerebelli; CR, crista cerebellaris; Dl, area dorsalis telencephali pars lateralis; DLT, nucleus dorsolateralis thalami; Dm, area dorsalis telencephali pars medialis; Dp, area dorsalis telencephali pars posterior; flm, fasciculus longitudinalis medialis; GR, corpus glomerulosum pars rotunda; H, hypophysis; HB, habenula; IQ, inferior oblique muscle; IR, inferior rectus muscle; LI, lobus inferior; LR, lateral rectus muscle; MR, medial rectus muscle; nIII, oculomotor nerve; NIII, oculomotor nucleus; nIV, trochlear nerve; NIV, trochlear nucleus; nVI, abducens nerve; NVI, abducens nucleus; NC, nucleus corticalis; NDLI, nucleus diffusus lobi inferioris; NE, nucleus entopeduncularis; NPC, nucleus of the posterior commissure; NRL, nucleus recessi lateralis; PC, posterior commissure; PRN, preoptic retinopetal nucleus; PS, nucleus pretectalis superficialis; RF, reticular formation; Rho, rhombencephalon; SAC, stratum album centrale; SC, suprachiasmatic nucleus; SFGS, stratum fibrosum et griseum superficiale; SGC, stratum griseum centrale; SM, stratum marginale; SO, stratum opticum; SQ, superior oblique muscle; SPV, stratum periventriculare; SR, superior rectus muscle; TE, telencephalon; TL, torus longitudinalis; TNG, ganglion of the terminal nerve; TO, tectum opticum; tro, tractus opticus; trod, tractus opticus pars dorsomedialis; trov, tractus opticus pars ventrolateralis; TS, torus semicircularis; VC, valvula cerebelli; Vv, area ventralis telencephali pars ventralis.

MATERIALS AND METHODS

Specimens

The Cab-Kyoto inbred strain, derived from the Southern population of the Japanese medaka, *Oryzias latipes* (Furutani-Seiki *et al*., 2004), were used in this study. The fish were raised in 9 L tanks (at a population density of 30 fish/tank) at 26°C±1, pH 7.0±0.5 with a 14 h light/10 h dark cycle. Fish were fed twice daily with live brine shrimp (*Artemia salina*) larvae and commercial dry fish food (Hikari Crest Guppy, Kyorin). A total of 17 adult medaka fish (over 3 months old, 2–3 cm of body length) were used for biocytin injection and analysis. To determine the peripheral distribution of cranial nerves innervating the oculomotor muscles, >5 adult medaka were used for each nerve.

Peripheral distribution of cranial nerves innervating extraocular muscles

Fish were anaesthetized for 5 minutes in chilled 0.04% 3-aminobenzoic acid ethyl ester (MS222, Sigma) in fish cultivation water (Furutani-Seiki *et al*., 2004). Fish were held on a dissecting stage with insect pins and then perfused through the conus arteriosus with 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4), using a small glass pipette under a dissection microscope (MZ12.5, Leica). Whole bodies were post-fixed with a fresh solution of the 4% PFA in 0.1 M phosphate buffer for longer than 2 days.

The parietal, frontal, and occipital bones were removed under the dissection microscope prior to the overnight immersion of fish in dimethyl sulfoxide (DMSO)-methanol (DMSO: methanol=1:1). After rinsing in 4% PFA in 0.1 M phosphate buffer, the peripheral distribution of the nerves was observed under the dissection microscope.

General experimental scheme of tracer experiments

Fish were anaesthetized as described above for the cranial nerve distribution study. Under anesthesia, fish were held on a dissecting stage with insect pins. The body was covered with a wet paper (Kimwipe, Kimberly-Clark Corporation) to avoid drying. The procedure for the injection of biocytin into the cranial nerve was performed under a dissection microscope. After tracer injection (described below), fish were kept on the stage for 30 minutes, wrapped in a wet Kimwipe and occasionally sprinkled with water to maintain hydration. Fish were then returned to tanks and reared for 1 day.

Labeling of optic nerve

In order to expose the optic nerve, the left eyeball was turned ventrally after incising the skin at the orbital margin, superior oblique muscle, superior rectus muscle, and the optic nerve (the number of fish specimens, n=7). After wiping the orbit with a piece of dry Kimwipe, a small crystal of biocytin (ε-biotinoyl-L-lysine, Molecular Probe) was placed on the distal end of the optic nerve.

Labeling of oculomotor nerve

In order to expose the oculomotor nerve, the left eyeball was turned dorsonasally after incising the skin at the orbital margin, inferior oblique muscle, inferior rectus muscle and lateral rectus muscle (n=4). After wiping the orbit with a piece of dry Kimwipe, 1–2 μl of biocytin solution (3% biocytin in 0.05 M Tris-buffer, pH 7.2) was pressure-injected into the oculomotor nerve using a PLI-100 Pico-Injector (Harvard/Medical Systems). The oculomotor nerve was injected with tracer just before the nerve branched in the temporal area of the orbit, in order to trace innervation of the superior rectus, inferior oblique, inferior rectus and medial rectus muscles.

Labeling of trochlear nerve

In order to expose the trochlear nerve, the left eyeball was

turned slightly ventrally after incising the skin of the orbital margin (n=5). Pressure injection was used to inject the biocytin solution into the trochlear nerve running through the dorsal area of the orbit.

Labeling of abducens nerve

In order to expose the abducens nerve, the left eyeball was turned dorsonasally after incising the skin of the orbital margin, then cutting the inferior oblique, inferior rectus and lateral rectus muscles at their insertion to the eyeball (n=1). Biocytin solution was pressure-injected into the abducens nerve running through the temporal area of the orbit.

Fixation

Fish were anaesthetized and perfused as described above. The brain was excised and post-fixed in a mixed solution of 4% PFA and 0.5% glutaraldehyde in 0.1 M phosphate buffer for 1 day or longer at 4°C.

