Taste and somatosensory neurons in sea catfish *Plotosus japonicus*: Morphology, distribution in the ganglion and central projections.

Nurdiyana Ahmad Denil

Graduate School of Science and Engineering,

Kagoshima University, Japan

2013

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Chapter 1: Introduction

Chemoreception plays an important and indispensable role in the behaviour of fishes. It is involved in the procurement of food, recognition of sex, discrimination between individuals of the same or different species, in defense against predators, in parental behaviour, in orientation and in many other ways.

Vertebrates possess three major classes of chemosensory systems; the gustatory organs, the olfactory organs, and the common chemical senses. Gustation is defined as the chemical sense that is mediated by specific receptors of gustatory cells within taste buds. Olfactory responses are undisputedly mediated through specific protein receptors expressed in the receptor neuron membranes, and therefore are structurally and functionally in strong contrast with the gustatory and common chemical sense. The common chemical sense is all other chemical senses that are perhaps mediated by nonspecific nervous structures such as free nerve endings. All three sensory systems are stimulated by chemical substances, whether specific or non specific, and prone to mechanical stimulation.

Gustation or the taste system mediates one of the most fundamental processes for the survival of individuals and species, which is feeding. The relation of gestation to food intake is manifested in all vertebrates, but many aspects of the complex integration of chemosensory input with antecedent or concomitant physiological activities remain mostly unclear. The evolutionary development of the chemosensory systems associated

with feeding followed two different courses, one in invertebrates and one in vertebrates. In invertebrates, gustatory cells preferentially sensitive to food materials can be found almost anywhere on the body surface, for example, on antennae, tentacles, or legs. In vertebrates, the presence of specialized taste buds in the oral cavity has been taken evidence of an ability to taste. Taste perception is responsible for basic food appraisal and bestows the organism with valuable discriminatory power.

In fish, the taste buds are usually bulbiform, basally swollen and distally tapered. They vary in size (45-75 μm in height and 30-50 μm in width) depending on the thickness of the epithelial layer, and orientated perpendicular to the skin surface (Iwai, 1964; Kiyohara *et al.,* 1980). Taste buds vary in size, especially height, considerably among species and location even in the same species, as shown as in the minnow *Pseudorasbora parva* (Kiyohara *et al.,* 1980). In channel catfish, for example, taste buds innervated by vagal nerves are taller and more slender than those innervated by facial nerves (Eram & Michel, 2005). In *P. japonicus*, the barbels possess the largest buds, suggesting their importance as taste organs. The taste buds are distributed across the entire epithelium of the barbel along its entire length. At each level of the barbel, taste bud density is highest on the rostral surface, moderately high on the caudal surface, but notably low on the intermediate epidermis of the barbels. Each taste bud contains at least three types of taste bud cells- tubular (t) or light cells, filamentous (f) or dark cells, and basal cells, according to the characteristics of their cytoplasm or their position in the taste buds (Fig. 1) (Reutter, 1978). Tubular and filamentous cells are longitudinally elongated. Tubular cells, which are assumed as taste receptor cells, have rod-shaped process (large receptor villus) at the apex and are usually surrounded by filamentous

cells with microvillar processes. The taste receptor cells do not have axons, but do synapse onto peripheral ends of sensory ganglion cells of the taste cranial nerves. The number of taste cells in taste buds varies considerably, for example, from five in *Pomatoschistus* (Gobiidae) to sixty seven in *Corydoras* (Callichtyidae) (Jakubowski & Whitear, 1990). The number of taste cells in taste buds also varies according to the location of taste buds in the mouth or on the body surface.

As in other vertebrates, the gustatory system in fish comprises two parts, the peripheral and the central taste system. The peripheral part includes the taste organs, the so called taste buds, as well as their afferent and efferent nerves, whereas the central part consists of the nuclei of these nerves in the medulla oblongata and some other nuclei in higher brains. In fish, taste buds are distributed in five subpopulations, to a varying degrees: (1) oral, (2) palatal and laryngeal, (3) branchial (gills), (4) cutaneous, and (5) barbels.

Certain fish groups, the cyprinids (~2700 species including carps, minnows, goldfish and so on), and silurids (~2300 species including catfishes) have evolved a vast system of external gustatory receptors over virtually the entire body surface, along with an elaborately organized complex neural organization. Catfishes (order Siluriformes) are notable exceptions to the general conditions among teleosts, as they evolved thousands of extra taste buds over virtually the entire body surface, which is a result of the independent evolution that occurred a number of three times among teleosts (Northcutt, 2005).

The distribution of taste buds varies considerably among species reflecting their feeding habitat and the relative importance of the taste sense for their respective feeding behaviours (Kiyohara *et al.*, 1980; Gomahr *et al*., 1992; Fishelson & Delarea, 2004). For example yellow bullhead catfish *Ictalarus natalis* (Lesueur 1819) have more than 175,000 taste buds on their entire body surface alone (Atema, 1971). On the gill arches or gill rakers of the *I. natalis*, the number of taste buds is estimated at 12,500 (Atema, 1971). Carp (*Cyprinus carpio*) and goldfishes (*Carassius auratus*) on the other hand, have evolved a highly sophisticated food separation system on the roof of the mouth (palatal organ) that is studded with thousands of taste buds (Sibbing and Uribe, 1985). In rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) a visual feeder, the highest taste bud density is found intraorally, on the palatal organ, totalling 3000-4000 taste buds (Hara et. al., 1993). One area that has been overlooked is the gill arches where the total number of taste buds is estimated at 12,500 in bullhead (Atema, 1971).

It is well known that fish taste buds, in contrast to most mammalian taste buds, are not only sensitive to the popular sweet, sour, salty and bitter tastants (Glaser, 1966), but also to a great variety of amino acids, nucleotides, bile acids and carbon dioxide (Yoshii *et al*., 1979, 1980; Hara *et al*., 1984; Yamamori *et al*., 1988; Yamashita *et al*., 1989; Marui & Caprio, 1992). The sensitivity of fish taste buds is generally much higher than that of in other vertebrates. However, the relative stimulatory effectiveness of these chemicals varies extensively among fish species. Apart from chemical senses, the taste nerves of fish also carry tactile information, through connections of Merkel-like basal cells of the taste buds to intragemmal fibers. In fish, taste buds respond to both chemical and sometimes mechanical stimuli and the information is

conducted to the primary taste center of the medulla (Finger, 1983; Kiyohara & Tsukahara, 2006; Hara, 2007) as impulses via one of three cranial nerves, facial (VIIth), glossopharyngeal (XIth) and vagal (Xth). Generally, all cutaneous taste buds on the body surface and oral regions are innervated by the facial (VIIth) nerve, while taste buds located within the buccal and pharyngobranchial regions are innervated by the glossopharyngeal (VIIth) and vagal (Vth) nerves with a rostrocaudal order.

The primary taste center of vertebrates in organized as a pair of special visceral sensory columns located within the medulla oblongata (Herrick, 1905; Ariens- Kappers *et al.*, 1936). Each column receives input in a rostrocaudal manner from the facial, glossopharyngeal and vagal nerves, respectively. Fish are unique compared to other vertebrates, in that they have anatomical elaborations of the primary taste center. This primary taste center in particular species of fish with highly developed taste systems, such as cyprinids and catfishes, is subdivided into facial (FL) and vagal (VL) lobes. Behavioural experiments in catfish indicate that the FL and VL have different functions (Atema, 1971). The FL, which receives input from the facial nerve innervating taste buds located across the entire external body surface and rostral oral regions, functions in appetitive (food search and ingestive) behaviours. The VL, which receives input from glossopharyngeal (more rostral region) and vagus nerves that innervate taste buds exclusively within the oral cavity and pharynx, functions in consummatory (swallowing and rejection) behaviours (Kiyohara & Caprio, 1996). The functional difference between the FL and VL is also supported by anatomical findings showing different reflex connections of both lobes within the brainstem (Finger & Morita, 1985).

The primary taste center of *P. japonicus* is located as a pair of longitudinal columns that is divided into facial and vagal lobes. Histologically, the FL is subdivided by fascicles of nerve fibers into five distinct lobules containing five longitudinal columns. They are termed from medial to lateral the medial mandibular lobule (MML), lateral mandibular lobule (LML), maxillary barbel lobule (MXL), nasal barbel lobule (NBL) and the trunk tail lobule (TTL). The three gustatory nerves from different regions of the body thus enter the nucleus in approximately the same order as they are located in the periphery, that is, axons from more rostral regions of the mouth and or body surface enter the more rostral portions of the nucleus (Finger, 1987; Butler & Hodos, 1996). Therefore, distinct somatotopic and viscerotopic organizations are present in the FLs and VLs, respectively. For example, in catfishes, such as *Arius felis* (L. 1766) (Kiyohara & Caprio, 1996), *Ictalurus punctatus* (Rafinesque 1818) (Kanwal & Caprio, 1987; Hayama & Caprio, 1989), *Plotosus lineatus* (Thunberg 1787) (Marui *et al.*, 1988; Kiyohara *et al.*, 1996), taste buds distributed on the external body surface and internal surface are mapped continuously in the FL and VLs showing barbels as sharply defined enlarged lobules extending rostrocaudally.

The complex gustatory system as has been described makes itself even more complex by the fact that the facial nerve is closely allied to the trigeminal (Vth) nerve. In vertebrates, the trigeminal nerve generally carries the sensory nerves from the jaws and head, as well as the motor neurons that control the jaw muscles (Hara, 2007). It is responsible for sensation, including pain, temperature, touch and proprioception. The trigeminal system is best developed in animals with prominent snout, especially on vibrissae (whickers). The sensory part of the trigeminal is exclusively of carrying tactile

stimuli from the same region innervated by the facial nerve (Herrick, 1905; Luiten, 1975). Barbels of catfishes are heavily innervated by trigeminal nerves mixed with the facial, both of which project to the facial lobe by three major branches, ophthalmic, maxillary and mandibular.

The cell bodies of the primary taste neurons are located in the peripheral ganglia of the three cranial nerves. Though a topolological organization in the primary taste center has been well established in many species of fishes, little is known whether such a organization occurs in the taste ganglia of these fishes. Early work in bullhead catfishes, *I. nebulosus* and *Ictalurus natalis* (Lesueur 1819) examined the relation between the barbels and specific ganglion regions, but topographic relation of the facial neurons was not clearly examined since the facial neurons are mixed with trigeminal and lateral line neurons in the anterior ganglion (Finger, 1976). In rocklings, such as *Ciliata mustela* (L. 1758), no somatotopy was detected within the geniculate ganglion between different branches of the recurrent nerve (Kotrschal & Whitear, 1988). In amphibians, such as axolotl *Ambystroma mexicanum*, cell bodies of the glossopharyngeal nerve are mainly distributed in the rostral part of the glosopharyngeal-vagal ganglion, but a few cells were also distributed in the middle and caudal parts, indicating an undifferentiated topographic organization of the glossopharyngeal nerve within the ganglion (Nagai & Oka, 1991). The researches regarding the organization of trigeminal nerve fibers to the primary taste center is also well established in fish. For example, in the channel catfish, the trigeminal fibers terminate in the principal and spinal trigeminal nuclei, as well as throughout the facial lobe, with the exception of the lateralmost lobule which contains a representation of

taste buds innervated by the recurrent branch of the of the facial nerve (Kiyohara *et al.*, 1999). The trigeminal fibers of channel catfish are coarser than those of the facial which terminate within the same structural loci, and its input to the primary gustatory complex is restricted to those portions of nucleus receiving sensory inputs from the face and barbells. Contrary to its central projections, virtually nothing is known about the peripheral termination of the trigeminal nerve in fishes, except for the observations that in sea catfish and goatfish some nerve fibers terminate inside taste buds perigemmally without ending on gustatory cells (Sakata *et al*. 2001; Kiyohara *et al.*, 2002). A recent study by Kerem *et al.* (2005) using biocytin applications to the three trigeminal branches (ophthalmic, maxillary and mandibular nerves) revealed that trigeminal ganglions in tilapia, *Oreochromis niloticus* were somatotopically distributed in the ganglion reflecting the dorsoventral order of the three branches.

The polarities of taste neurons were extensively examined in mammals, such as mouse (Boudreau, 1977). The results established that most of the taste neurons are pseudo-unipolar, which emit a single axonal process dividing into central and peripheral branches. Neurons located in the glossopharyngeal-vagal taste ganglia of the axolotl were also examined with histochemistry of neural tracers (Nagai & Matsushima, 1990). In the previous study, a single process from each cell body was clearly labelled and the majority of the neurons were assumed to be pseudo-unipolar without showing ramification of the process. Few studies in fishes deal with the polarity of taste neurons. The early work by Finger (1976) suggests the presence of unipolar and bipolar neurons in the anterior ganglion of *I. nebulosus* and *I. natalis*. The majority of ganglion cells

observed in trigeminal ganglion of *O. niloticus* were pseudounipolar with large round cell bodies, while minority was bipolar with oval cell bodies (Kerem *et al.*, 2005).

The present study selected the sea catfish *Plotosus japonicus* Yoshino & Kishimoto 2008 (Fig. 2), as the experimental model since they possess four pairs of barbels or whiskers; nasal, maxillary, lateral mandibular and medial mandibular, which are especially well-endowed with taste buds and serve as important exploratory organs utilized for localization of food objects. The taste buds are distributed across the entire epithelium of the barbel along its entire length and density of buds increases toward the tips of the barbel (Kiyohara & Tsukahara, 2006). *P. japonicus* do not actively trail food substances with their barbels, but instead, they usually keep the barbels passively extended forward (Kiyohara & Tsukahara, 2006). These barbels, which possess the largest taste buds among the rest of the body, are innervated by mixed nerves containing branches of the trigeminal (Vth) and the facial (VIIth) fibers. The trigeminal nerve conveys general cutaneous and proprioceptive information, while the facial nerve innervates taste buds and convey chemosensory as well as mechanosensory information (Kiyohara *et al*., 1999). Though the trigeminal and facial fibers are intimately mixed in the ganglia, it is now possible to determine the distribution of the fibers in the anterior complex ganglion, with the advance of post mortem tracing application of carbocyanine dyes. Besides having abundance of taste buds on its barbels, *P. japonicus* also possess taste buds on its entire body surface and fins. These taste buds on the body and fins are innervated by the recurrent branch of the facial nerve. The facial recurrent ganglion is independent of the anterior complex ganglion. This anatomical feature allowed us to focus on the polarities in addition to the general morphology of the taste neurons and

their organization in the anterior complex ganglion and the independent recurrent ganglion.

The present study revealed that the double labelling experiments using two different fluorescent carbocyanine dyes to the descending trigeminal root and facial lobe, respectively in isolated, paraformaldehyde-fixed brains suggested that both the cell bodies of the trigeminal (red) and facial (green) nerves of *P. japonicus* are bipolar neurons. The cell bodies of trigeminal and facial nerves are distributed in separated area in the ganglion; the trigeminal and facial cell bodies are distributed mainly in the rostral and caudal parts of the anterior complex ganglion, respectively, with minor overlappings. This showed that distinct somatotopical organization in the trigeminofacial complex ganglion of *P. japonicus*. The present work also revealed that the recurrent facial neurons are frequently observed to be bipolar with thick peripheral and thin central fibers. Besides, it is observed that the trunk and pectoral fin cell bodies are independently located in the ganglion, appearing to form groups of various shapes and sizes. Therefore, no distinct somatotopical organization is present in the recurrent ganglion of *P. japonicus*. The trunk and pectoral fibers of the recurrent nerve terminate in the anterolateral and intermediate part of the trunk-tail lobule, respectively, showing distinct somatotopy in the primary taste center. To reveal the somatotopic map in the peripheral regions of the facial ganglion, two different dyes, DiA and DiI and were applied to the nasal barbel lobule (NBL) and to either the maxillary lobule (MXL) or medial mandibular lobule (MML), respectively. In the facial ganglion, the cell bodies of the nasal barbel and medial mandibular barbel are distributed in separate areas. The nasal barbel cell bodies are located at a more peripheral location, while the medial

mandibular cell bodies are located at a more central location. Minor overlapping is observed between the two groups of cell bodies

Chapter 2: Materials and Methods

2.1 Experimental animals

Sixty *P. japonicus*, ranging in standard body length from 10.3 – 18.5 cm and in body weight between 19.8 - 56.6 g were used in this study. The fish were collected with net traps at the coast near Nagashima, Kagoshima Prefecture, Japan and transported to the Division of Chemistry and Bioscience, Graduate School of Science and Engineering, Kagoshima University. The fish were held in aquaria filled with recirculating artificial seawater at 22-24ºC under a 12:12 light:dark cycle.

2.2 General morphological experiments

The fish were anaesthetized with trichaine methanesulfonate (MS222; approximately 0.01 % w/v) and perfused through the conus arteriosus with 4 % paraformaldehyde in 0.1M phosphate buffer for light microscopy and mixtures of 2% glutaraldehyde and

1.6% paraformaldyhyde in the same buffer for Epon sections. The brains and/or recurrent nerve ganglia were removed from the fixed fish under a surgical microscope and were processed with routine methods of light or electron microscopy (Kitoh *et al.*, 1987). For transmission electron microscopy, the target tissues were postfixed in 2 % osmium tetra oxide buffered with 0.005 M veronal acetate. They were then stained in block by uranyl acetate, dehydrated in ethanol and embedded in epoxy resin. Sections were stained with uranyl acetate and lead cirate, and observed on the JEPL 100 CX electron microscope. Semi-thin sections of about 0.5 μm were cut and stained with toludine blue.