Sectioning and staining

Fixed brains were immersed in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C, then embedded in 15% (w/v) gelatin and 5% (w/v) sucrose in 0.1 M phosphate buffer at 37°C. After freezing at –20°C, brains were cut serially on a cryostat (50 μm-thick transverse sections), and sections were divided into wells in a 48-well plate and processed for visualization of the tracer using free-floating conditions.

Sections were washed in 0.1 M phosphate-buffered saline for 10 minutes, then immersed in 70% methanol containing 3% H_2O_2 for 10 minutes. After two rinses in 0.1 M phosphate- buffered saline, sections were incubated for 3 hours in a 1% avidine-biotin complex (ABC: Streptavidin Biotin Complex Peroxidase kit, Nacalai Tesque) solution containing 0.4% Triton-X-100 in 0.1 M phosphate-buffered saline. After washing 3 times in 50 mM Tris-HCl for 5 minutes, sections were incubated with diaminobenzidine (DAB) solution (6×10^{-4}% DAB, 0.4% ammonium nickel (II) sulfate hexahydrate and 3×10^{-4}% H_2O_2 in distilled water) until specimens developed color (approximately 5–10 minutes). To terminate the reaction, sections were washed 3 times for 5 minutes in 100 mM Tris-HCl. After washing in distilled water, sections were serially mounted in rostrocaudal order on silan-coated slides (Super Frost White MAS, Matsunami), counterstained with 0.1% cresyl violet (Nissl staining), then analyzed using microscopy (AX80, Olympus).

Figure production

Images of appropriate sections were captured using a DP70 digital camera (Olympus) and imported into a computer. Line drawings were made using a camera lucida.

RESULTS

We first analyzed the optic nerve by examining 1) the crossing pattern at the optic chiasm, 2) anterograde-labeling pattern or retinofugal system, and 3) retrograde-labeling pattern or retinopetal system. We then describe the cranial nerves that innervate the extraocular muscles and extraocular nerves, focusing on 1) peripheral distribution and 2) distribution of extraocular motor neurons.

Optic Nerve

The optic nerve was labeled by placing a small biocytin crystal on the distal stump of the nerve at the margin of eyeball.

1) Optic chiasm

Labeled fibers of the optic nerve were followed to the optic chiasm, where all labeled fibers crossed the midline. After crossing, labeled fibers continued to the tractus opticus and enter the contralateral brain. This indicates that the optic nerve of medaka is of a complete decussation type.

2) Retinofugal system

a. Preoptic area

Immediately after crossing the optic chiasm, a thin fiber bundle emerged from the tractus opticus contralateral to the injected optic nerve and coursed dorsally to enter the brain. This fiber bundle terminated in the suprachiasmatic nucleus located immediately dorsal to the optic chiasm (Figs. 1C, 2A). Terminals in the nucleus distributed from the level of the nucleus entopeduncularis to that of habenula. At the level of the habenula, a portion of the labeled fibers in the suprachiasmatic nucleus crossed the midline again through the commissura minor of Ishikawa *et al*. (1999) and terminated in the suprachiasmatic nucleus, ipsilateral to the injected optic nerve (Fig. 1C).

b. Thalamus and pretectum

Caudal to the chiasm, labeled fibers in the tractus opticus coursed dorsocaudally to provide terminals to the thalamus and pretectum, and finally to the tectum opticum. The tractus opticus split into 2 branches: the tractus opticus pars dorsomedialis that gave off terminals to the thalamus, dorsal pretectum and tectum opticum, and the tractus opticus pars ventrolateralis that gave off terminals to the ventral pretectum, accessory optic nucleus and tectum opticum.

At the rostral level of the habenula, a region with dense terminals was observed in the nucleus pretectalis superficialis located dorsolateral to the tractus opticus pars dorsomedialis (Figs. 1C, 2B).

At the same level, a fiber bundle branched from the tractus opticus pars dorsomedialis, and proceeded dorsomedially. This bundle gave off terminals to the nucleus dorsolateralis thalami (Fig. 1C). At a more caudal level, where the habenula appeared the largest, the terminal field in the nucleus dorsolateralis thalami extended maximally in the dorsoventral axis, where terminals appeared to be the densest (Figs. 1D, 2C).

Labeled fibers in the nucleus dorsolateralis thalami continued caudally and formed a terminal field in nucleus of the posterior commisure (NPC), located ventrolateral to the posterior commissure (Figs. 1E,F, 2F). A fraction of fibers crossed the midline though the posterior commissure and terminated in the NPC, ipsilateral to the injected optic nerve (Fig. 1F).

Some fibers of the tractus opticus pars dorsomedialis formed terminals in the area pretectalis pars dorsalis (APd) before they reached the tectum opticum (Figs. 1D, 2D). Ventral to the APd, another group of terminals was provided from the tractus opticus pars ventrolateralis, which is the terminal field in area pretectalis pars ventralis (APv) (Figs. 1D,E, 2E).

c. Accessory optic nucleus

Some fibers in the APv passed through the nucleus and extended caudomedially. These labeled fibers terminated in the accessory optic nucleus (Figs. 1F, 2G).