2.3 Experiments for revealing the distribution of trigeminal and facial bodies in the anterior complex ganglion

The superficial ophthalmic, upper lip, maxillary barbel, lower lip, mandibular barbel, and hyomandibular rami are heterogenous, containing mixed populations of trigeminal and facial fibers (Fig.3). In order to reveal the morphology and distribution of trigeminal and facial sensory neurons in the anterior complex ganglion, two different fluorescent carbocyanine dyes, DiI and DiA (Molecular Probes, OR, USA) were applied to the descending trigeminal root and facial lobe, respectively in isolated, paraformaldehyde-fixed brains (Fig. 4). The fish were anesthetized with the diluted MS222. They were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and the brains were removed with the trigeminofacial complex root attached. The brains were embedded in 3% agar, keeping the position of the root perpendicular to the brain. Small crystals of DiI and DiA were applied to the

descending trigeminal root and facial lobe, respectively. The agar was poured to the surface of the brain to keep the dyes in place and to prevent accidental diffusion of dyes to other regions of the brain. The agar blocks were hardened overnight in the fixative. They were then placed in a 37 centigrade oven for 10-60 days to promote the diffusion of dyes. After the diffusion period, the brain was removed from the block, trimmed off and embedded in egg yolk. The egg block was hardened overnight in the same fixative. The hardened block was serially sectioned on a vibratome and the sections were examined under epifluorescence microscope.

2.4 Experiments for revealing the somatotopy organization of the facial neurons in the anterior complex ganglion

In order to reveal the morphology and distribution of the facial sensory neurons of the barbel nerves in the facial ganglion, two different fluorescent carbocyanine dyes, DiI and DiA (Molecular Probes, OR, USA) were applied to either maxillary or medial mandibular and nasal barbel lobules, respectively in isolated paraformaldehyde-fixed brains (Fig. 5). The procedures for labelling the barbel neurons with the tracers are essentially the same as described previously. Small crystals of DiI and DiA were applied to maxillary and nasal barbel lobules in two specimens and to medial mandibular and nasal lobules in three specimens.

2.5 Experiments for labelling the cell bodies of recurrent neurons and their central axons

The recurrent nerve sends fibers to the entire trunk surface and fins via two major rami, trunk and pectoral fin branches (Fig. 3). Dextran amine-tetramethylrhodamine (D-Rho, MW3000, Molecular Probes), dextran amine-Alexa Fluor 488 (D-Alexa, MW10000, Molecular Probes) and horseradish peroxidase (HRP, Toyobo Grade1-C) were used as neural tracers. The procedures for labelling the recurrent neurons with the tracers are essentially the same as described previously (Kiyohara & Caprio, 1996). In brief, the fish were anesthetized with the diluted MS222, covered with wet paper and placed on a plate in a Plexiglas chamber. Aerated, artificial sea water was perfused through the mouth and over the gills during the surgery. Supplemental MS-222 was introduced into the perfusion solution as necessary to maintain a constant level of anaesthesia. In six fish, dextran- tetramethylrhodamine or HRP was applied in crystals to the central cut stumps of the whole recurrent nerve (Fig. 6). In nine fish, the trunk and pectoral branches were simultaneously exposed and cut. D-Rho and D-Alexa were applied to the cut stumps of the trunk and pectoral branches, respectively (Fig. 7). In three fish, the trunk branch was exposed at a level near the caudal fin (Fig. 8) and cut. D-Alexa was applied to the central cut ends.

After application of the tracers, pieces of gelfoam (Astellas, Tokyo, Japan) were inserted into the wound and the skin was closed with silk thread sutures. The fish were returned to the aquaria and maintained for 3-7 days. After this period, the fish were again anaesthetized deeply with MS-222 and perfused transcardially with the fixative as mentioned above. The brains were surgically removed with the recurrent facial nerve intact from the fixed specimen, embedded in egg yolk, postfixed overnight in the

fixative, and immersed in cold buffer with 20% sucrose. The egg yolk blocks were serially sectioned at 40 or 50 μm in horizontal or transverse planes on a freezing microtome, and sections were collected in 0.1 M phosphate buffer. Sections treated with the dextran amines were thawed on microslides, and, after drying, the slides were mounted. Sections treated with HRP were incubated for the demonstration of HRP reaction product according to the tetramethyl benzidine method (Mesulam, 1978) and processed as mentioned above.

2.6 Observation of histological preparations and analysis of data

The anterior complex ganglion, whole recurrent ganglion and brains labelled with the fluorescent carbocyanine dyes, DiI and DiA, and dextran amine were viewed under a dissecting epifluorescence microscope (MZFL111; Leica Heidelberg GmbH, Mannheim, Germany) with a rhodamine or dextran- Alexa filter. Series of sections were viewed under Nikon (Eclipse 80i; Tokyo, Japan) with a rhodamine or dextran-Alexa filter. The sections were also examined on confocal laser microscopes (Nikon A1si-90i, Tokyo, Japan and Leica TCN NT; Leica Heidelberg GmbH, Mannheim, Germany). Series of confocal pictures of the ganglion sections taken with 1 μm interval were processed with an image analysis system (Quantiment 500, Leica Microsystems) to measure cell size and to obtain a three-dimensional reconstruction.

Chapter 3: Peripheral Trigeminal and Facial Sensory Pathways

Prior to the nerve-tracing experiments, the peripheral distribution of the trigeminal and facial nerve and the course of the trigeminal, facial and facial recurrent nerves inside the cranium were examined under a dissecting microscope in two freshly killed and three fixed specimens.

Eight peripheral rami were identified in *P. japonicus* as the major pathways that project into the medullary FL (Fig. 3). The terminology for these individual branches reflects the taste receptive fields of the body that each branch innervates. The major rami include the superficial ophthalmic, palatine, upper lip, maxillary barbel, lower lip, mandibular barbel, hyomandibular, and recurrent rami. The superficial ophthalmic distributes fibers to taste buds above the eye. The palatine, maxillary (divides into two branches) and mandibular rami (divides into four branches) innervate taste buds of the rostral palate, upper lip and lower lip, respectively. The three rami of the hyomandibular trunk innervate taste buds on the operculum, branchiostegal rays, and in the lower cheek region. A facial recurrent ramus distributes fibers to taste buds on the trunk and pectoral fin via two rami, the lateral recurrent ramus and pectoral recurrent ramus; the general cutaneous innervation to this territory comes from segmental spinal nerves as in other vertebrates (Herrick, 1901). All rami except the palatine and recurrent are heterogeneous, containing not only mixed populations of fibers serving taste and other modalities, but also sometimes motor axons.

3.1 Anterior complex ganglion

The organization of the anterior complex ganglion is complicated in teleosts. Herrick (1901) described that the anterior ganglion advocate a separate ontogeny and position for the different components of the complex; trigeminal (gasserian or trigeminal ganglion), facial (geniculate ganglion) and lateralis (acoustico-lateral ganglion). Similar organization has been observed in the sea catfish, where the trigeminal and facial ganglion are intimately associated and along with the anterior lateral line ganglion are described as forming an anterior ganglionic complex (Fig. 9, 10) (Finger, 1976; Kiyohara et. al., 1999). However, despite the complex, interdigitated nature of the ganglion, the central roots of the trigeminal, facial and lateral line nerves are distinct and penetrate the brainstem separately to project at each primary center in the medulla of *P. japonicus* (Fig.11).

3.2 Independent recurrent facial ganglion

The recurrent ramus consists of pure facial fibers at its point of exit from the skull although it receives spinal sensory fibers at each dermatome as described in siluroid catfishes (Herrick, 1901; Finger *et al.*, 1991). The recurrent ramus supplies taste buds distributed along the whole trunk surface caudal to the operculum and fins via two major divisions, the trunk and pectoral fin branches (Fig. 3: point 8a and 8b).

After entering the skull through a foramen, the recurrent ramus exists as a single root above the most caudal region of the FL and runs rostrolaterally over the FL. After

passing over the entrance level of the anterior ganglion root to the medulla (Fig.11a), the ramus turns dorsoventrally approximately 0.5 mm rostral to the anterior limit of the FL in 15-cm long specimens. The ramus then penetrates between the optic tectum and trigeminal root and anastomoses the facial root at its dorsal portion. The recurrent fibers turn caudally and travel along with other facial fibers to enter ventrodorsally in the medulla, caudal to the entrance of trigeminal nerve, as the name of "ramus recurrent" indicates. The entire pathway of the recurrent root inside the cranium was also confirmed with nerve tracing experiment.

The major part of the recurrent facial root is recognized as a horizontally flat-shaped structure to form the ganglion (Fig.11b). The maximum width and thickness of the ganglion are approximately 1370 μm and 800 μm, respectively, in a 20-cm-long specimen (Fig. 11a, b). A dorsal surface view of the whole ganglion stained with toulidine blue shows that cell bodies are visible in some parts of the ganglion (Fig. 11b). However, an intraganglionic observation using serial sections of 15- and 18-cm long specimens shows that the cell bodies are scattered throughout the 2000-2500 μm length of the recurrent root.

Chapter 4: Morphology of Sensory Neurons in *P. japonicus*

4.1 Morphology of the trigeminal and facial sensory neurons in the anterior complex ganglion

To identify the location of the trigeminal and facial cell bodies in the anterior complex ganglion, two carbocyanine dyes, DiI and DiA were applied to the facial sensory root and descending trigeminal root, respectively in eight preparations. DiI applied to the facial sensory root labelled the facial cell bodies, while DiA applied to the descending trigeminal root labelled the trigeminal cell bodies in the anterior complex ganglion. The results of the double-labeling experiments suggest that the cell bodies of the trigeminal and facial nerves are distributed mainly in the rostral and caudal parts of the ganglion, respectively, with minor overlapping. The labeled trigeminal cell bodies have various shapes of cell bodies, showing round and elongated oval shapes (Fig.12). On the other hand; the labeled facial cell bodies are round or egg- like in shape (Fig.13). Majority of the trigeminal and facial cell bodies are predicted to be bipolar neurons. In this present study, most labeled facial neurons in the anterior complex ganglion are frequently observed to send thick peripheral and thin central fibers. The trigeminal neurons are also frequently observed to send coarse peripheral fibers, however it was difficult to detect the thin central fibers. The diameter of the peripheral and central fibers of the trigeminal neurons is also observed to have a bigger diameter as opposed to those of facial neurons.

The size of the trigeminal and facial cell bodies are measured in four different preparation specimens (17.2-, 20.0- 20.5-, 21.2-cm long). In each fish, three hundred labelled trigeminal and facial cells with complete nucleoli were selected in horizontal sections of the ganglion and diameters were measured. The diameter of the trigeminal cell bodies ranged between 14.7 – 45.1 μm, with the average long and short diameters of 26.1±5.8 μm and 21.6±5.4 μm (n-600), respectively (Fig.14). On the other hand, the diameter of the facial cell bodies ranged between 16.1 – 27.6 μm, with the average long and short diameters of 22.5±2.2 μm and 18.9±2.6 μm (n-600), respectively (Fig.14). The profiles of the long diameter for the trigeminal and facial cell bodies showed non-unimodal distribution in the trigeminal cell bodies but a unimodal distribution in the facial cell bodies, respectively (Fig.15).

4.2 Morphology of the recurrent facial neurons in the independent recurrent ganglion

To specify the location of the cell bodies of the recurrent neurons, the tracers were applied to the central cut end of the whole recurrent ramus in five preparations (Fig. 3, point 8). In each preparation, retrogradely labelled cell bodies were observed only in the recurrent ganglion (Fig. 16a) and not in any regions of the anterior ganglion. The labelled cells are round or oval in shape and the majority was classified as bipolar neurons. Labelled cells were frequently observed to send thick and thin fibers from opposite poles (Fig. 16b). The poles are arranged to correspond with the rostrocaudal axis of the fish body. For most labelled cells, the caudal extensions were easily observed and their courses from the cell bodies were traced in serial sections. No

ramification was detected. The rostral fibers were not detected in some labelled cells; however, the thin rostral fibers were possibly removed or separated from the cell bodies during histological manipulation of the tissue. The diameters of peripheral and central fibers without myelination are 3-6 μm and less than 2 μm, respectively.

The size of the cell bodies was examined in three different preparation sizes (10.3-, 15.1-, 18.3-cm long). In each fish, one hundred labelled cells with complete nucleoli were selected in horizontal sections of the ganglion and diameters were measured. The long and short diameters are 22.8±2.2 μm and 18.5±2.9 μm in the small fish (10.3 cm), 25.9±1.9 μm and 21.1±1.7 μm in the intermediate fish (15.1 cm) and 25.1±1.7 μm and 19.8±2.0 μm in the large fish (18.3 cm). In each fish, linear relationships between the long and short diameters of the cells were observed (Fig. 17). The profiles of the long diameter for the three fish showed a unimodal distribution and a shift towards the right to some extent in the two larger fish than in the small one (Fig. 18), indicating that taste neurons increase in size as the fish grow larger at least up to 15.2 cm in total length.

To further examine the morphology of the recurrent cell bodies, five ganglia were examined in Epon sections with light and electron microscopy. Most of the recurrent ganglion cells were again confirmed to be bipolar neurons. Observations of serial sections show the presence of thin central and thick peripheral processes in the majority of cells. The cell bodies send peripheral thick processes from the caudal pole and central thin processes from the rostral pole. With the exception of their initial segments, both types of processes are myelinated. The diameter of central fibres is less than 2 μm as

observed in labelled neurons. The cell bodies contain large, central, round nuclei. The nuclear envelope is either smooth or wavy. One nucleolus is usually noted in the nucleus and seen in the centre or close to the periphery. In the cytoplasm of the cells, prominent granular endoplasmic reticulum, mitochondria and Golgi apparati are present. The cells frequently make clusters where cell bodies are adjacent to each other. Each cell is completely wrapped by thin structures composed of several membranous layers of satellite cells. The nucleus of a satellite cell is located in the exterior surface of the soma or the proximal part of peripheral fibers.

Chapter 5: Organization of Sensory Neurons in the Ganglion of *P. japonicus*

5.1 Organization of the trigeminal and facial neurons in the anterior complex ganglion

To determine the peripheral distribution of the trigeminal and facial sensory neurons in the anterior complex ganglion, DiI and DiA were applied simultaneously to the descending trigeminal root and facial sensory root, respectively in eight specimens. The anterogradely labelled cell bodies of the trigeminal and facial neurons were examined in series of horizontal sections of each specimen. The cell bodies of trigeminal (red) and

facial (green) nerve are distributed in one continuous cell aggregation in the anterior complex ganglion; the trigeminal cell bodies at a more central while the facial cell bodies at a more peripheral location with some minor overlapping (Fig.19). Each group is independent and thus does not contain both types of cells; although in some regions the two types of cells are intermingled (Figs.20 & 21). No double-labelled cells (yellow) were observed, although some cells appeared yellow in part due to the overlapping of red and green cells in a 50 μm section. Distal to their ganglia, the trigeminal and facial nerve branches fused and ran toward the periphery.

5.2 Somatotopical organization of the facial neurons in the facial ganglion

To determine whether somatotopic organization occurs in the facial ganglion, DiI and DiA were applied to either the maxillary or medial mandibular and nasal barbel lobules, respectively, in isolated paraformaldehyde-fixed brains. The anterogradely labeled cell bodies of the trigeminal and facial neurons were examined in series of horizontal sections of each specimen. When DiA and DiI were applied to the nasal barbel and medial mandibular barbel lobules, respectively, in two specimens, labeled innervating the nasal barbel are distributed towards the peripheral of the ganglion, while the cell bodies innervating the medial mandibular barbel are distributed towards the more central region of the ganglion (Fig.22). Some overlapping was observed in the facial ganglion. Labeled facial neurons sending fibers to the nasal or mandibular barbels were also bipolar (Fig.23).

In another three specimens, when DiA and DiI were applied to the nasal barbel and maxillary barbel lobules, respectively, similar distribution of cell bodies were observed in the facial ganglion of *P. japonicus*. Labeled cell bodies innervating the nasal barbel are distributed towards the peripheral of the ganglion, while the cell bodies innervating the maxillary barbel are distributed towards the more central region of the ganglion. Some overlapping was observed in the facial ganglion. According to these results, topography organization was observed within the facial ganglion of *P. japonicus*.

5.3 Organization of recurrent facial neurons in the independent recurrent ganglion

To determine whether a somatotopic organization occurs in the recurrent ganglion, D-Rho and D-Alexa were applied simultaneously to the trunk and pectoral recurrent rami, respectively, in six specimens. The retrogradely labelled cell bodies of the trunk and pectoral neurons were examined in series of horizontal or coronal sections of each specimen. Labelled trunk (red) and pectoral (green) cell bodies appeared throughout the ganglion as shown in twelve serial horizontal sections (Figs. 24 & 25). No double-labelled cells (yellow) were observed, although some cells appeared yellow in part due to the overlapping of red and green cells in a 50 μm section (Fig. 24). The trunk (red) and pectoral (green) cell bodies are mainly arranged in groups of irregular shape and distribution. Each group is independent and thus does not contain both types of cells, although in some regions the two types of cells are intermingled as shown in Fig. 25 (sections 8-12). The groups are intermingled with each other throughout the

recurrent ganglion. It was difficult to segregate the labelled cells into two distinct populations. When the tracer was applied in three specimens to the main trunk ramus at a level near the caudal fin (Fig. 26), labelled cells were found to scatter from the rostral to caudal end of each ganglion. No somatotopy was evident within the recurrent facial ganglion.

Chapter 6: Topographical Projections of the Recurrent Nerve Fibers to the Trunk Tail Lobule in the Facial Lobe of *P. japonicus*

The facial, glossopharyngeal and vagal sensory fibers project to visceral sensory areas in the medulla oblongata to form a primary taste center. When facial taste buds are numerous, the anterior portion of the medulla is enlarged to form a facial lobe (FL). On the other hand, an increase in the number of TBs along the gills and pharyngeal cavity results in a hypertrophy of the posterior portion of the medulla into a vagal lobe (VL). The FL varies in appearance and organization among fish species. Histological examination in various species of fish has revealed at least four types of FL organization: 1) simple columnar in fish with a poorly developed sense of taste; 2) fused type in cyprinids; 3) lobular type in catfish; and 4) laminar type in goatfish (Kiyohara, 1988).

The primary taste center of *P. japonicus* is located in two pairs of extraordinary enlargements, the FL and the VL, on the dorsal surface of the medulla oblongata, (Figs. 27, 28A). The FL and VL receive projections from the facial, and glossopharyngeal-vagal nerves, respectively. Previous study by Kiyohara *et al.* (1996) provided anatomical evidence that the FL of *P. japonicus* is more developed than that of any other species of catfish yet studied. Histological examination showed that the FL of *P. japonicus* is subdivided by fascicles of nerve fibers into five distinct lobules constituting five longitudinal lobules, which extends rostrocaudally in the medulla oblongata; nasal barbel (NBL), maxillary (MXL), lateral mandibular (LML), medial mandibular lobules (MML) and trunk-tail lobule (TTL) (Fig. 28A) as reported previously (Marui *et al.*, 1988; Kiyohara *et al.*, 1996). The TTL is located dorsolateral to the barbel lobules and is dorsoventrally flattened.