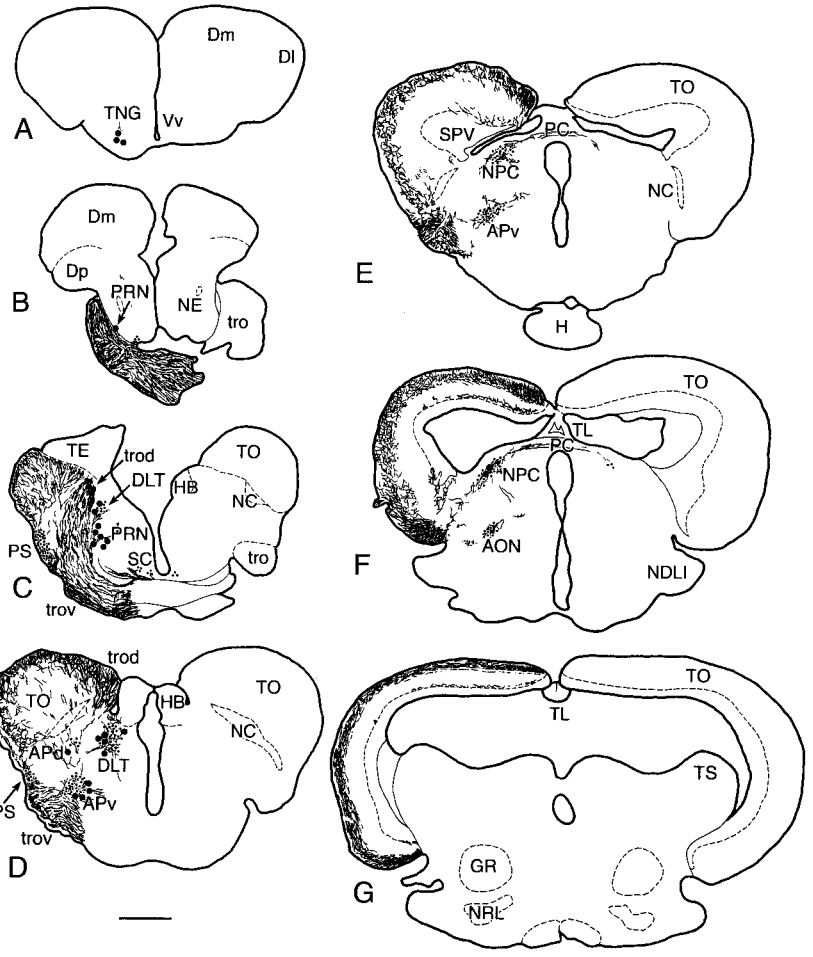

Fig. 1. Representative frontal sections from the telencephalon (**A**) to the mesencephalon (**G**) showing distribution of retinal projections and retinopetal neurons. Left optic nerve was injected with biocytin. Labeled fibers (thin lines), terminals (small dots) and retrogradely labeled cells (large dots) are illustrated. Scale bar=200 μm.

d. Tectum opticum

The largest of the retinal targets was the tectum opticum. The tractus opticus pars dorsomedialis entered the tectum from its dorsomedial aspect and the tractus opticus ventrolateralis from the ventrolateral aspect (Figs. 1D–F). The tectum opticum of medaka has 6 layers, a common feature in teleosts (Ishikawa *et al.,* 1999). Labeled fibers ran mainly through the stratum opticum and stratum fibrosum et griseum superficiale, terminating at this point (Fig. 3A). A fraction of labeled fibers ran deeper to terminate in the stratum album centrale. The stratum fibrosum et griseum superficiale appeared to be divided into a superficial layer with denser fibers and a deeper layer containing less dense fibers.

3) Retinopetal system

In medaka, neurons projecting to the retina were identified in 6 nuclei: ganglion of the terminal nerve (TNG) in the telencephalon, preoptic retinopetal nucleus, nucleus dorsolateralis thalami, APd, APv and NPC. The latter 4 nuclei are retinorecipient nuclei as described above.

The TNG was a cluster of large neurons located in the area ventralis telencephali pars ventralis (Fig. 1A). Labeled cells were piriform or oval in shape (Fig. 3B). Fibers of labeled neurons in the TNG extended dorsocaudally, turned ventrolaterally at the rostral level of the habenula, and then a subpopulation of fibers merged with the tractus opticus (Fig. 1C) toward the optic nerve.

Approximately 20-labeled neurons in the preoptic retinopetal nucleus were observed in the boundary region between the preoptic area and the tractus opticus (Fig. 1B). Labeled cells of the preoptic retinopetal nucleus were oval-shaped. At more caudal levels, labeled neurons in the nucleus were distributed more dorsally and reached the nucleus dorsolateralis thalami (Figs. 1C, 3C). Labeled neurons in the nucleus dorsolateralis thalami thus appeared as a dorsocaudal continuation of labeled neurons of the preoptic retinopetal nucleus (Figs. 1C,D, 3D). Labeled cells were piriform or oval-shaped in the nucleus dorsolateralis thalami. Small numbers of labeled, oval cells were also observed in the NPC (Fig. 3E), positioned immediately caudal to the nucleus dorsolateralis thalami. A few neurons were seen lateral to the preoptic retinopetal nucleus, and labeled, oval neurons continued to the APv (Fig. 1D). One single, labeled, oval neuron was also observed in the APd (Fig. 1D).