In order to reveal the central projections of the whole recurrent nerve to the trunk tail lobule in the FL, dextran- tetramethylrhodamine or HRP was applied in crystals to the central cut stumps of the whole recurrent nerve. The central projections of the whole recurrent nerve were clearly traced in six fish. In each specimen, following the application of tracers to the whole recurrent nerve, heavily labelled fibers were found in the ipsilateral side of the medulla, but cell bodies were never labelled. The labelled fibers enter the FL by way of the dorsolateral part of the facial sensory root and terminate anteroposterioly in the TTL. No labelled fibers were found in the primary afferent areas of the brain stem other than the FL. The terminal field of the recurrent nerve clearly revealed a rostrocaudal extension of the TTL at its caudal end (Fig. 28B).

The TTL lies approximately in the anterior half of the FL. The recurrent ramus distributes fibers to taste buds on the trunk and pectoral fin via two rami, the trunk and pectoral recurrent ramus. Following simultaneous application of D-Rho and D-Alexa to the trunk and pectoral fin branches, respectively in the same specimen, results showed distinct topographic projections into the TTL (Fig. 29). The trunk fibers (red) are located rostral and lateral to the pectoral fibers (green) in the sensory root of VII (Figs. 29, 30). The terminal field of the trunk branch begins at the anterior tip of the TTL and extends through the lateral half of TTL. Application of D-Rho to the trunk branch at a caudal position near the tail (Figs. 3, point 8c) labelled only the anterior portion of the terminal filed of the trunk branch. The terminal field of the pectoral fin branch starts more caudally than that of the trunk ramus and covers the medial half in the caudal TTL (Fig. 31).

Chapter 7: Discussion

The present study shows five principal findings concerning taste neurons in the *P. japonicus*: (1) The trigeminal, facial and facial recurrent neurons are bipolar with thick peripheral and thin central processes; (2) Distinct separations between the trigeminal and facial cell bodies in the anterior complex ganglion (3) Distinct separation between facial cell bodies innervating different barbel lobules in the facial ganglion (4)

No distinct separation between the location of trunk-tail and pectoral fin neurons occurs in the recurrent ganglion; and (5) the trunk-tail and pectoral fin neurons project somatotopically to the TTL of the FL.

7.1 Type of sensory neurons in *P. Japonicus*

According to S. R Cajal, somatosensory neurons in the spinal ganglia are bipolar in fish while they are pseudo-unipolar in other vertebrates (Mannen, 1992). The ontogenesis of pseudo-unipolar neurons is well-documented (Kandel, 1991). Pseudo-unipolar neurons initially appear as bipolar cells, but central and peripheral processes fuse to form a single process that emerges from the cell body and splits into two processes; one process courses to the periphery, the other to the spinal cord. The pseudo-unipolar neurons conduct action potentials without passing through the perikaryon along a single fiber to the central nervous system faster than bipolar neurons. Ariens-Kappers *et al*. (1936) summarized that visceral (taste) afferent neurons are pseudo-unipolar in most vertebrates. A bifurcation of the single process originating from each soma of taste neurons in the ganglia occurs in various species of mammals, such as mouse *Mus musculus* (Boudreau *et al.*, 1971), guinea pig *Cavia porcellus* and monkey *Macaca irus* (Kitamura *et al.*, 1982). Nagai & Matsushima (1990) successfully labelled cell bodies and their single processes in the glossopharyngeal-vagal ganglia of the axolotl with cobaltic-lysine method. They assumed that the majority of the neurons to be pseudo-unipolar without showing any ramification of the process to send central and peripheral extensions. The present study in *P. japonicus* shows that the thick peripheral and thin central processes frequently originate from the trigeminal, anterior facial and

facial recurrent cell bodies at opposite poles (Figs. 16, 22) and concludes that the majority of taste neurons are bipolar in accordance with the phylogenetic principle of sensory neurons, suggesting that action potentials slow down after reaching the cell bodies. However, somatosensory neurons in the trigeminal ganglion of goldfish *Carrasius auratus* are reported to be large, pseudounipolar cells (Puzdrowski, 1988). In another study in tilapia *Oreochromis niloticus* (L. 1758), the trigeminal ganglion cells are reported to be mostly pseudo-unipolar with minor populations of bipolar neurons (Kerem *et al*., 2005). In *O. niloticus*, the single processes originating from the cell bodies are clearly shown, but their ramification to send peripheral and central fibers, which is direct evidence of being pseudo-unipolar, is not shown. Therefore, the polarity of the somatosensory neurons in fish remains to be further clarified. The greater diameter of the peripheral fibers than the central ones in *P. japonicus* coincides with the results of physiological recordings in the puffer *Takifugu pardalis* (Temminck & Schlegel 1850) where neural activities recorded from the peripheral facial nerve, palatine ramus, are much greater in amplitude than those recorded from the central facial root (Kiyohara *et al*., 1975, Kiyohara *et al*., 1985).

The present study supports the classical idea that the primordial type of sensory neurons is a bipolar cell with peripheral thick and central thin fibers in vertebrates. The present and previous findings suggest two possible evolutional pathways of the neurons that enable them to propagate impulses to the brain with a faster speed. One is from the primordial type to the pseudo-unipolar type and the other is from the primordial type to the lateral line and auditory types. They are present throughout vertebrates and bipolar, but both peripheral and central fibers are thick and sheathed by rich myelination.

7.2 Relation between the geniculate and recurrent facial ganglia in fish

The term geniculate ganglion in vertebrates is defined as a neural tissue mass that includes somata of facial sensory neurons. Independence of the recurrent ganglion from the geniculate ganglion or the anterior complex ganglion in *P. japonicus* is the anatomical base for the present experiment to examine the ganglionic organization. This was first shown in siluroid catfish by Herrick (1901) who first named this nerve "ramus lateralis accessorius" and described its intracranial or recurrent course from the medulla to the posterior foramen of skull. According to Herrick, the rostral end of the distribution of the cell bodies of the recurrent nerve is closer to the main facial sensory root or geniculate ganglion in siluroid than in *P. japonicus* where the rostral end is 0.5 mm or more distal to a level where the recurrent ramus arises from the dorsal portion of the facial root. The distinct separation of the recurrent ganglion from the geniculate ganglion is also shown in another catfish, *Silurus asotus* L. 1758 (Kiyohara & Kitoh, 1994), although a separate description of the two ganglia is not fully shown in either *I. nebulosus* and *I. natalis* (Finger, 1976) or *I. punctatus* (Northcutt *et al*., 2000) catfishes. The separation of these two ganglia is suggestive that a somatotopical relationship is present between the head and trunk-tail regions. In contrast to catfishes, gadids, such as *Gaidropsarus mediterraneus* (L. 1758), have a single tissue mass as a geniculate ganglion which contains both ordinal and recurrent somata of facial neurons (Herrick, 1900; Kotrschal and Whitear, 1988). Species without taste buds on the body surface and fins lack the recurrent root or ganglion (Freihofer, 1963).

7.3 Organization of the trigeminal and facial ganglion in the anterior ganglion

In previous studies, the trigeminal and facial ganglions of teleosts are regarded as part of the trigeminofacial ganglionic complex, whereas the trigeminal and geniculate ganglion cells are of discrete populations in the adult siluroid catfish (Herrick, 1901). Later, it was reported that the trigeminal, facial and anterior line ganglion are part of the anterior ganglion (Ladacre, 1910; Finger, 1976; Luiten, 1979; Puzdrowski, 1988). The idea of the anterior ganglion is derived from the study by Ladacre (1910) that mentioned that the trigeminal ganglion fuses early in embryogenesis with the facial and anterior line ganglia to form the anterior ganglion which is found in the adult catfish (Silurodei, Siluriformes). It has also been reported that the anterior ganglion of the adult catfish (Finger, 1976) and goldfish (Puzdrowski, 1988) can be distinguished on the basis of cell size, their segregated positions and connections to their roots by Nissl preparations. In a recent study in tilapia, Kerem *et. al*, (2004) reported that the trigeminal, facial and anterior lateral line ganglia can be distinguished easily as they are independent from each other in the anterior ganglion. The trigeminal ganglion cells of tilapia formed one cell aggregate, which is not continuous with the facial and anterior line ganglia.

Following application of DiI and DiA to the descending trigeminal tract and facial sensory root, respectively, results showed that labelled trigeminal and facial ganglion cells formed one cell aggregate which is continuous in the anterior complex ganglion of *P. japonicus*. The trigeminal ganglion cells are distributed mainly in the central part of the anterior ganglion, while the facial ganglion cells are distributed more

peripherally in *P. japonicus* (Figs.19, 20). A minor overlap between the trigeminal and facial cell bodies was observed. This result showed that the trigeminal and facial ganglia in *P. japonicus* is not separately located as observed in tilapia, but formed one continuous cell aggregation in the anterior complex ganglion. This result may also suggest that separation into the two groups is observed between the trigeminal and facial cell bodies in the anterior ganglion of *P. japonicus*.

Despite the apparent overlap of the gustatory (facial) and tactile (trigeminal) system in the anterior complex ganglion, the trigeminal and facial fibers have very different functions. The trigeminal nerve fibers are distributed in the dermis, and convey mechanosensory as well as proprioception information. On the other hand, the facial nerve fibers innervate taste buds, convey chemosensory and mechanosensory information. The latter is also sensitive to potent chemical stimuli (proline, betaine and alanine) and minute pH changes in the water. It is also reported by Kiyohara *et. al*., 1999 that regardless of the complex, interdigitated nature of the anterior ganglion, the central roots of the trigeminal, facial and the anterior lateral line nerves are distinct and penetrate the brainstem in the usual order and arrangement.

7.4 Somatotopic organization in the anterior facial ganglion

In previous study by Kiyohara et. al., 1985, it was observed that highly distinct topographical arrangement in the facial lobe of *P. japonicus* when central projections of seven facial nerve branches (nasal, barbel, palatine, maxillary, maxillary barbel, mandibular, mandibular barbel and recurrent) were examined. The facial fibers of each

branch ended in specific area of the lobe, medial mandibular, lateral mandibular, maxillary and nasal barbel respectively, showing somatotopical organization representing each of the barbells, lips and head.

The present study showed that somatotopical organization occurred in the facial anterior ganglion of *P. japonicus*. The findings in *P. japonicus* indicates that the nasal, medial mandibular, and maxillary barbel ganglion cells distribute in different areas of the facial ganglion with minor over lapping. This results shows that the somatotopical organization of the facial barbel lobules are maintained in the peripheral ganglion level of *P. japonicus.*

7.5 Organization of the recurrent facial ganglion

The present study shows that trunk and pectoral fin cell bodies in *P. japonicus* are independently located throughout the recurrent ganglion and each type of cell appears to distribute as different groups of various shapes and sizes (Fig. 25). However, spatial segregation could not be detected within the ganglion of the trunk-tail versus pectoral fin somata. Somata innervating the caudal one-fourth of the body are also scattered widely throughout the ganglion. These results suggest that no distinct somatotopy occurs in the recurrent ganglion of *P. japonicus*. This result is consistent with previous attempts to discern such topography in the taste ganglia of fishes, such as for gadids (Kotrschal & Whitear, 1988) and blenniids (von Bartheld & Meyer, 1985) and for amphibians, such as the frog *Rana catesbeiana* (Hanamori & Ishiko, 1983) and the axolotl (Nagai & Matsushima, 1996). In the mouse, ganglion cells innervating the

tongue and palate were differentially concentrated in lateral and rostral regions of the geniculate ganglion, respectively (Zaidi & Whitehead, 2006). Within the tongue or palatal regions of the ganglion, however, no distinct topographical relation was present. The results in the mouse and *P. japonicus* may suggest that somatotopy in the taste ganglion occurs between the larger peripheral regions, but not between small adjacent regions.

For the trigeminal ganglion which sends fibers to head regions along with facial fibers, a relatively distinct somatotopical organization is observed in hagfish *Eptatretus burgeri* (Girard 1855) (Nishizawa *et al*., 1988), lampreys *Lampetra japonica* (Martens 1868) (Koyama *et al.*, 1987) and *O. niloticus* (Kerem *et al*., 2005). For example, Kerem *et al*., (2005) examined the presence of somatotopy in the trigeminal ganglion of *O. niloticus* by labeling the ophthalmic, maxillary and mandibular nerves with bioacytin. They revealed that the trigeminal ganglion cells were somatotopically organized following the dorsoventral order of the three branches with minor overlapping of the maxillary and mandibular cells.

7.6 Somatotopical projections of the recurrent nerve fibers to the facial lobe

In contrast to the organization in the recurrent ganglion, the trunk and pectoral fin in *P. japonicus* are represented separately in the TTL of the FL (Figs. 29 & 31) as shown in other species of catfishes, such as *A. felis* (Kiyohara & Caprio, 1996) and *I. punctatus* (Hayama & Caprio, 1989). The terminal filed of the pectoral fin branch is caudal to that of trunk branch in the three species of catfishes examined suggesting an antero-posterior

body axis is represented in the postero-anterior extent of FL. Separate representation of the trunk and pectoral fins is also revealed in the FL of gadids (Kotrschal & Whitear, 1988) and carp (Fukusako *et al*., 1993).

7.7 Cell body size of sensory neurons

Herrick (1901) differentiates the trigeminal (gasserian) ganglion and facial (geniculate) ganglion on the basis of cell size. According to Herrick (1901), the trigeminal ganglion contains a mixture of large and small cells, while the facial ganglion is composed of small cells only. In the bullhead catfish, both large and small cells were observed in the anterior ganglion which contains a mixture of elements from the trigeminal, facial and anterior lateral line nerves. Following the classical scheme (Ladacre, 1910), the small ganglion cell masses constitute the facial (chemosensitive) nerve portion while the mixed size population of cells is trigeminal (tacto-sensitive) (Finger, 1976). However, the result of Finger (1976) did not provide the basis of separation of trigeminal from facial nerve components in the anterior ganglion of bullhead catfish.

In this present study, by using two different fluorescent carbocyanine dyes, DiI and DiA, which were applied to the descending trigeminal root and facial lobe, respectively, provided the basis of separation between the trigeminal and facial sensory neurons in the anterior complex ganglion of *P. japonicus*. In the anterior ganglion of *P. japonicus,* the trigeminal cell neurons varies in size, some small and some big, ranging from 14.7-45.1 μm in diameter (Figs. 12, 14, 15). On the other hand, the facial cell neurons showed a unimodal distribution of cell size, ranging between 16.1-27.6 μm in

diameter (Figs. 13, 14 15). This result is in concordance with Herrick (1901) and Ladacre (1910). The present result in *P. japonicus* supports the neuroanatomical principle that the mechanosensory neurons have larger somata compared to those of chemosensory neurons. The mechanosensory cells, for example the octaval and lateral line nerve cells have equally thick peripheral and central fibers, hence the big cell bodies (Figs. 32, 33). On the other hand, the chemosensory cells usually have thick peripheral and thin central fibers and smaller cell bodies. The lateral line neurons of *P. japonicus* have big cell bodies, ranging from 32.2-62.5 μm with thick central and peripheral fibers (Fig. 33).

The recurrent facial neurons in *P. japonicus* (24.8 μm long diameter on average) are characteristic of a unimodal distribution in cell size (Figs.17, 18). A similar distribution is also shown in taste neurons in the axolotl (Nagai & Matsushima, 1996). In the geniculate ganglion of gadids, three different sizes of cell bodies, small (6-15 μm in diameter), medium (18-24 μm) and large (> 25 μm), are present (Kotrschal & Whitear, 1988). *P. japonicus* lack the small size of somata which are assumed to supply in the gadids the vibratile anterior dorsal fin, a specialized chemosensory organ containing many solitary sensory cells (Kotrschal & Whitear, 1988). Since there is a positive correlation between fiber diameter and cell size (Kotrschal & Whitear, 1988), a unimodal distribution is also likely in the diameters of recurrent fibers. Finger et al. (1991) examined the postlarval grows of recurrent fibers in channel catfish and showed that the myelinated fibers increased in size as the fish grew. This result supports the present finding that cell bodies of the recurrent nerve are greater in size in the large specimen of *P. japonicus* than in the small one (Fig. 17). The facial fibers of the *I.*

punctatus (Davenport & Caprio, 1982) and *T. pardalis* (Kiyohara *et al*., 1985) contain at least two groups of functionally different afferents, i.e. chemosensitive and mechanosensitive fibers. The two functional groups do not correspond to the present anatomical analysis of the recurrent neurons (Figs. 17, 18) and the two functional groups of fibers might show a unimodal distribution although physiological recordings show that mechanosensitive fibers cause greater amplitudes of impulses than chemosensitive fibers (Davenport & Caprio, 1982; Kiyohara *et al*., 1985).

Abstract

Chapter 1: Introduction

Certain fish groups have evolved a vast system of external gustatory receptors over virtually the entire body surface, along with an elaborately organized complex neural organization. For example, the sea catfish *Plotosus japonicus* are densely supplied with external taste buds over the entire body surface from the lips to the caudal fin. This catfish possess four pairs of equal length of barbels, which are well-endowed with taste buds and serve as important exploratory organs utilized for localization of food objects. They are innervated by mixed nerves containing branches of both the trigeminal (Vth) and facial (VIIth) fibers. The taste buds on the body surface and fins are innervated by recurrent facial nerve (VIIth). This chapter is the overview of the sensory system in the sea catfish and recent findings regarding this system will be briefly discussed.

Chapter 2: Materials and methods

This study used sea catfish, *Plotosus japonicus* to study the polarities of sensory neurons and their organization in the anterior and recurrent ganglion. The trigeminal and facial cell bodies and their distribution in the anterior complex ganglion were studied by using fixed brains with attached anterior ganglion and the fluorescent carbocyanine dyes, DiI and DiA (Molecular Probes, OR, USA). On the other hand, the cell bodies of the recurrent nerve and their central axons were examined by means of neural tracing techniques using dextran amine tetramethylrhodamine (MW3000, Molecular Probes), dextran amine-alexa fluor 488 (MW10000, Molecular Probes) and horseradish perxidase (HRP, Toyobo Grade 1-C). The anterior complex ganglion, recurrent ganglion and brain labeled with neurotracers were viewed under dissecting epifluorescence microscope (MZFL111; Leica Heidelberg GmbH, Mannheim, Germany). Series of sections were viewed under Nikon (Eclipse 80i; Tokyo, Japan) and confocal laser microscopes (Nikon A1si-90i, Tokyo, Japan and Leica TCN NT; Heidelberg GmbH, Mannheim, Germany).

Chapter 3: Peripheral trigeminal and facial sensory pathways in the head and trunk

This chapter describes the peripheral distribution of the trigeminal and facial nerves. Eight peripheral rami were identified as the major pathways to project into the facial lobe in the medulla, which includes the superficial ophthalmic, palatine, upper lip,

maxillary barbel, lower lip, mandibular barbel, hyomandibular and recurrent rami. All of these rami except the palatine and recurrent are heterogenous, containing mixed populations of fibers. The recurrent ramus supplies taste buds on the trunk surface and fins via two different branches, the trunk and pectoral fin. The recurrent ganglion consists only of the facial recurrent neurons that innervate taste bud across the entire surface of the trunk and fins, and is independent from the anterior complex ganglion which consists of the trigeminal, facial and anterior lateral line neurons sending peripheral fibers to the head region.

Chapter 4: Morphology of sensory neurons in sea catfish

This chapter studies the morphology of 1) the trigeminal and facial sensory neurons in the anterior complex ganglion, and 2) the recurrent taste neurons in the independent recurrent ganglion, respectively. The present study shows that trigeminal, facial and facial recurrent sensory neurons of sea catfish are bipolar neurons, with thick peripheral and thin central fibers originating at opposite poles of the cell bodies. The peripheral fibers are greater in diameter than the central ones. The trigeminal neurons were bipolar with various shapes of cell bodies, some are round and some are elongated-oval shape. The facial neurons and recurrent facial neurons were bipolar with round or egg-like shaped cell bodies. It is also observed that the diameter of trigeminal cell bodies have a wider range compared to the facial and recurrent taste neurons.

Chapter 5: Organization of the sensory neurons in the ganglion

In this chapter, the organization of the sensory neurons in both the anterior complex ganglion and the recurrent ganglion are examined. In the anterior complex ganglion of the sea catfish, the trigeminal and facial sensory neurons are distributed mainly in the central and peripheral regions of the ganglion, respectively, with minor overlapping. In the recurrent ganglion, the trunk and pectoral fin cell bodies are mainly arranged in groups of irregular shapes and distribution. Each group is independent and does not contain both types of cell simultaneously. The two groups however, intermingled with each other throughout the entire ganglion. No somatotopy was detected within the recurrent facial ganglion.

Chapter 6: Central projections of the recurrent nerve

This chapter studies the central projections of the recurrent nerve fibers following the application of neurotracers to the recurrent nerve. The labeled fibers of the whole recurrent were observed to terminate anteroposteriorly only in the trunk tail lobule of the facial lobe. Following simultaneous application of two different tracers to the trunk and pectoral fin branches respectively, results showed that their fibers project topographically into the trunk tail lobule.

Chapter 7: Discussion

This chapter discussed the five principal findings concerning taste neurons in the *P. japonicus*: (1) The trigeminal, facial and facial recurrent neurons are bipolar with thick peripheral and thin central processes; (2) Distinct separations between the trigeminal and facial cell bodies in the anterior complex ganglion (3) Distinct separation between facial cell bodies innervating different barbel lobules in the facial ganglion (4) No distinct separation between the location of trunk-tail and pectoral fin neurons occurs in the recurrent ganglion; and (5) the trunk-tail and pectoral fin neurons project somatotopically to the TTL of the FL.

Acknowledgement

This thesis would not have been possible without the guidance and the help of numerous outstanding individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, I offer my sincerest gratitude to my supervisors, Dr. Sadao Kiyohara and Dr. Masanori Kasai who have supported me throughout my thesis with their patience and knowledge, whilst allowing me the room to work in my own way. I attribute the level of my doctorate degree to their encouragement and effort and without

them this thesis, too, would not have been completed or written. One simply could not wish for better or friendlier supervisors.

In my daily work I have been blessed with a friendly and cheerful group of fellow students. I thank Dr. Masato Kirono, Emi Yamashita, Masaya Matsubara, Mami Shimohara, Kentarou Enoki, Ryota Shimura, Yusuke Nagata, Ryuichi Morishige, Shiori Uezono, Toshifumi Nobori and Takahiro Higashi for generous support of experiments and good laughs throughout my three years stay in Kagoshima. I also offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

Finally, I would like to thank my parents and my siblings, who through my childhood and study career had always encouraged me to follow my heart and inquisitive mind in any direction this took me. My parents provided me with a loving home, one where an academic mind was celebrated, and my siblings' sharp sense of humour has been a gift in my life.

Major parts of this work were published as a paper in the Journal of Fish Biology (2013) 82, 1773 – 1788. The title of paper is "Recurrent facial taste neurons of sea catfish *Plotosus japonicus*: morphology and organization in the ganglion by N. A. Denil, E. Yamashita, M. Kirino and S. Kiyohara". I am grateful for the Journal (Elsevior) to use the figures published.

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List of Abbreviations

AG, anterior ganglion;

CB, cerebellum;

FL, facial lobe;

FLM, medial longitudinal fasciculus;

LL, lateral line lobe;

LML, lateral mandibular lobule;

MML, medial mandibular lobule;

MXL, medial maxillary lobule;

NBL, nasal barbel lobule;

Nuc, nucleus;

NV 111, octaval nerve;

OT, optic tectum;

RDV, descending trigeminal root;

RG, recurrent facial ganglion;

RSVII, sensory root of facial nerve;

S, spinal cord;

T, telencephalon;

TGS, ascending secondary gustatory tract;

TTL, trunk tail lobule;

VL, vagal l

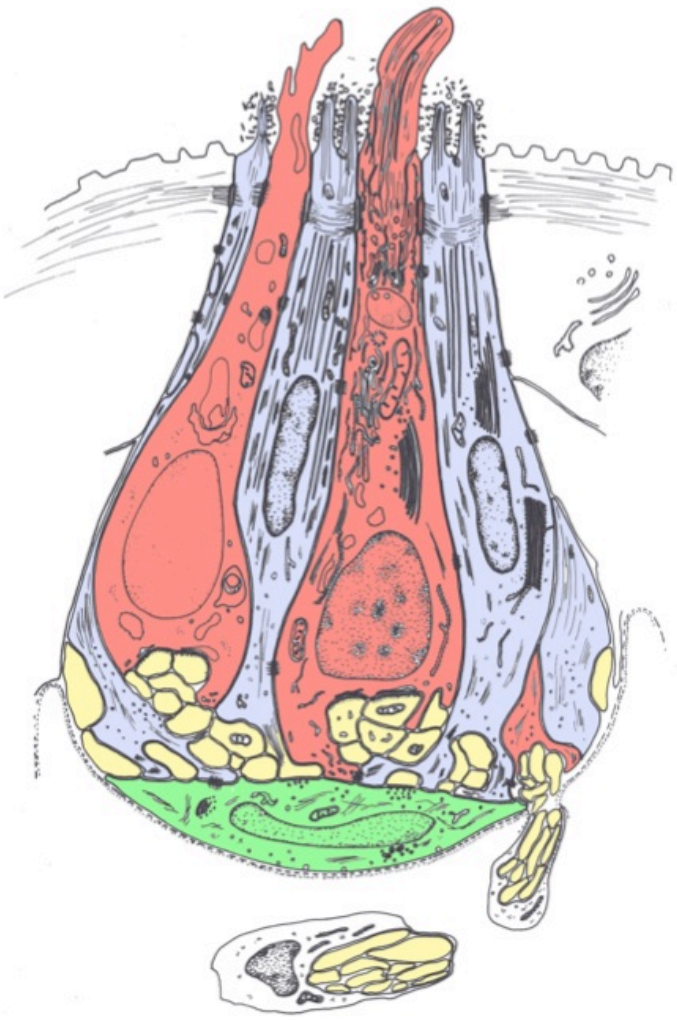


Figure 1 Schematic representation of fish taste buds showing t-cell(light cell, red), f-cell (dark cell, blue), basal cell (green) and nerve fibers (yellow) of the bud's nerve fiber plexus. Reproduced from Kitoh et al. (1987).

Figure 2. The sea catfish, *Plotosus japonicus* possess vast numbers of taste buds distributed within the mouth, on four pairs of barbels and across the entire body surface. Corresponding to many buds in the peripheral regions, they have extremely enlarged primary and higher taste centers in the brain.

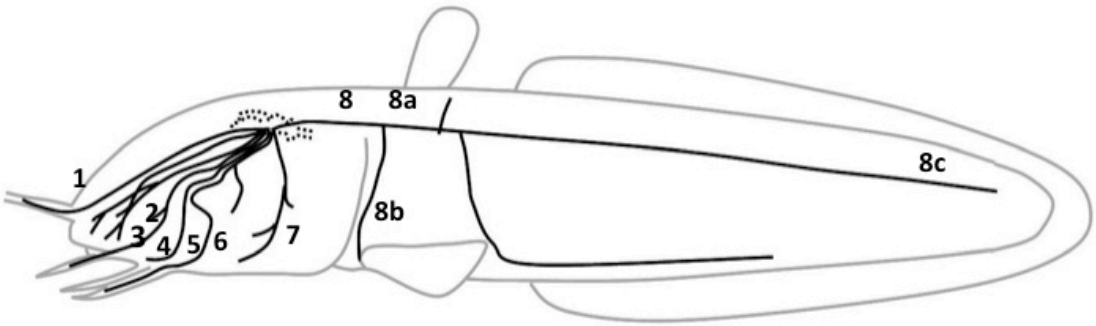


Figure 3. Lateral view of the catfish illustrating the peripheral course of the trigeminal and facial fibers. All peripheral rami except the palatine (2) and recurrent (8) are mixed with the trigeminal and facial nerve fibers. 1, superficial ophthalmic; 2, palatine; 3, upper lip; 4; maxillary barbel; 5, lower lip; 6, mandibular barbel; 7, hyomandibular; 8, recurrent. Reproduced from N. A. Denil et al. (2013)

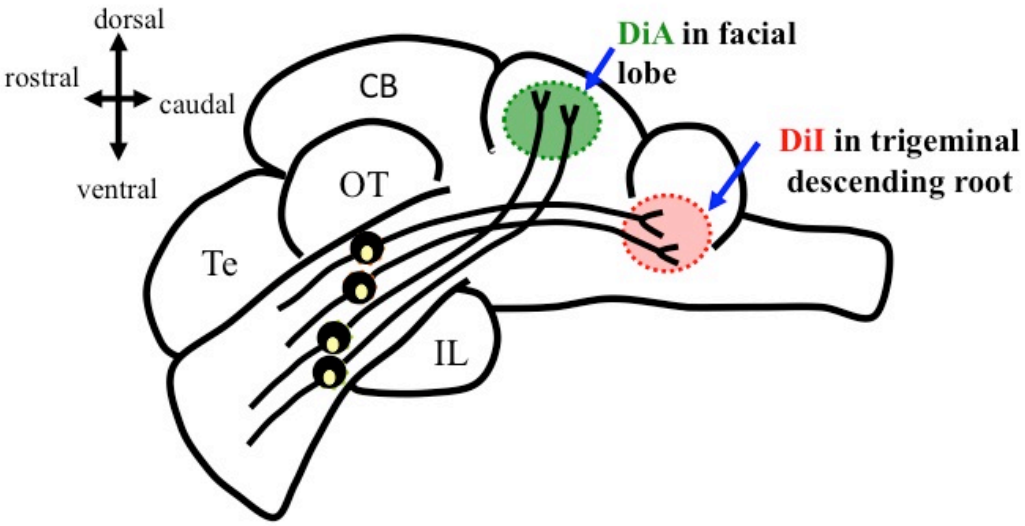


Figure 4. Selective labeling of trigeminal and facial neurons in the anterior ganglion with two dyes. First, DiI was applied to the descending trigeminal root, let diffused through the root to reach the trigeminal cell bodies in the anterior ganglion. DiA was applied to the facial lobe and was then carried through the facial sensory root to reach the facial cell bodies in the anterior ganglion.

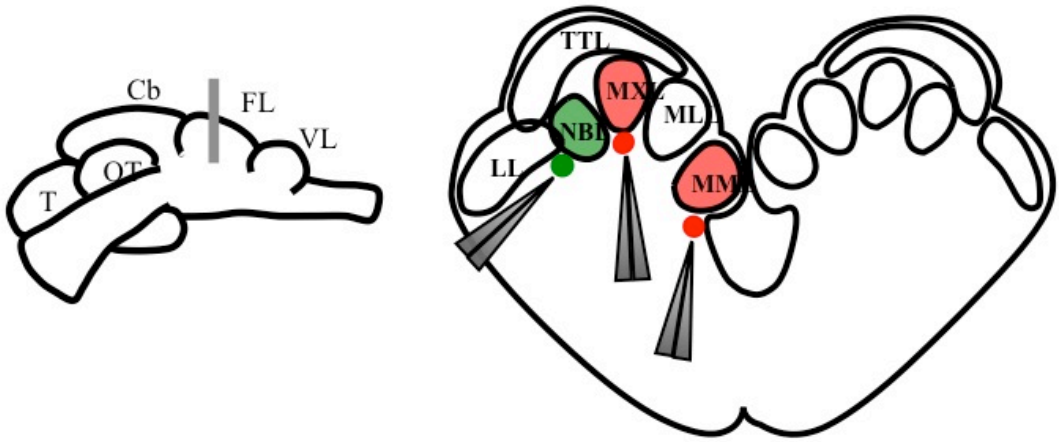


Figure 5. Selective labeling to the barbel lobules in the facial lobe with two different dyes. To reveal the somatotopic map in the peripheral regions of the facial ganglion, two different dyes, DiA and DiI and were applied to the nasal barbel lobule (NBL) and to either the maxillary lobule (MXL) or medial mandibular lobule (MML), respectively.

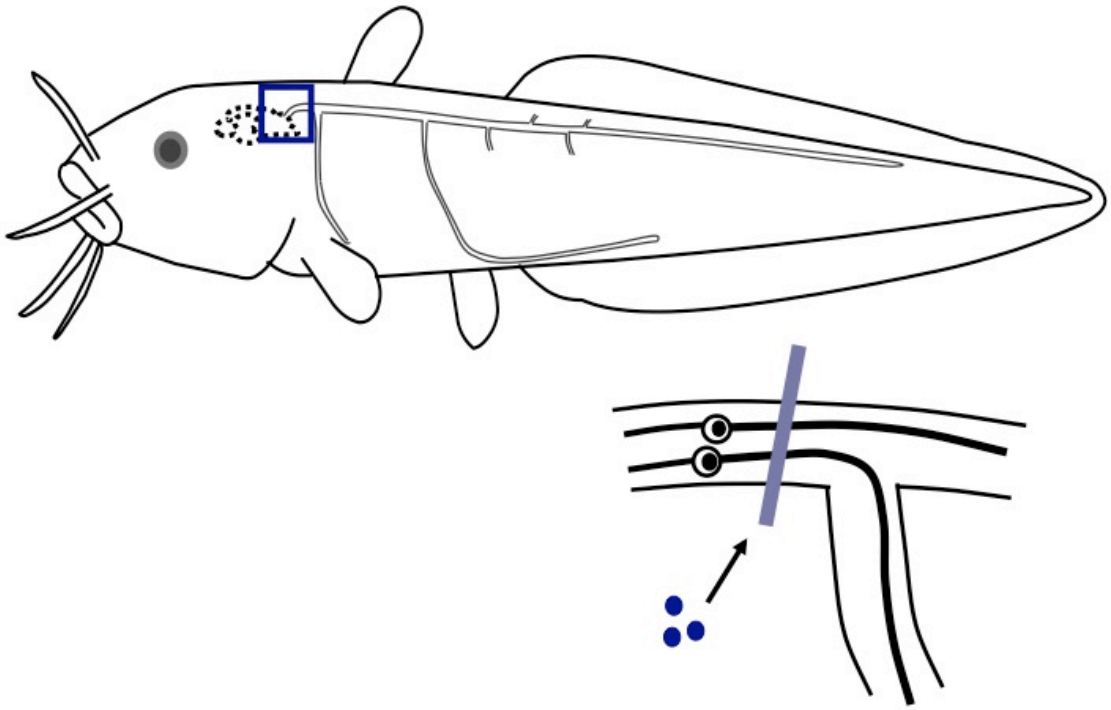


Figure 6. The whole recurrent nerve sends fibers to the entire trunk-tail surface and fins via two major rami, trunk and pectoral branches. In six fish, dextran amine – tetramethylrhodamine or HRP was applied to the central cut stumps of the whole recurrent nerve.