Cranial nerves that innervate extraocular muscles

1) Peripheral distribution

Under the dissection microscope, the oculomotor nerve

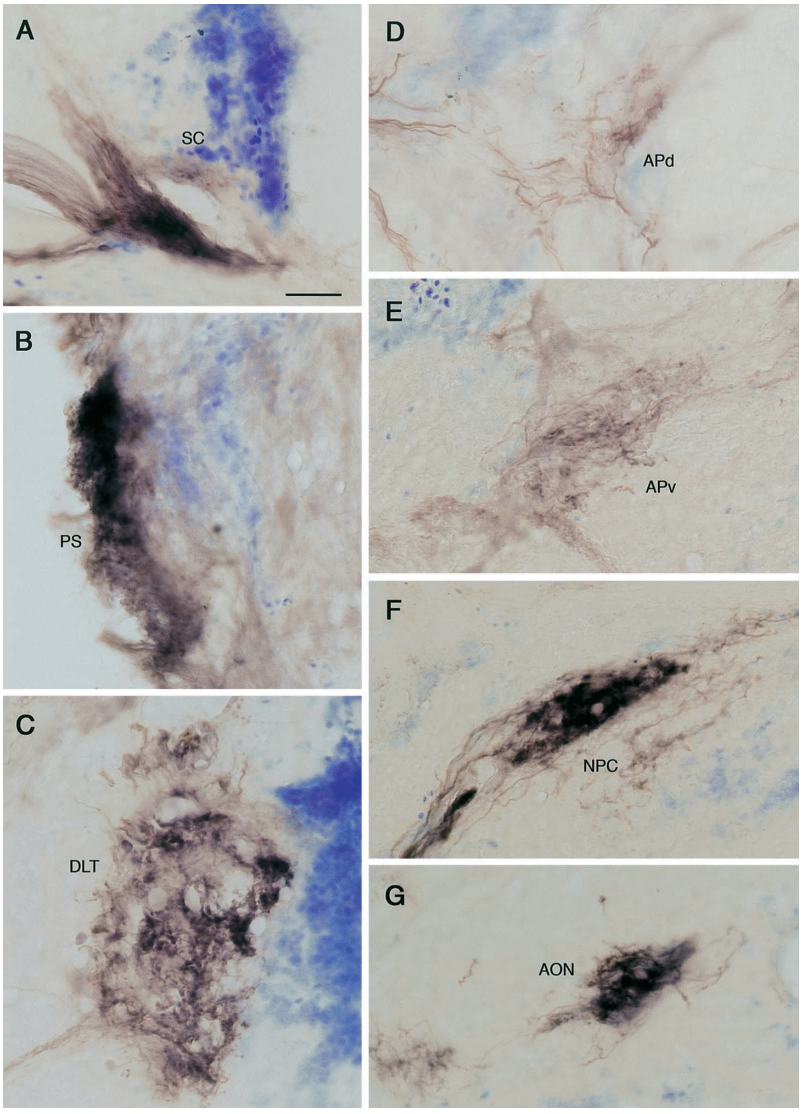

Fig. 2. Biocytin-labeled retinofugal fibers and their terminals in suprachiasmatic nucleus (**A**), nucleus pretectalis superficialis (**B**), nucleus dorsolateralis thalami (**C**), area pretectalis pars dorsalis (**D**), area pretectalis pars ventralis (**E**), nucleus of the posterior commissure (**F**), and accessory optic nucleus (**G**) in frontal sections. Sections were counterstained with cresyl violet (blue). Scale bar=25 μm.

appeared to exit the brain from the ventral side of the mesencephalon and run rostroventrally along the lateral surface of the brain. The oculomotor nerve exited the cranium through a foramen located in the caudal basement of the parasphenoid bone (Iwamatsu, 1997) that constitutes the rostroventral wall of the cranium. The oculomotor nerve then split into 4 branches in a caudal area of the orbit to innervate the superior rectus muscle (SR), medial rectus muscle (MR), inferior oblique muscle (IQ) and inferior rectus muscle (IR) (Fig. 4A,B). The origin of SR was an articulation-like portion at the center of a parasphenoid located slightly caudal to the optic chiasm. The SR extended rostrodorsally and inserted into the dorsal part of the eyeball (rostrocaudally central portion). The MR originated from a central position of the parasphenoid bone and inserted into the rostral part of the eyeball (dorsoventrally central portion). The IQ originated from the medial end of the dorsal part of the palatine bone, extended caudoventrally and inserted to the ventral part of the eyeball (rostrocaudally central portion). The IR originated from the center of the parasphenoids, similar to the SR, and extended rostroventrally to insert into the ventral part of the eyeball (rostrocaudally central portion).

The trochlear nerve exited the brain from a position dorsocaudal to the exit of the oculomotor nerve. The trochlear nerve extended rostroventrally along the lateral surface of the brain and exited the cranium from a foramen in the caudal basement of the parasphenoid bone. The nerve then traveled along the dorsal side of the eyeball and innervated the superior oblique muscle (SQ) (Fig. 4A,B). The SQ originated from the medial end of the dorsal part of the palatine bone, extended dorsocaudally and inserted into the dorsal part of eyeball (rostrocaudally central portion).

The abducens nerve exited the brain from a ventromedial part of the rhombencephalon, then extended rostrally and slightly laterally along the ventral aspect of the brain. The abducens nerve left the cranium from a foramen in the caudal basement of the parasphenoid bone and innervated the lateral rectus muscle (LR) (Fig. 4A,B). The LR originated

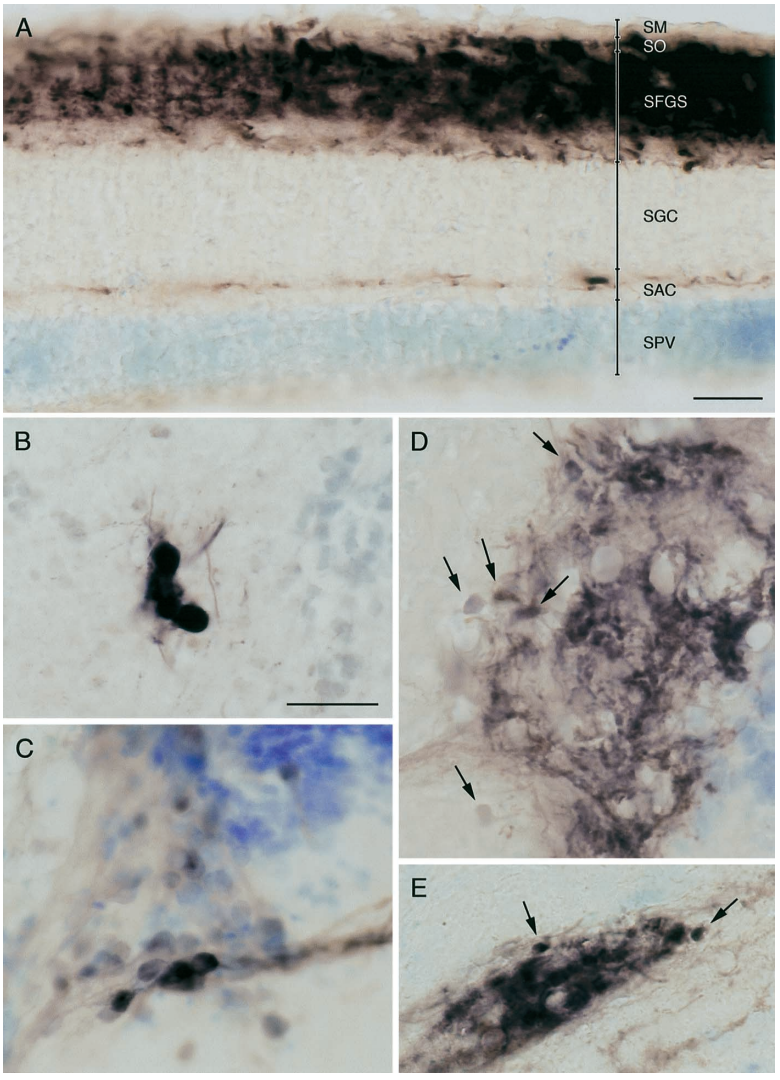

Fig. 3. Biocytin-labeled retinofugal fibers (**A**) and retinopetal neurons (**B**–**E**). (**A**) Frontal section showing retinofugal fibers and their terminals in the tectum opticum. Fibers are mainly observed in the stratum opticum and stratum fibrosum et griseum superficiale (SFGS). Labeled fibers are dense in superficial portions of SFGS and less dense in the deeper portions. Much fewer fibers and terminals are found in the stratum album centrale. Retinopetal neurons in the ganglion of the terminal nerve (**B**), preoptic retinopetal nucleus (**C**), nucleus dorsolateralis thalami (**D**) and nucleus of the posterior commissure (**E**). Arrows in (**D**)–(**E**) point to select retinopetal neurons. Scale bar=25 μm.

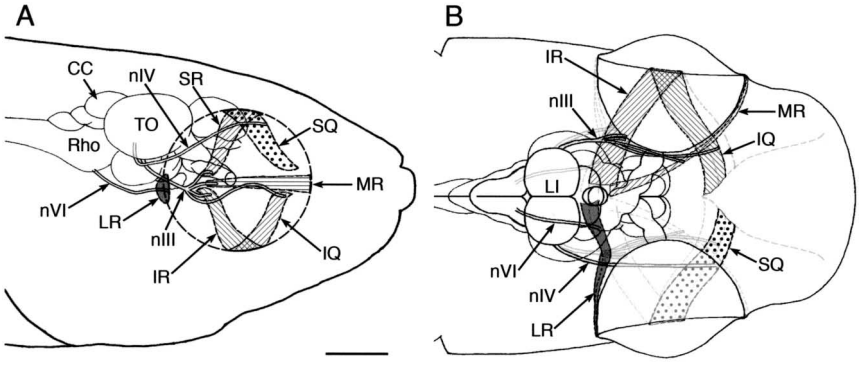

Fig 4. Schematic drawings indicating innervation patterns of extraocular muscles by oculomotor, trochlear and abducens nerves. (**A**) Right lateral view of the head of medaka. The circle drawn with a broken line indicates the right eyeball. (**B**) Ventral view of the medaka head, upper-half (right-half of the head) showing oculomotor nerve and muscles innervated by this nerve (inferior rectus muscle, inferior oblique muscle, medial rectus muscle, but superior rectus muscle not shown). The lower-half shows trochlear and abducens nerves, and superior oblique muscle and lateral rectus muscle innervated by the nerves, respectively. Scale bar=1 mm.

from the medial part of the parasphenoid bone, extended laterally and inserted into the caudal part of eyeball (dorsoventrally central portion).

2) Distribution of extraocular motor neurons

Extraocular motor neurons were labeled retrogradely by injecting biocytin solution into each cranial nerve. The ocu-

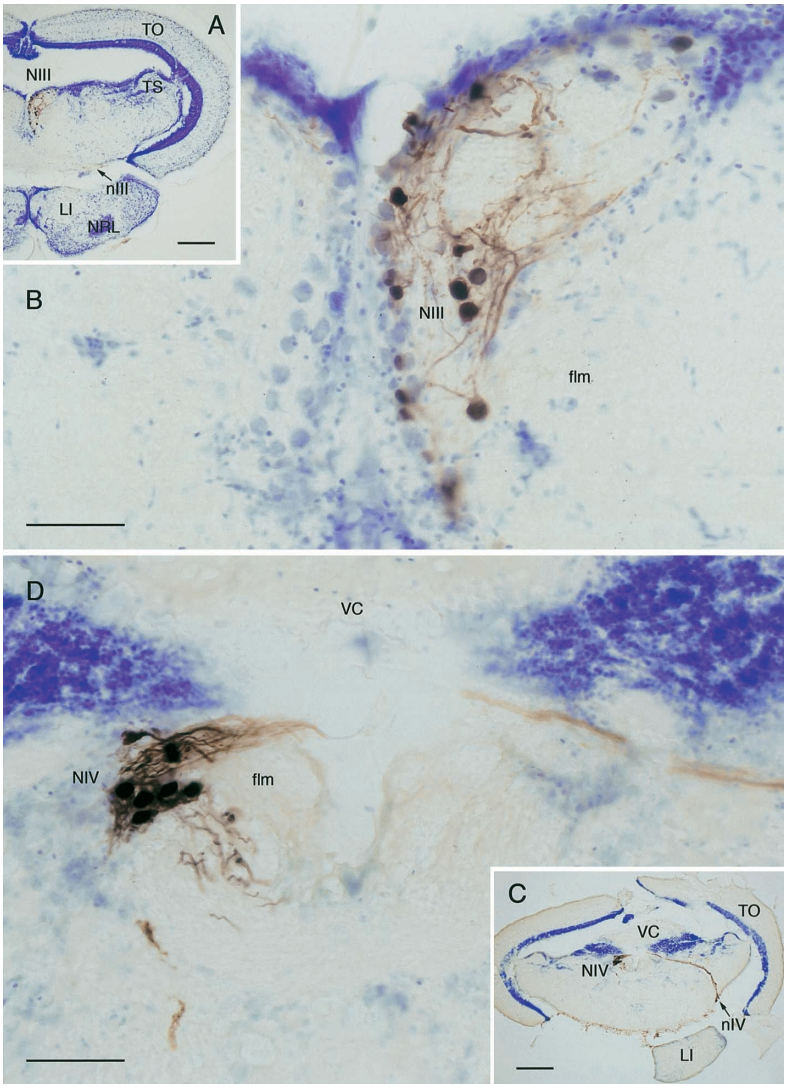

Fig. 5. (**A**) Frontal section at the level of oculomotor nucleus after unilateral injection of biocytin into the left oculomotor nerve. Scale bar=200 μm. (**B**) Increased magnification of oculomotor neurons from (**A**). Scale bar=50 μm. (**C**) Frontal section at the level of trochlear nucleus after unilateral injection of biocytin into left trochlear nerve. Scale bar=200 μm. (**D**) Increased magnification of trochlear neurons from (**C**). Scale bar=50 μm.