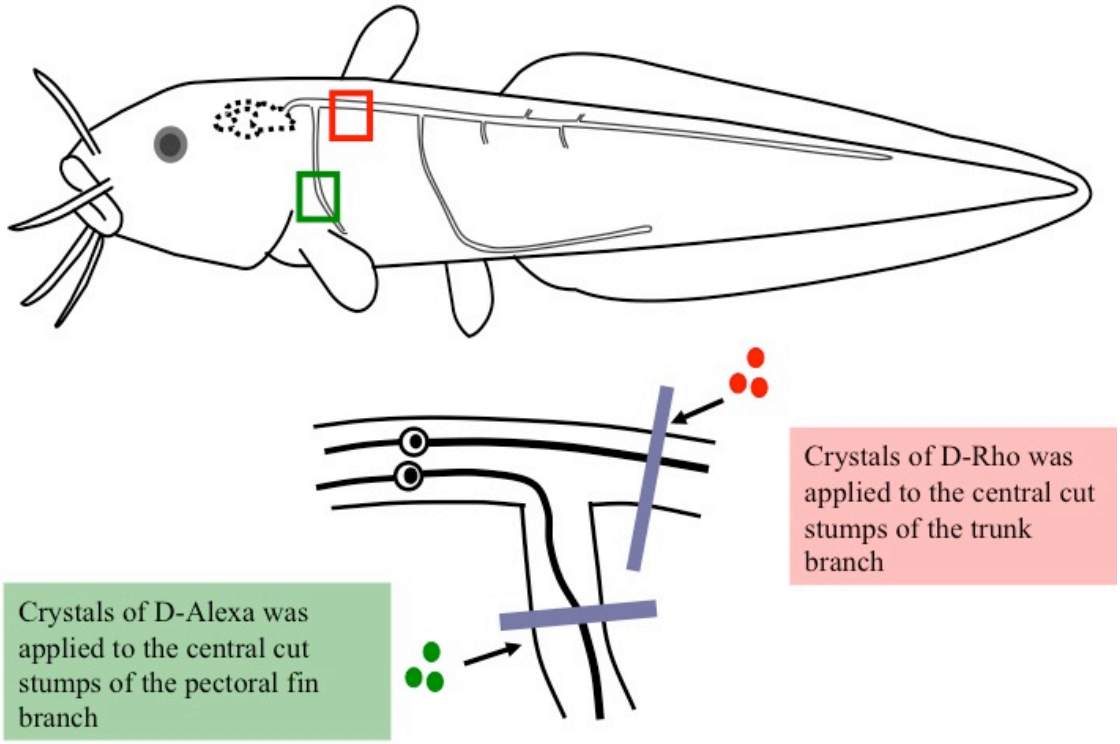


Figure 7. In nine fish, the trunk and pectoral branches were simultaneously exposed and cut. Dextran amine –tetramethylrhodamine and Dextran-Alexa were applied to the cut stumps of the trunk and pectoral branches, respectively.

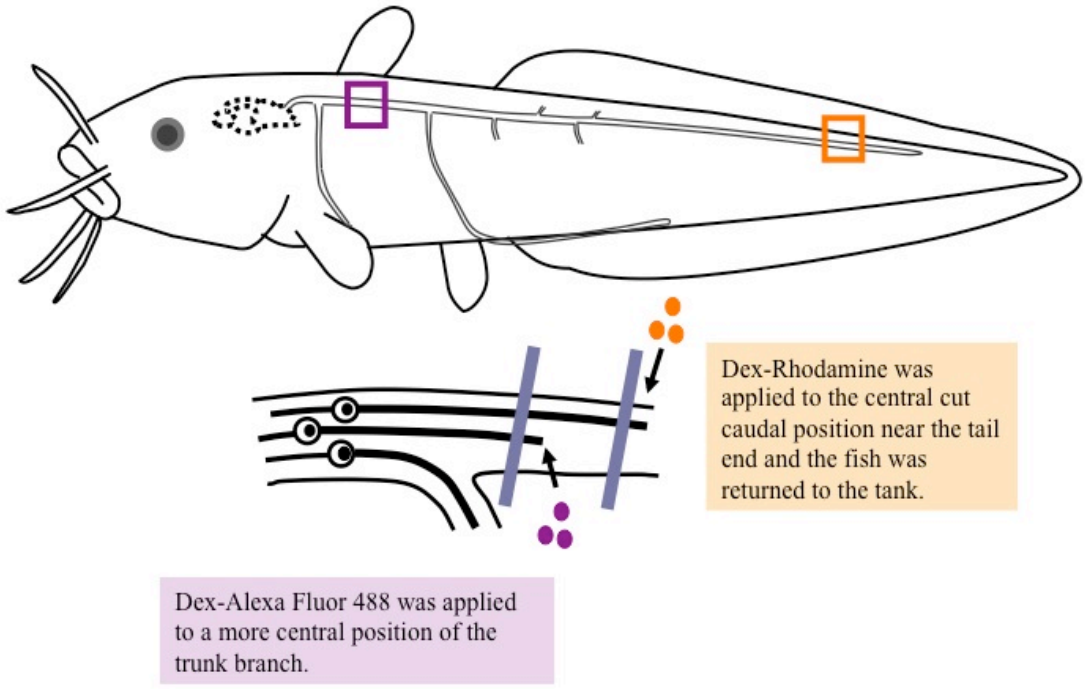


Figure 8. In three fish, the trunk branch was first exposed at the caudal position near the tail end and cut. Dex-Rhodamine was applied to the central cut ends and the fish was returned to the tank. After seven days, the fish were again anesthetized and the trunk recurrent rami were exposed at more central and cut. Dex-Alexa Fluor 488 was applied to the cut stumps.

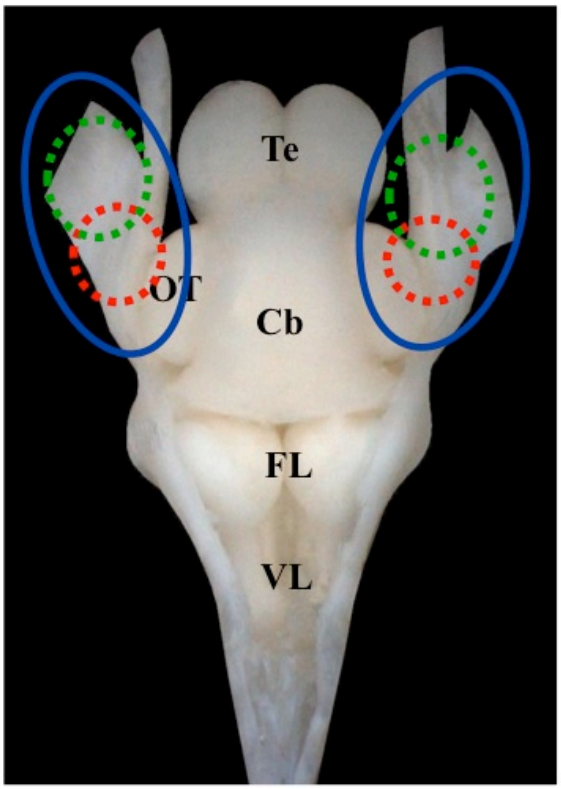


Figure 9. The trigeminal and facial ganglia are intimately associated in the catfish . Along with the anterior lateral line ganglion, they are known as the anterior ganglion. Each root of the trigeminal, facial and lateral line nerves originates from the ganglion to project each primary center in the medulla. Te : Telenchepalon FL : Facial lobe Cb : Cerebellum VL : Vagal lobe OT: Optic tectum

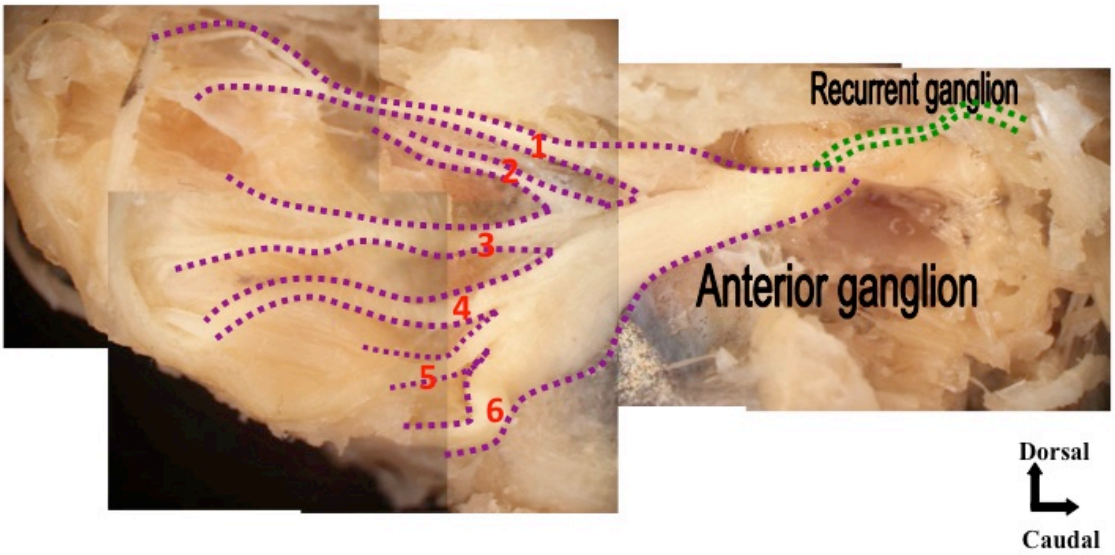
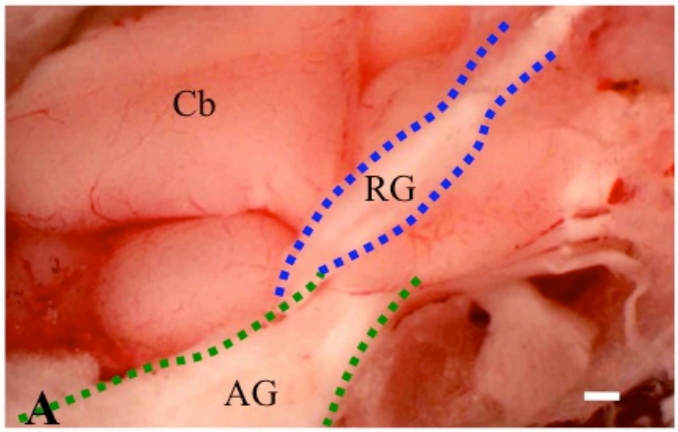
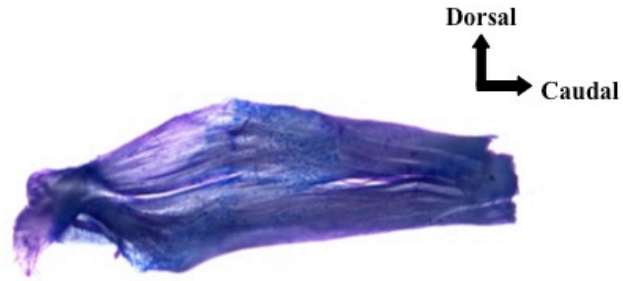


Figure 10. Two distinguished ganglia are visible under dissecting microscope; the anterior and the recurrent ganglia. The anterior ganglion contains mixed cell bodies of the trigeminal, facial and lateral line neurons.





B

Figure 11. A: Enlarged photograph showing the position of recurrent facial ganglion (RG). Note this ganglion is independent of the anterior ganglion (AG)containing trigeminal, facial and lateral line neurons. RG and AG are encircled with dotted lines. B: Horizontally flat recurrent nerve ganglion stained with Nissl technique. Reproduced from N. A. Denil et al. (2013)

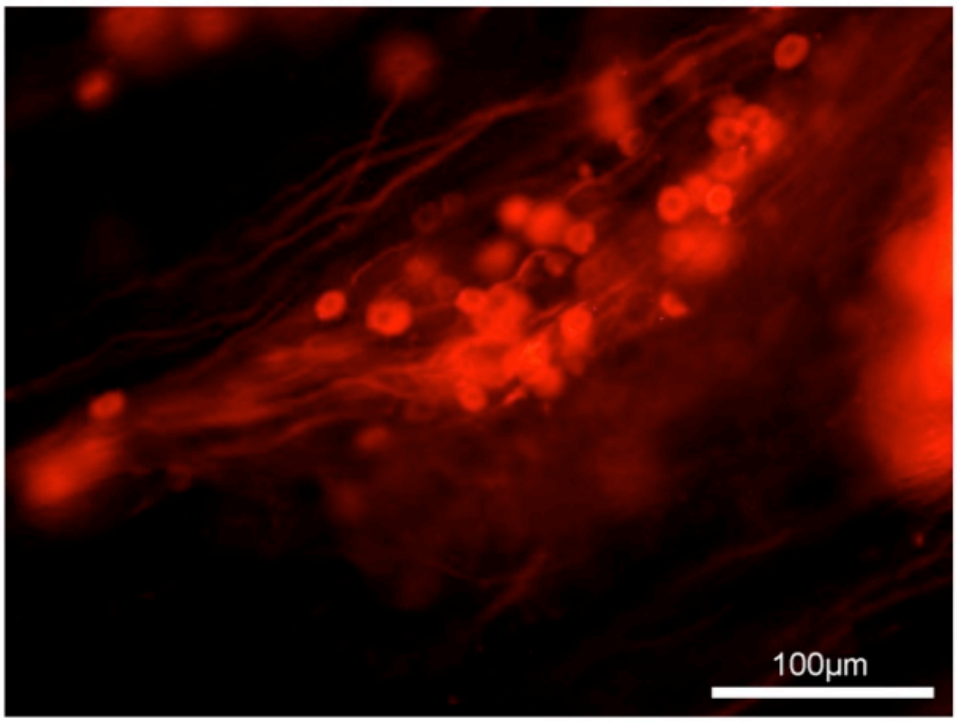


Figure 12. Horizontal section of trigeminofacial complex ganglion of *P. japonicus* showing trigeminal cell bodies labeled with DiI. The trigeminal neurons were bipolar with various shapes of cell bodies; some are round and some are elongated-oval shape. The long diameter of the cell bodies ranged between 14.7 – 45.1 μm (n=600).

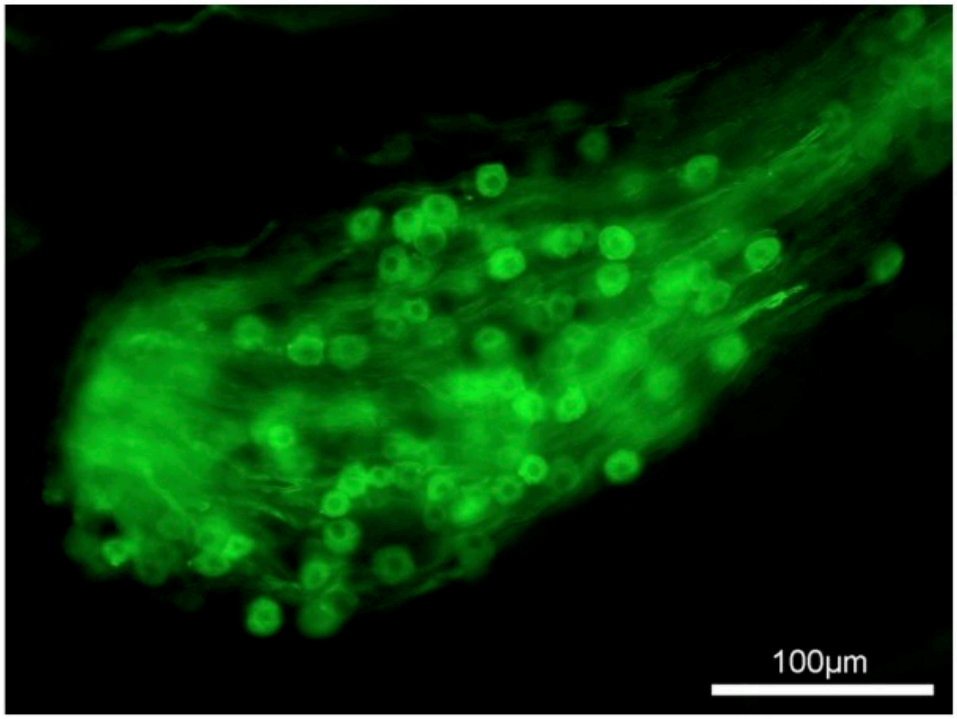


Figure 13. Horizontal section of trigeminofacial complex ganglion of *P. japonicus* showing facial cell bodies labeled with DiI. The facial neurons were bipolar with round or egg-like shaped cell bodies. The long diameter of the cell bodies ranged between 16.1 – 27.6 µm (n=600).

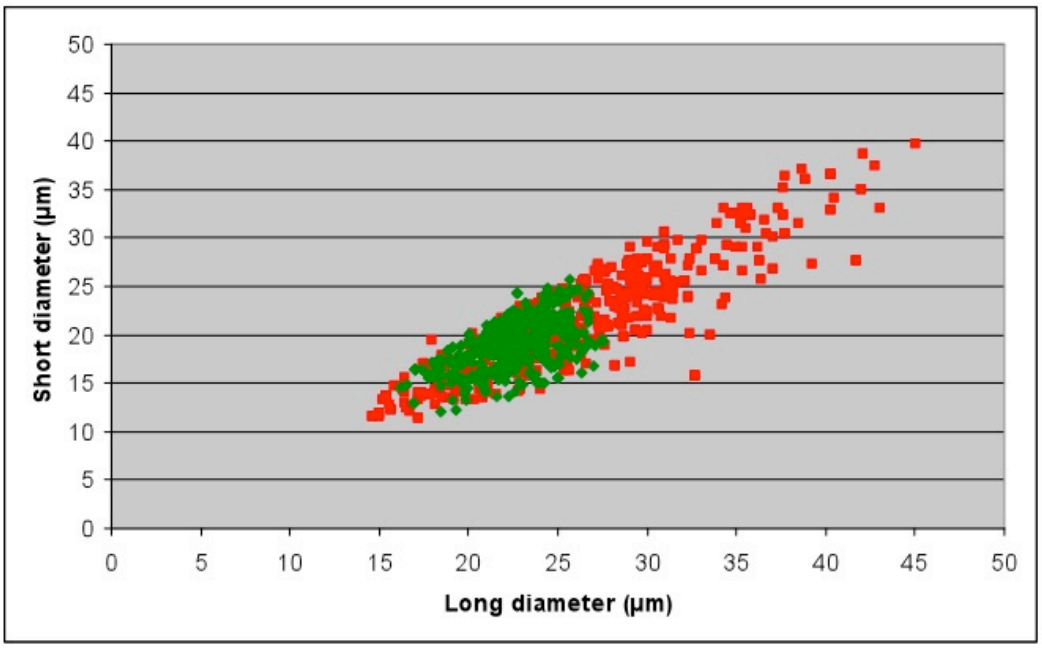


Figure 14. Scatter plot showing the relationship between long diameter and short diameter for trigeminal (red) and facial (green) cell bodies (n=600). The averages of long and short diameter for trigeminal cell bodies are 26.1±5.8 µm and 21.561±5.4 µm. The averages for facial cell bodies are 22.5±2.2 µm and 18.9±2.6 µm.