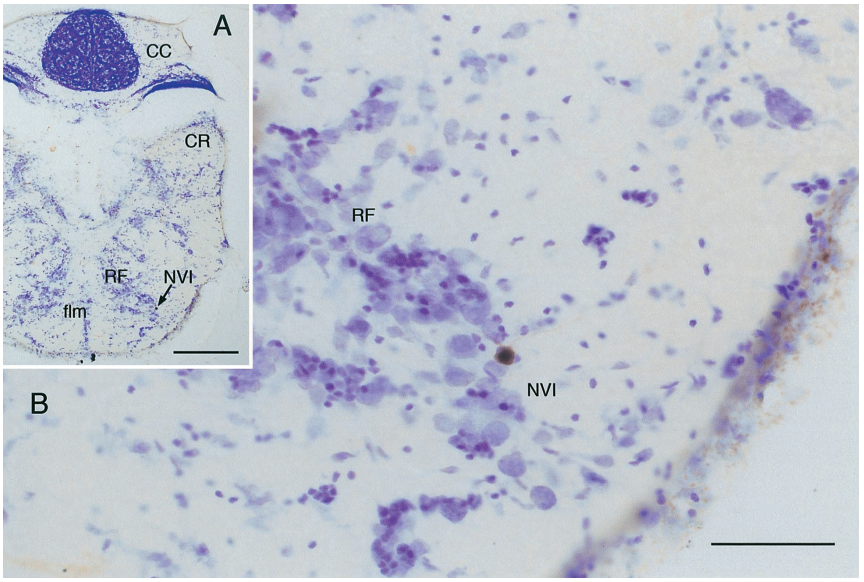

Fig. 6. (**A**) Frontal section at the level of abducens nucleus after unilateral injections of biocytin into left abducens nerve. Scale bar=200 μm. (**B**) Increased magnification of abducens neurons from (**A**). Scale bar=50 μm.

lomotor nucleus was located in the dorsal portion of the tegmentum mesencephali at the rostral level of nucleus recessi lateralis. The oculomotor nucleus was dorsomedially adjacent to the fasciculus longitudinalis medialis (Fig. 5A,B). Most of the labeled neurons were observed in the ipsilateral nucleus with only a few cells in the contralateral nucleus. Ipsilateral neurons were located dorsal and medial to the fasciculus longitudinalis medialis, while contralateral neurons medial to the fasciculus. The labeled cells in the oculomotor nucleus were fusiform or piriform in shape. The fibers derived from the labeled neurons extended ventrally to form a bundle. The bundle turned laterally and left the brain as the oculomotor nerve from the ventral aspect of the mesencephalon (Fig. 5A). A subpopulation of labeled fibers derived from labeled neurons in the oculomotor nucleus entered the fasciculus longitudinalis medialis and extended caudally as part of the fasciculus.

The trochlear nucleus was caudal to the oculomotor nucleus and located dorsolateral to the fasciculus longitudinalis medialis at the level of the nucleus interpeduncularis (Fig. 5C,D). Labeled cells were fusiform or piriform in shape. In its most rostral part, the trochlear nucleus was very close to the caudalmost neurons of the oculomotor nucleus. Labeled fibers of the trochlear neurons extended medially, running dorsal to the ventriculus quartus, forming a bow-shape tract, and passing through the commissura cerebelli to the contralateral side. These fibers extended further laterally, then turned ventrally and left the brain at a position caudolateral and dorsal to the oculomotor nerve exit (Fig. 5C). A subpopulation of labeled fibers derived from labeled neurons in the trochlear nucleus entered the fasciculus longitudinalis medialis and extended caudally through the fasciculus.

The abducens nucleus was located caudally away from the other extraocular motor nuclei, in a ventrolateral part of the rhombencephalic reticular formation. Labeled neurons in the abducens nucleus were oval or piriform-shaped (Fig. 6A,B).

DISCUSSION

To understand the central neural circuits in medaka, we initiated an analysis of cranial nerve projections and distribution of motor neurons using tract-tracing biocytin tracers. This study revealed a central distribution of retinofugal terminals, retinopetal neurons, and oculomotor, trochlear and abducens motor neurons in medaka. Medaka belongs to smegmamorphs, which together with the closely-related percomorphs, are regarded as highly-derived teleost groups (Nelson, 1994). We thus compared the present results in medaka with those of closely-related percomorphs and other more distant related teleosts.

Optic nerve

1) Retinofugal system

Retinal projections in medaka were contralateral dominant. Labeled terminals were found in 8 brain nuclei: the suprachiasmatic nucleus, nucleus pretectalis superficialis, nucleus dorsolateralis thalami (sometimes considered as a projection to the nucleus ventrolateralis thalami; e.g. Braford and Northcutt, 1983; Northcutt and Wullimann, 1988), APd, APv, NPC, accessory optic nucleus, and tectum opticum. On the ipsilateral side, small numbers of terminals were observed in the suprachiasmatic nucleus and NPC. These targets as well as terminal patterns in the tectum opticum are common to other teleosts (see review by Vanegas and Ito, 1983), but a projection to the nucleus ventromedialis thalami reported for percomorphs (e.g. Ito *et al*., 1984) was not detected in medaka. Contralateral dominance of retinal projections is also common to other teleosts (see review by Vanegas and Ito, 1983), except the piranha, in which abundant ipsilateral projections have been reported (Ebbesson and Ito, 1980). Projection of the optic nerve to the ipsilateral nucleus of the posterior commissure in medaka, as observed in this study, is also known to occur in ostariophysan teleosts, e.g. cyprinids (Reperant *et al*., 1976), piranha (Ebbesson and Ito, 1980) and goldfish (Springer and Gaffney, 1981).