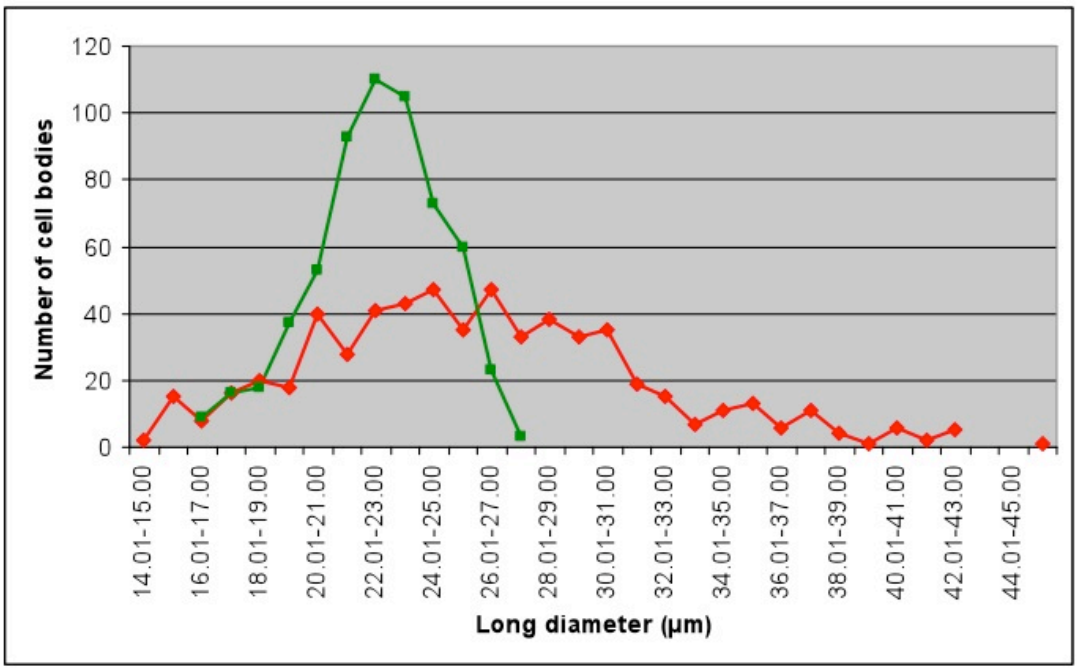


Figure 15. Distribution of the trigeminal (red) and facial (green) cell bodies in the anterior ganglion of *P. japonicus*. The trigeminal cell bodies varies in cell body size, while unimodal distribution of facial cell bodies size is observed in the anterior complex ganglia.

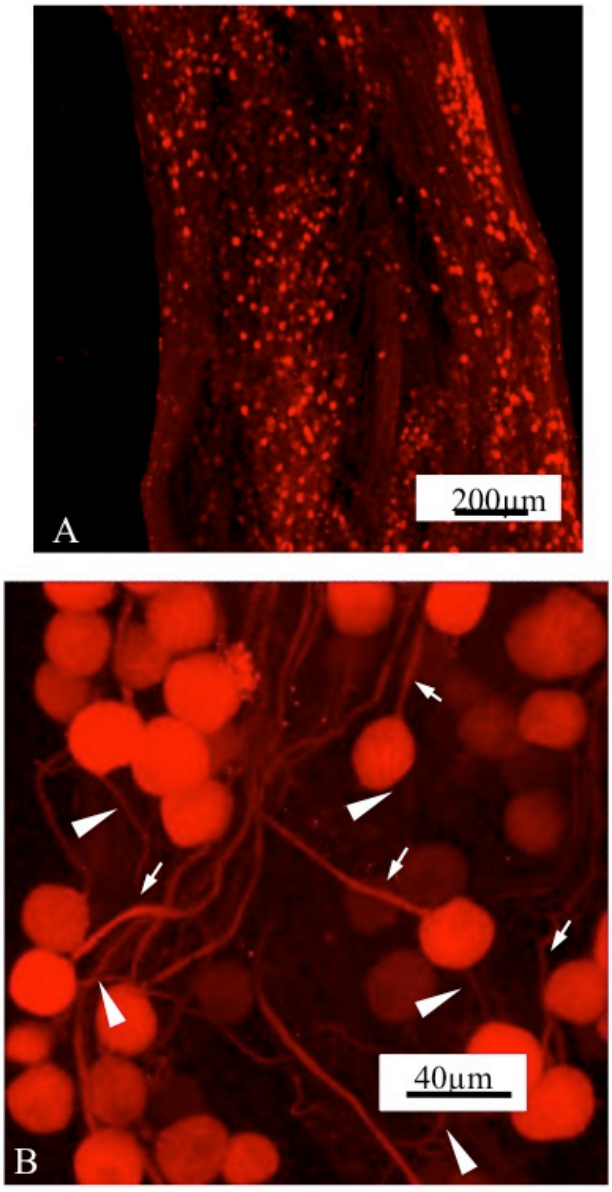


Figure 16. Labeled neurons in the recurrent ganglion after application of dextran amine rhodamine in vivo to the central cut stump of whole recurrent ramus. A: Labeled cell bodies scattered throughout the recurrent ganglion. B: enlargement of labeled neurons showing round shape cell bodies. Arrow and arrowheads indicate thick peripheral and thin central fiber, respectively. Reproduced from N. A. Denil et al. (2013)

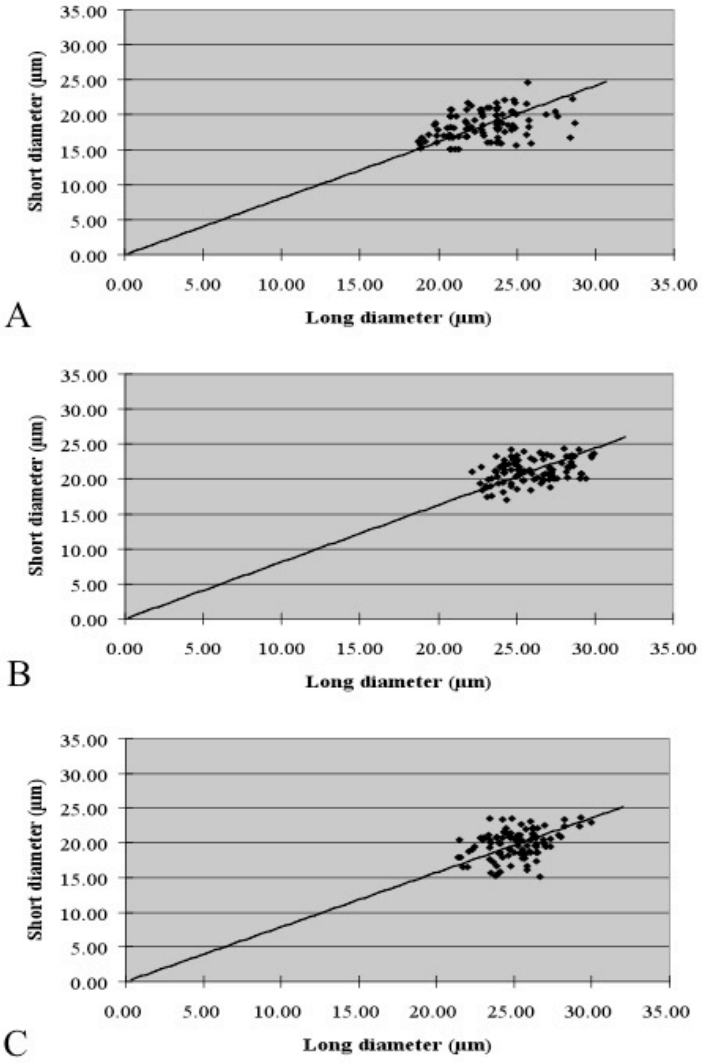


Figure 17. Scatter plots showing the relationship between the long and short diameters for the recurrent cell bodies (n=100) in 10.3 (a), 15.1cm (b), 18.3 (c) cm total length specimens. The averages of the long and short diameters are 22.8±2.2 µm and 18.5 ±2.9 µm in a 10.3 cm specimen, 25.9±1.9 µm and 21.1±1.7 µm in a 15.1 cm specimen, and 25. 1±1.7µm and 19.8±2.0µm in a 18.3 cm specimen. Reproduced from N. A. Denil et al. (2013) .

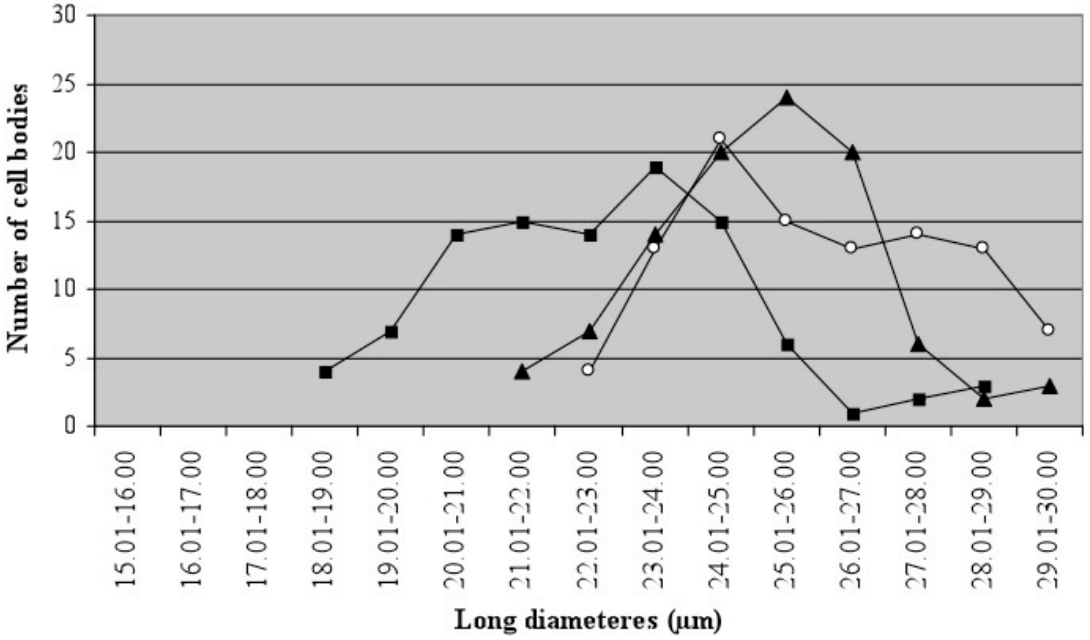


Figure 18. Distribution of the size of 100 cell bodies in three different sizes of fish. Long diameters are plotted in three ganglia of 10.3cm (solid square), 15.1 cm (open circle) and 18.3 cm (solid triangle) total length specimens. Reproduced from N. A. Denil et al. (2013)

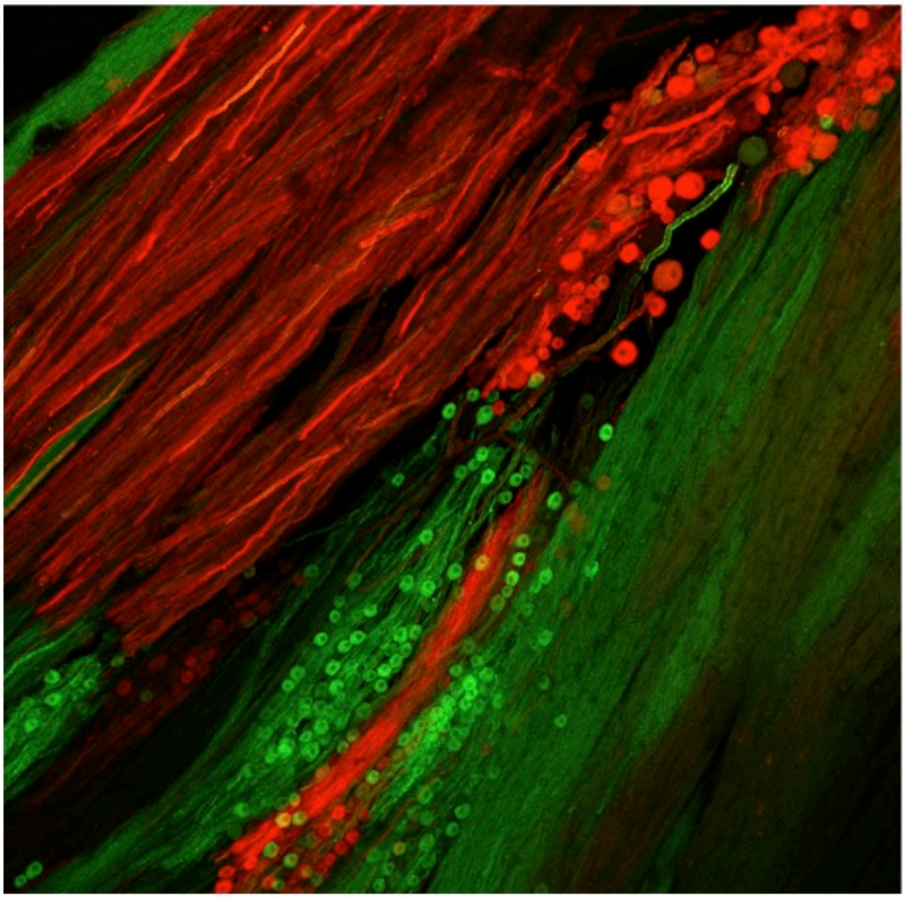


Figure 19. Labeled trigeminal (red) and facial (green) neurons in the anterior ganglion after application of DiI and DiA to the descending trigeminal root and facial sensory root, respectively. The trigeminal and facial neurons are distributed mainly in the central and peripheral regions of the ganglion, respectively, with minor overlapping.

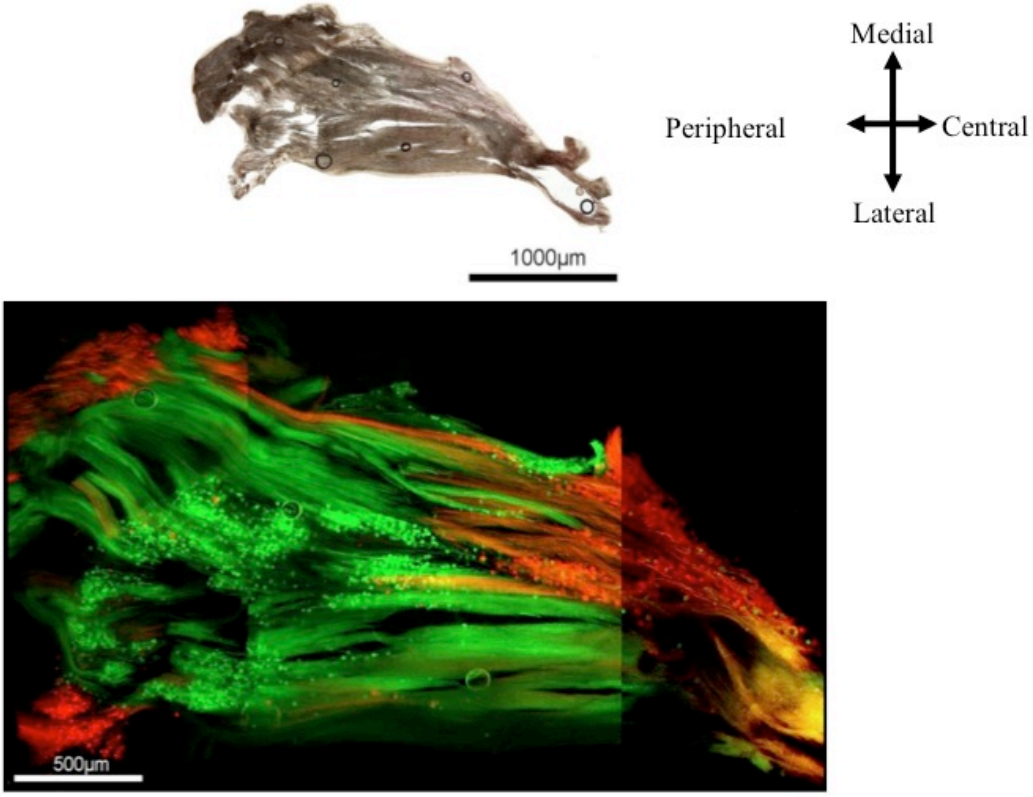


Figure 20. The labeled trigeminal (red) and facial (green) neurons are distributed in one continuous cell aggregation; the trigeminal cell bodies mainly in the central and the facial cell bodies in the peripheral regions of the ganglion, with minor overlapping.

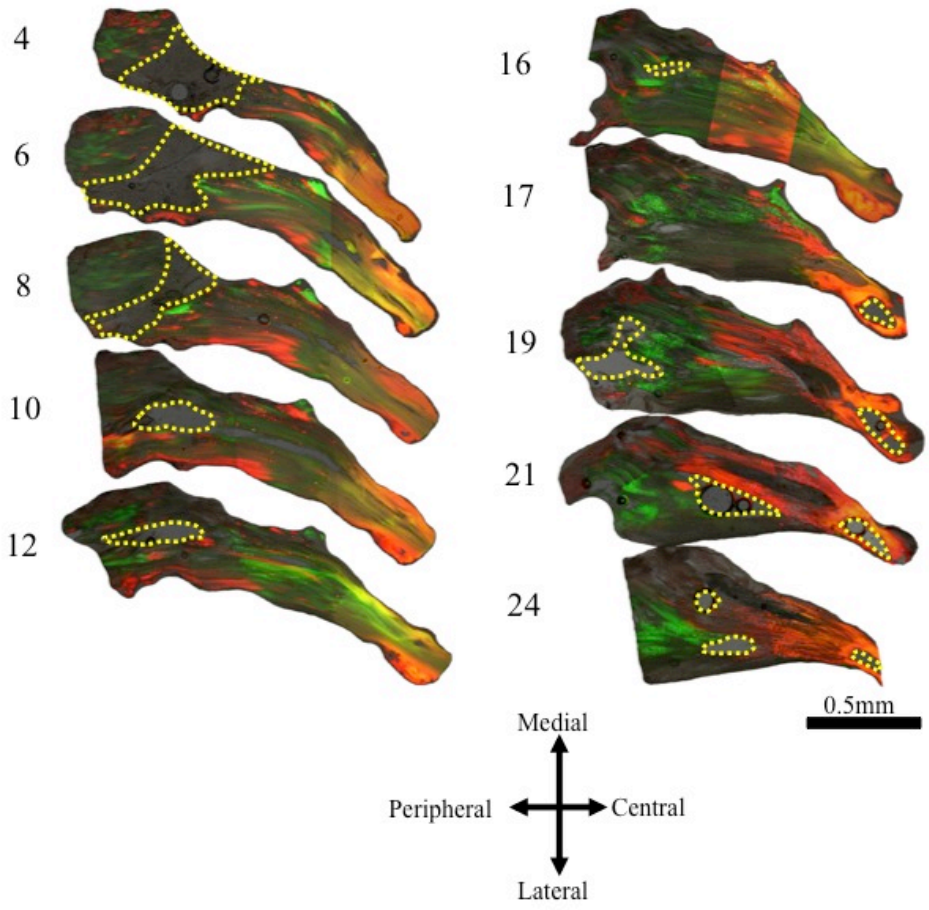


Figure 21. Photomicrographs of 10 serial-horizontal sections of the anterior complex ganglion from dorsal (4) to ventral (24) showing distribution of the trigeminal and facial cell bodies. The trigeminal and facial neurons are distributed mainly in the central and peripheral regions of the ganglion, respectively, with minor overlapping.

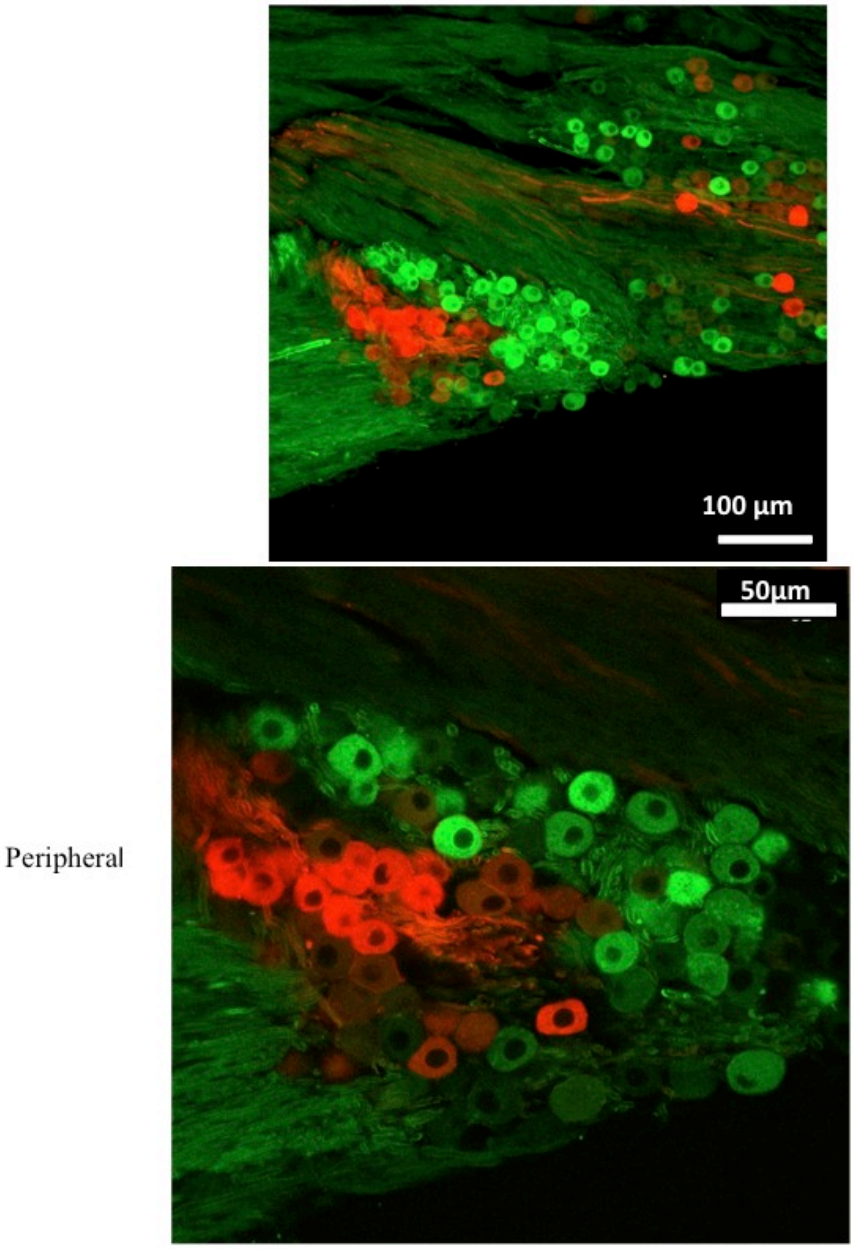


Figure 22. Labeled nasal barbel (green) and medial mandibular (red) facial neurons in the facial ganglion after application of DiI and DiA to the nasal barbel lobule and mandibular barbel lobule, respectively. The cell bodies innervating the nasal barbel and medial mandibular barbel are round in shape. The cells formed separate groups, but some over lapping were also observed.

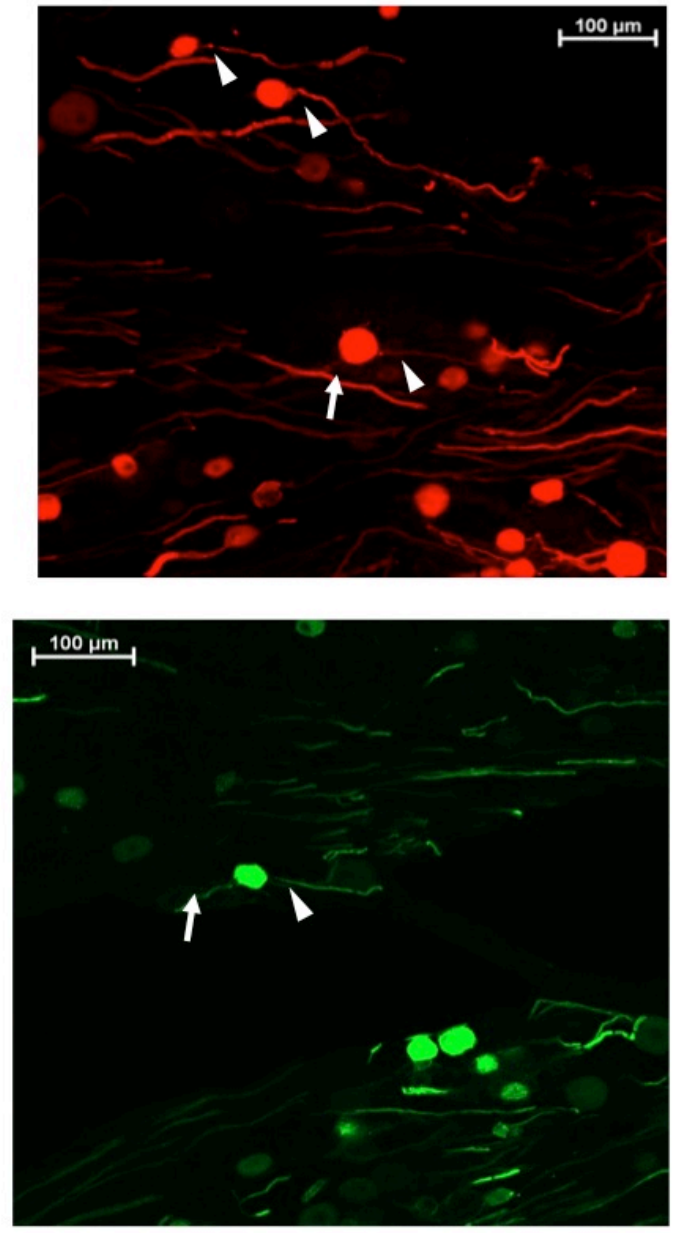


Figure 23. Labeled neurons in the facial ganglion after application of DiI and DiA to the nasal barbel lobule and mandibular barbel lobule, respectively. Bipolar nasal barbel and medial mandibular barbel cell bodies in the facial ganglion. Fine central fibers (▶) and thick peripheral fibers (→) were observed.

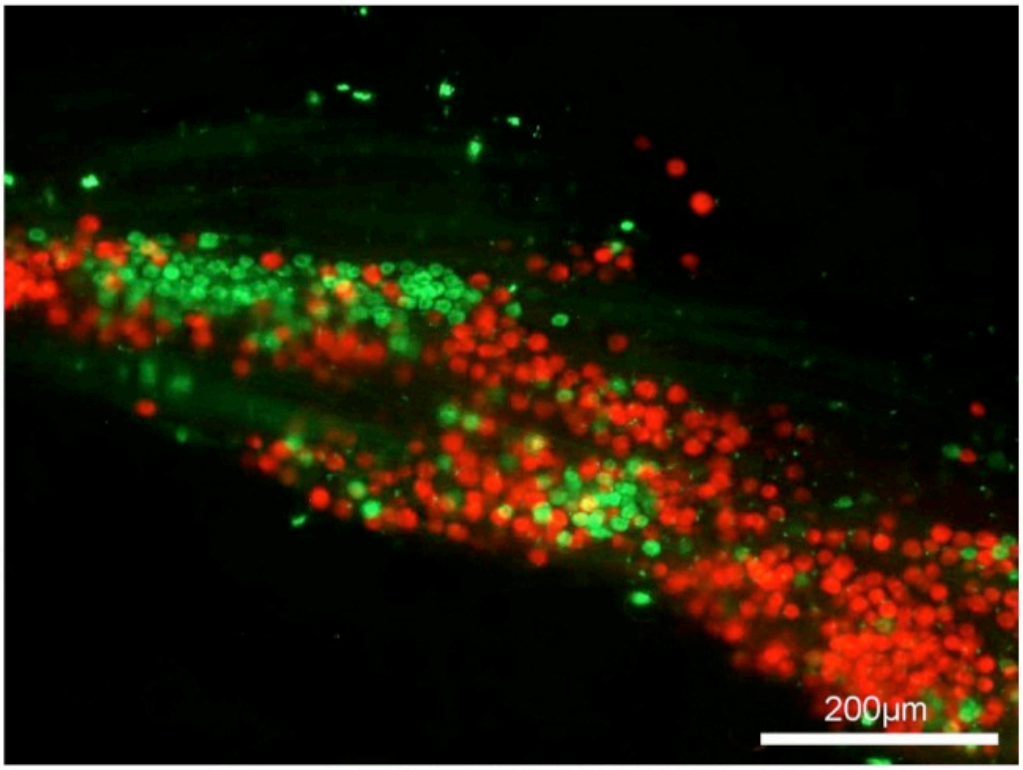


Figure 24. The trunk (red) and pectoral fin (green) cell bodies in *P. japonicus* are independently located throughout the recurrent ganglion. Each type of cell appears to distribute as different groups of various shapes and sizes.

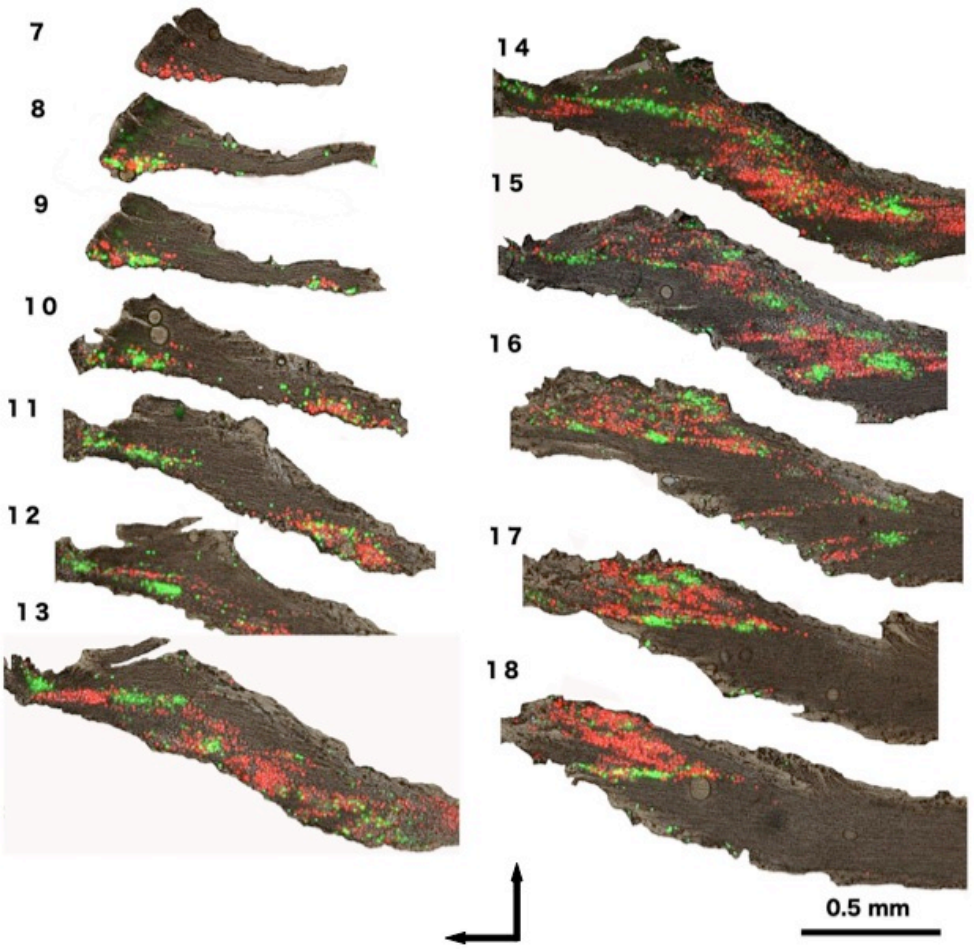


Figure 25. Photomicrographs of 12 serial-horizontal sections of a recurrent ganglion from dorsal (7) to ventral (18) showing the distribution of labeled trunk (red) and pectoral fin (green) cell bodies. Each section is 50μm in width; section 12 lacks its right half. The two kinds neurons are distributed in all sections except the first one (No.7). The groups are independent of each other, but intermingle throughout the sections, showing no distinct somatotopical organization in the recurrent facial ganglion. Reproduced from N. A. Denil et al. (2013) .

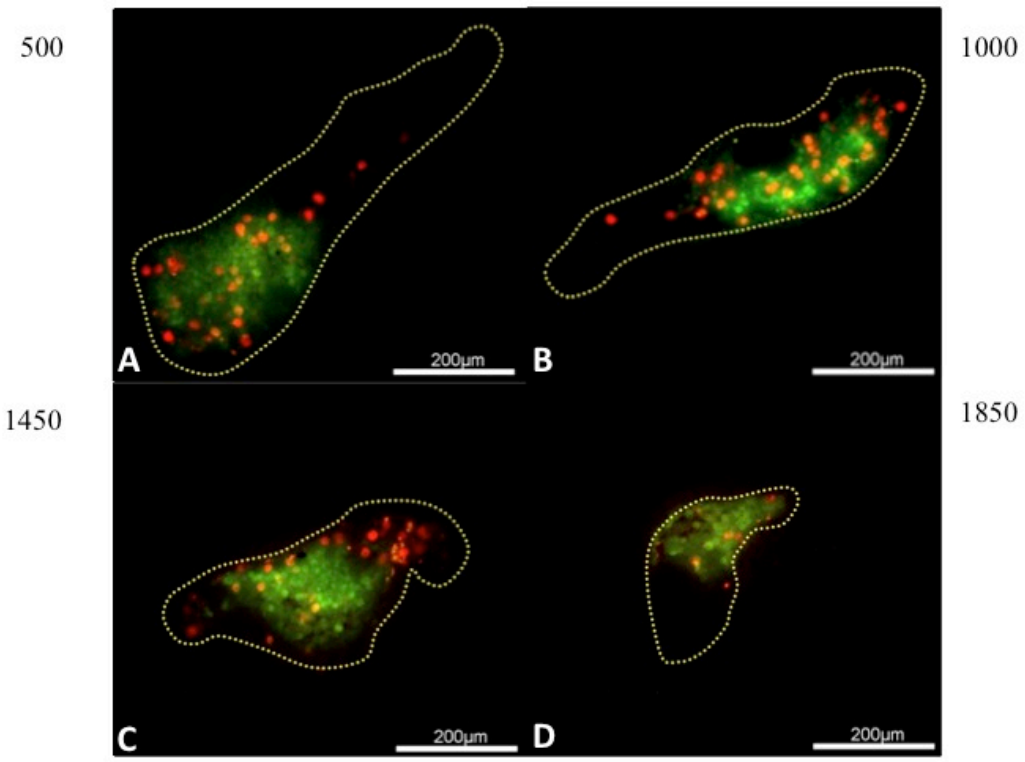


Figure 26. Transverse sections of recurrent ganglion from rostral (A) to caudal (D). Red or yellow somata innervating the caudal one-forth of the body are scattered wildly throughout the ganglion. The green cell bodies innervating the rest of the trunk surface are greater in number. This result suggests no distinct somatotopy in the recurrent ganglion.