As described above, retinal projections end in multiple nuclei. This implies that visual information is transmitted through multiple functional circuits to carry out parallel processing of visual information. The suprachiasmatic nucleus is located immediately dorsal to the optic chiasm and receives a projection of nerve tract fibers distinct from those projecting to other retinorecipient nuclei. This is analogous to the homonymous nucleus in mammals. Function of the suprachiasmatic nucleus in teleosts has not yet been determined, but the nucleus is likely involved in regulation of circadian rhythm, similarly to other vertebrates (Klein *et al*., 1997). Many pretectal nuclei receive retinal projections and project to the cerebellum (Meek and Nieuwenhuys, 1998). In filefish that belong to percomorphs, the APd, APv, and accessory optic nucleus are known to project to the oculomotor nuclear group, in addition to the cerebellum (Uchiyama *et al*., 1988). These nuclei may constitute neural circuits regulating oculomotor kinetics to stabilize visual images on the retina.

In filefish, the NPC sends information to the ciliary ganglion relayed by the accessory oculomotor nucleus (or Edinger-Westphal nucleus) and regulates visual accommodation (Somiya *et al*., 1992). The nucleus dorsolateralis thalami, although belonging to the thalamus, projects little to the telencephalon in most teleosts (Striedter, 1990; Yamamoto and Ito, 2000). In the visual system of percomorphs and holocentrids, the tectum opticum sends abundant fibers to the nucleus prethalamicus, which in turn projects to the telencephalon (Ito *et al.,* 1980; Ito and Vanegas, 1983, 1984). The nucleus pretectalis superficialis projects to the nucleus intermedius, which in turn sends fibers to the corpus glomerulosus, an afferent origin to the inferior lobe (Murakami *et al*., 1986; Sakamoto and Ito, 1982; Shimizu *et al*., 1999; Yamamoto and Ito, 2002). In percomorphs, the inferior lobe receives gustatory and viscerosensory inputs (Yoshimoto *et al*., 1998), and electrical stimulation of the

lobe evokes feeding behavior (Demski, 1983). It is possible that the visual signals through the nucleus pretectalis superficialis participate in the regulation of feeding behavior.

In the present study, no terminals of retinofugal projection were found on the cell bodies of nucleus corticalis neurons, a finding similar to the situation in percomorphs (Ito *et al*., 1984; for review see Vanegas and Ito, 1983). The nucleus corticalis develops well in percomorphs but is not present (or at least is undetectable) in ostariophysans or salmonids, both of which are considered to have been derived at a relatively early stage in teleost phylogeny (Ito and Yoshimoto, 1991; Yamamoto and Ito, 2002). The nucleus corticalis is also present in medaka, a smegmamorph consistent in its close relationship with percomorphs (Figs. 1C–E). In percomorph teleosts, there is electrophysiological evidence that the nucleus corticalis receives input from the retina (Rowe and Beauchamp, 1982). The nucleus corticalis neurons extend huge dendrites to the stratum fibrosum et griseum superficiale and stratum griseum centrale of the tectum opticum (Sakamoto and Ito, 1982; Shimizu *et al*., 1999). Dendrite morphology of the nucleus corticalis neurons is not described in medaka, but from an analogy with related percomorph teleosts, it is likely that the nucleus corticalis neurons receive retinal projections on their dendrites in medaka as well. The nucleus corticalis projects to the corpus glomerulosum, an afferent origin to the inferior lobe. Hence, visual information relayed by the nucleus corticalis may modulate feeding behavior, as discussed above for the nucleus pretectalis superficialis.

In teleosts, the tectum opticum is not only a relay center for visual input to the telencephalon but also an area that receives input of other modalities and sends fibers to various targets, including descending projections to the rhombencephalon (for review see Vanegas and Ito, 1983; Meek and Nieuwenhuys, 1998; Yamamoto and Ito, 2002). The tectum opticum is thus one of the most important centers for sensory-motor co-ordination.

Neural circuits related to vision that have been described for percomorphs are probably present in smegmamorphs medaka because of their close relationship. The present study provides a basis for further investigation into the visual circuits from a morphological, functional, behavioral, and genetic viewpoint.

2) Retinopetal system

In medaka, neurons projecting to the retina were identified in 6 brain nuclei on the contralateral side: the TNG, preoptic retinopetal nucleus, nucleus dorsolateralis thalami, APd, APv and NPC. To our knowledge, this is the first report in teleosts of retinopetal neurons located in the NPC.

Subsequent to the first discovery of projection from the TNG to the retina (Münz and Claas, 1981), this pathway has been confirmed in various fish species (for review see Uchiyama, 1989). Position of the TNG is variable depending on the species. In species with a stalked-type olfactory bulb, where the olfactory bulb is located close to the olfactory epithelium and distant from the telencephalon, the TNG is located in a rostral region of the olfactory bulb. In species with a pedunculated olfactory bulb-type, where the olfactory bulb is positioned immediately rostral to the telencephalon, the TNG is usually located at the boundary between the olfactory bulb and telencephalon (for review see Demski, 1993). Medaka, a pedunculated olfactory bulb-type species, had the TNG at the boundary of the olfactory bulb and telencephalon, in concordance with a previous immunocytochemical study (Ishikawa *et al*., 1999). The neurons of TNG, also called the nucleus olfactoretinalis, receive olfactory, visual and general somatosensory inputs, and project to wide areas in the brain (Yamamoto *et al*., 1995; Yamamoto and Ito, 2000) and produce gonadotropin-releasing hormone (GnRH) (for review see Demski, 1993; Yamamoto, 2003), presumably functioning as a neuromodulatory system (for review see Oka, 1997). GnRH derived from the TNG affects the excitability of retinal neurons (Stell *et al*., 1984; Walker and Stell, 1986; Umino and Dowling, 1991). At the behavioral level, in a percomorph (dwarf gourami), the TNG is involved in regulation of the nest-building behavior of male fish (Yamamoto *et al*., 1997; Yamamoto, 2003). Study of medaka will contribute to the further understanding of neuromodulatory functions of the TNG.

In medaka, retinopetal nuclei were observed not only in the telencephalon but also in the diencephalon, a common feature in percomorphs and other closely-related teleost groups (Uchiyama, 1989; Butler and Northcutt, 1992). In ostariophysans, no retinopetal neurons have been observed in the diencephalon; in zebrafish, only the TNG or nucleus olfactoretinalis have been described (Burrill and Easter, 1994). The reason for the difference is unknown, but considering the fact that a sturgeon, which branched earlier than teleosts, has retinopetal neurons in the diencephalon (Hofmann *et al*., 1993; Ito *et al*., 1999), the lack of diencephalic retinopetal neurons is likely a derived feature in ostariophysans.

Retinopetal neurons of medaka were distributed in a continuous zone and found in several nuclei of the diencephalon. A similar distribution of retinopetal neurons in the preoptic retinopetal nucleus and nucleus dorsolateralis thalami has also been observed in percomorphs (Ito *et al*., 1984). Retinopetal neurons in the diencephalon of medaka were generally 4–6 μm in size. These retinopetal neurons may, in fact, represent a homogeneous population of neurons. It is possible that the distribution of diencephalic retinopetal neurons is spread in a more rostrocaudally-elongated fashion in medaka, resulting in the inclusion in a number of nuclei. It is important to determine the connections of these neurons other than their retinal projections and to identify their neurotransmitters in order to solve the issue of the identity of these neurons in multiple nuclei. A sub-group of percomorphs, the Tetraodontiformes fish, which includes *Navodon modestus* and *Stephanolepis cirrhifer*, have highly-developed visual systems, and presumably as its consequence develop the preoptic retinopetal nucleus to contain as many as 8000 to 10000 neurons per side (Uchiyama *et al*., 1981; Uchiyama and Ito, 1984). In tetraodontiformes, the

preoptic retinopetal nucleus receives projections from the tectum opticum (Uchiyama *et al*., 1986) and may be involved in visual attention shifts on the retina (Uchiyama, 1989). Although medaka also has a well-developed visual system, the preoptic retinopetal nucleus is less developed than tetraodontiformes. The medaka showed approximately 20 labeled retinopetal neurons in the presently described tract-tracing experiments. Even though there might be neurons that were not labeled, the total number of neurons in the nucleus would not be significantly larger. Archer fish, another type of percomorph with a well-developed visual system, possesses very few neurons in the preoptic retinopetal nucleus (Tanaka, Yoshimoto, Yamamoto, Ito and Somiya, unpublished observation). These fish seek food on or above the water surface, while tetraodontiformes see objects in water, frequently by binocular vision. The difference in the development of the preoptic retinopetal nucleus may reflect a difference in the way visual information is used among fish species. This hypothesis can be examined by quantitatively analyzing neurons of the preoptic retinopetal nucleus in a variety of fish species.

Cranial nerves that innervate extraocular muscles

In medaka fry, gross peripheral distributions of cranial nerves innervating extraocular muscles have been previously described (Ishikawa and Hyodo-Taguchi, 1994). In the present study, adult medaka was investigated and the overall distribution of extraocular cranial nerves was in accordance with those of medaka fry. The present study extended further the investigation to include the distribution of motor neurons innervating the extraocular muscles by use of tracer experiments, the first time conducted in medaka. Projections of these motor neurons were unilateral dominant, namely ipsilateral dominance of the oculomotor neurons, contralateral dominance of the trochlear neurons and ipsilateral dominance of the abducens neurons. This pattern is also observed in other teleost fish (carp: Luiten and Dijkstra-de Vlieger, 1978; stargazer (a percomorph): Leonard and Willis, 1979; goldfish: Graf and McGurk, 1985; flatfish (a percomorph): Graf and Baker, 1985; weakly electric fish (an osteoglossomorph): Szabo *et al*., 1987; file fish: Somiya *et al*., 1992). In these studies, the oculomotor nucleus is subdivided into sub-nuclei depending on the muscles in which they innervate. According to these studies, labeled neurons in the contralateral oculomotor nucleus in the present study appear to correspond to those innervating the SR. Labeling the four individual branches of the oculomotor nerve should provide more detailed information. In carp (Luiten and Dijkstra-de Vlieger, 1978), goldfish (Sterling and Gestrin, 1975; Sterling, 1977; McGurk and Graf, 1984; Graf and McGurk, 1985; Cabrera *et al*., 1992; Pastor *et al*., 1991), stargazer (Leonard and Willis, 1979) and flatfish (Graf and Baker, 1985), neurons of the abducens nucleus are divided into rostral and caudal groups. In the present tract-tracing study of medaka, the distribution of these specific neurons remained elusive, but considering that neurons of the abducens nucleus form separate anterior and posterior bundles in the brain (data not shown), it is likely that in medaka, the abducens nucleus is also divided into rostral and caudal compartments.

Recently, physiological and behavioral studies have been carried out in medaka (Beck *et al*., 2004). The present study will provide an anatomical basis for these types of studies.

Concluding remarks

In the present study, central projection of the optic nerve, distribution of retinopetal system and distribution of motor neurons innervating the extraocular muscles were characterized in medaka. Analysis of other cranial nerves is currently ongoing. These findings are essential for understanding central neural circuits in both morphological and physiological terms. The use of medaka as an experimental animal serves a number of advantages and will be an important tool for future investigations into genes involved in the elicitation and regulation of behavior.

ACKNOWLEDGEMENTS

The authors would like to thank members of the ERATO Kondoh Project for encouragement and discussion. This work was partly supported by the SORST program of the Japan Science and Technology Agency and by grants from the Ministry of Education, Culture, Sports, Science and Technology (12CE2007) of Japan and the Human Frontier Science Program (RGP0040/2001-M) to HK.

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(Received November 30, 2004 / Accepted December 21, 2004)