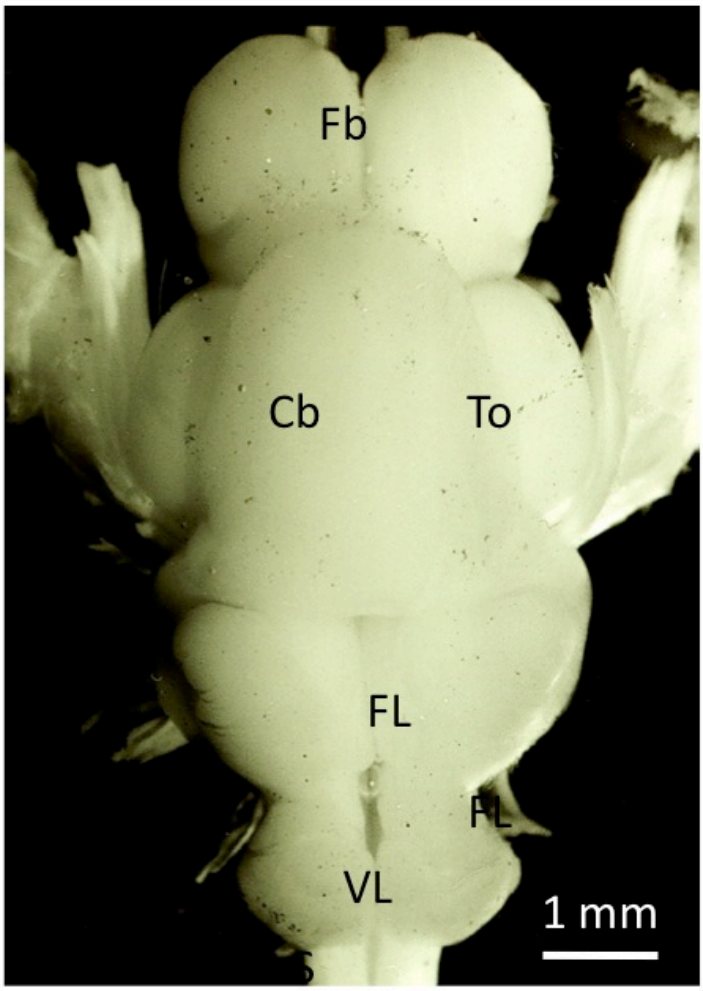


Figure 27. Dorsal view of brain of the sea catfish, *Plotosus japonicus*. Fb , forebrain; To, optic tectum; Cb, cerebellum; S, spinal cord,

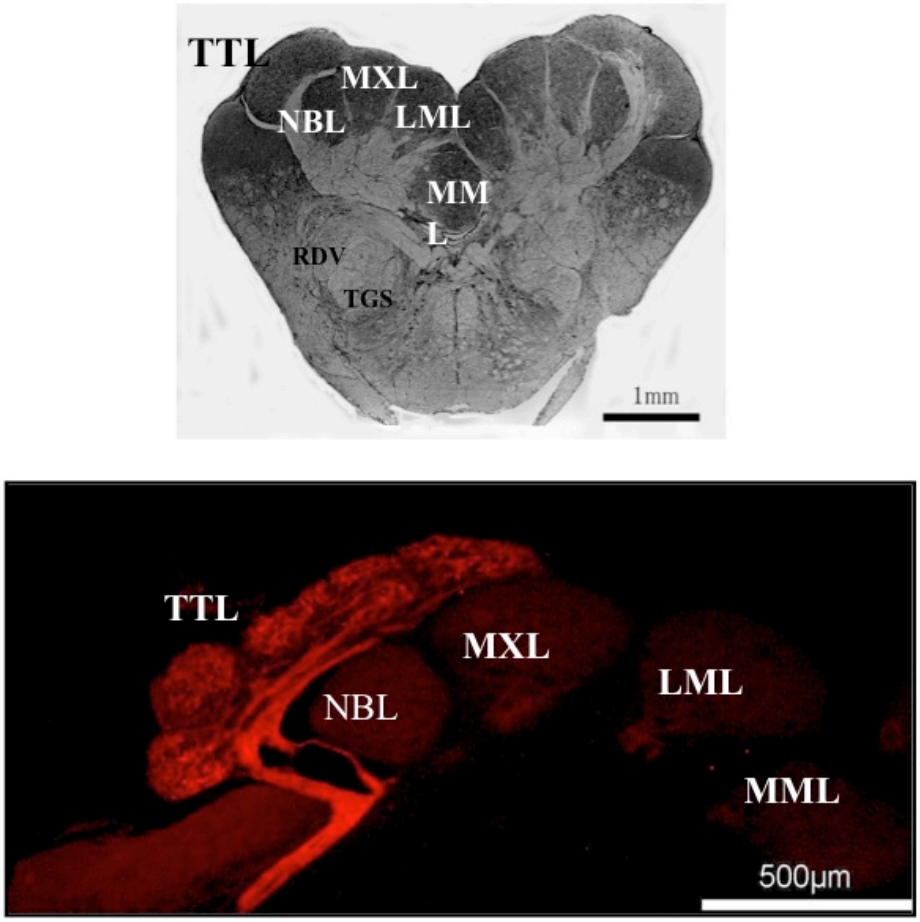


Figure 28. Central projections of the whole trunk branch to the facial lobe in the *P. japonicus*. Upper: Photomicrograph of the cross section showing the five distinct lobules extending rostrocaudally in the facial lobe. Lower: The trunk tail lobule (TTL) is located dorsolateral to the barbel lobules and is dorsoventrally flattened. Fibers of the recurrent ramus, labeled with rhodamine dextran were found in the most dorsolateral portion of the sensory root of the facial nerve and terminated in the entire TTL.

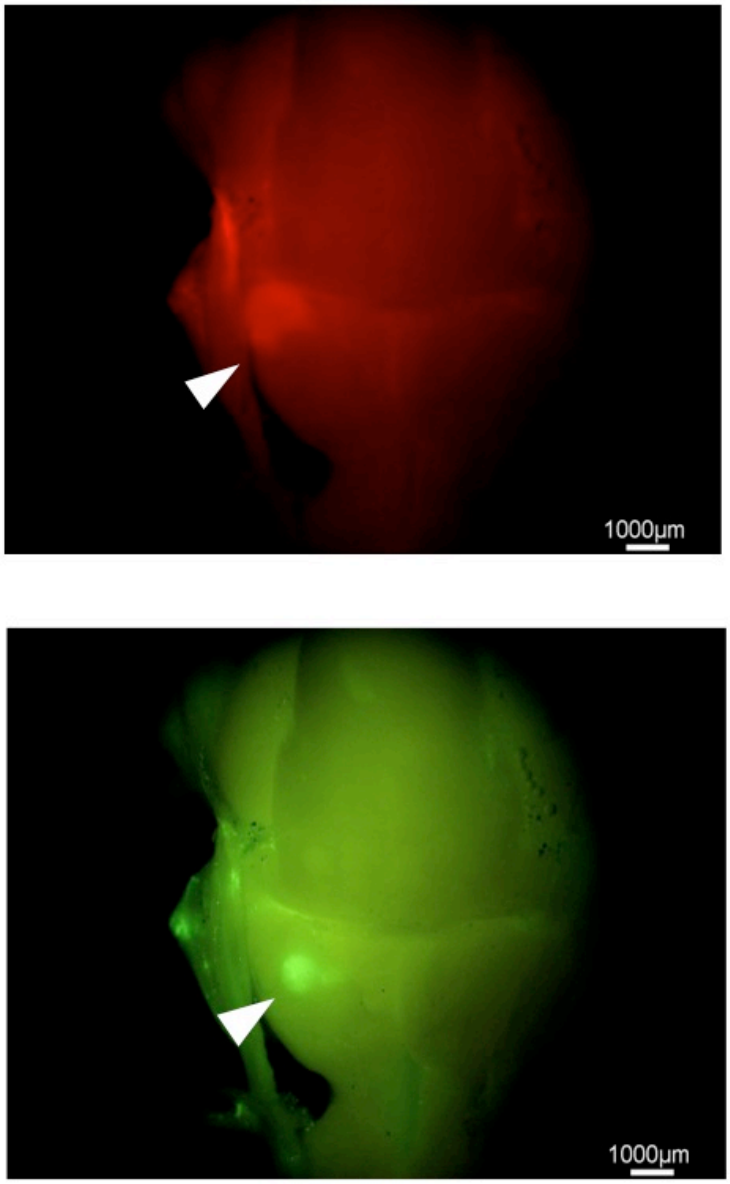


Figure 29. Dorsal view of whole brain after simultaneous labeling of trunk and pectoral fin ramus with two tracers. The labeled area with red (upper, trunk ramus projection) and that with green (lower, pectoral fin ramus projection) were observed at the most anterolateral part of the facial lobe and its intermediate part, respectively.

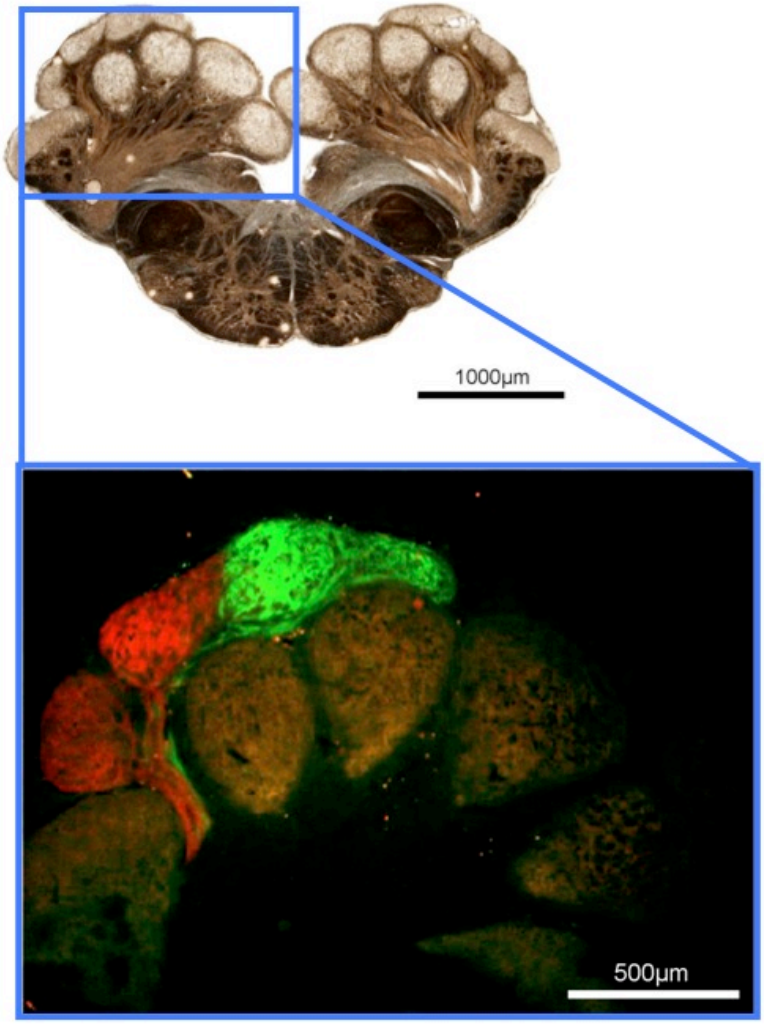


Figure 30. Central projection of trunk and pectoral fin rami on dorsal lobule of facial lobe. Fluorescent photomicrograph (B) of the area squared in A showing distinct topographic projections of the trunk (red) and pectoral fin (green) fibers into the trunk tail lobule in the facial lobe of *P. japonicus.* Reproduced from N. A. Denil et al. (2013) .

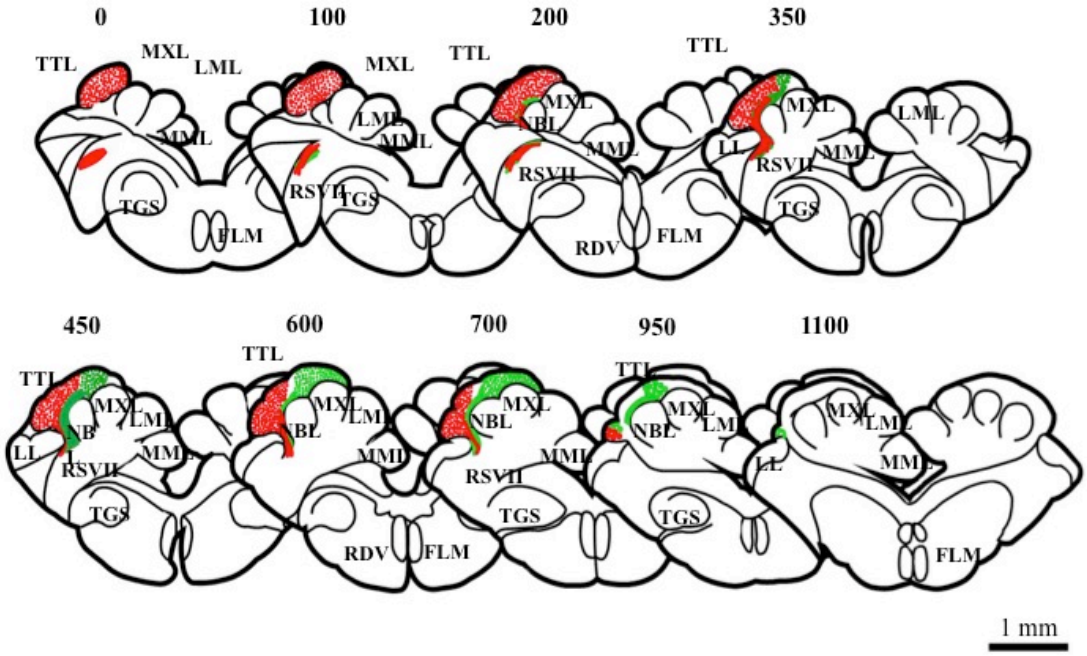


Figure 31. Line drawing illustrating the topographic projection of the trunk and pectoral fin recurrent fibers to the trunk-tail lobule in the catfish *Plotosus japonicus*. Transverse sections of the FL from rostral to caudal. The number at the left above each of the sections indicates the distance from the first section of the FL. The red lines and dots indicate the labeled trunk fibers and terminals. The green solid and dots indicate the labeled pectoral fibers and terminals. Reproduced from N. A. Denil et al. (2013) .

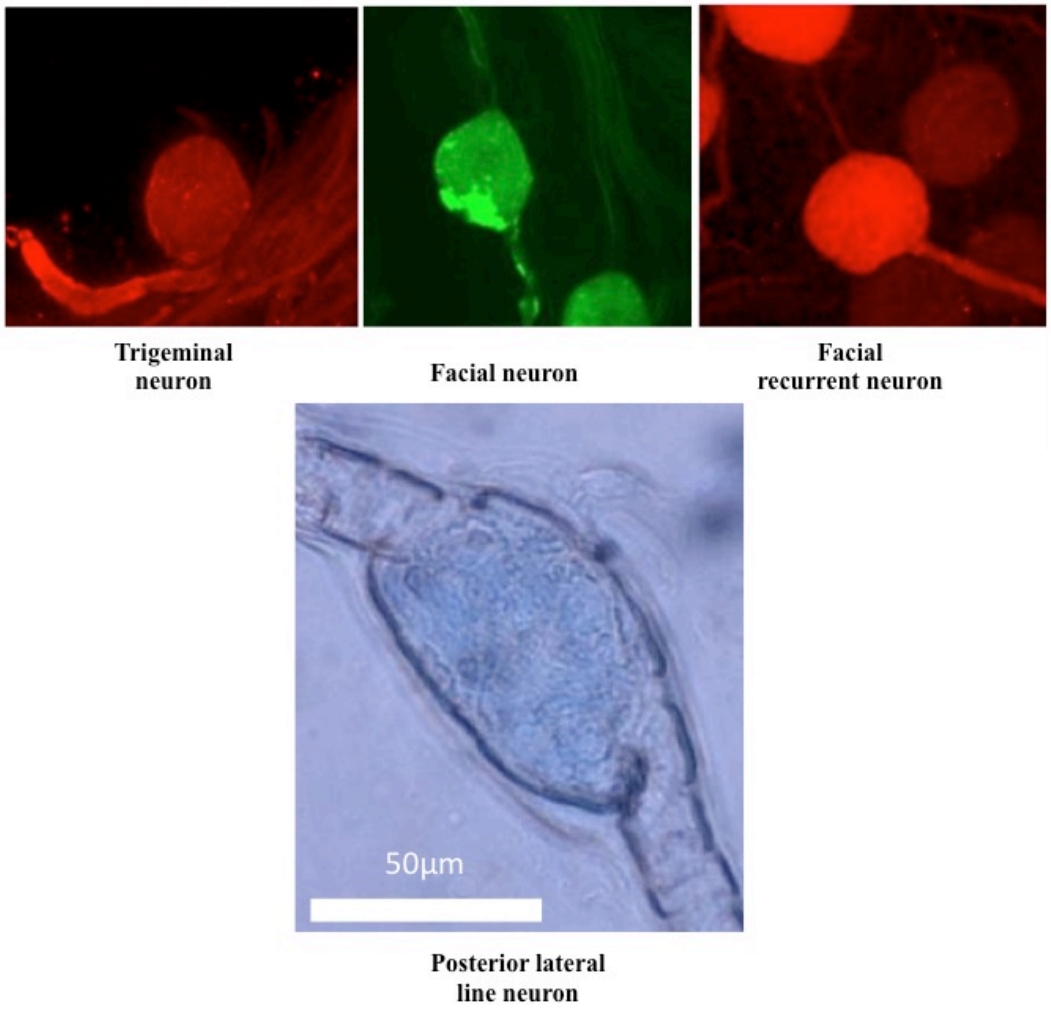


Figure 32. Comparisons of sensory neurons in *P. japonicus*. The present study showed that the trigeminal, facial, recurrent facial, lateral line auditory neurons are bipolar.

	Trigeminal	Anterior Facial	Recurrent Facial	Lateral line
Polarity	Bipolar	Bipolar	Bipolar	Bipolar
Shape of cell body	Round and elongated oval shaped	Round or egg-like shaped	Round shape	Elongated - oval shaped
Diameter range	14.7 – 45.1µm	16.1 – 27.6µm	18.84 - 29.95µm	32.21 – 62.47µm
Average diameter of cell bodies	(LD) 26.1±5.8µm (SD) 21.561±5.4	(LD) 22.5±2.2µm (SD) 18.9±2.6µm	(LD)22.8±2.2µm (SD)18.5 ±2.9µm	(LD)47.7±6.3µm (SD)36.6 ±5.5µm
Fibers	Thick peripheral & thin central fibers	Thick peripheral & thin central fibers	Thick peripheral & thin central fibers	Thick peripheral and thick central fibers

Figure 33. Morphological comparisons of various sensory neurons in *P.japonicus*