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The Autonomic Innervation of the Ovary of the Dab, *Limanda yokohamae*

Kazumasa Uematsu

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Abstract The autonomic innervation of the ovary of the dab was studied histologically and physiologically. The ovary receives a branch of nerve bundles that emerge into the abdominal cavity at the postero-ventral end of the kidney and can be traced back to the sympathetic chain in the vicinity of the 5th vertebra. Almost all the nerve fibers are AChE-positive, and some of them also emit adrenergic fluorescence. Electrical stimulation of the ovarian nerves caused ovarian contractions, and administration of ACh elicited contractions of the ovary preparations, supporting the hypothesis that the ovary is innervated by excitatory cholinergic fibers. In the ovarian nerve bundles, many AChE-positive and non-fluorescent ganglion cells are scattered. Ultrastructural studies suggest that nerve endings situated on the ovarian smooth muscle and on ganglion cells are cholinergic. These results also suggest that the cells are the post-ganglionic neurons of the cholinergic innervation and the axons of the cells reach to the muscle cells. On the other hand, the adrenergic fluoresecent fibers possibly participate in the inhibitory innervation, since the presence of inhibitory beta-adrenoceptors were demonstrated by pharmacological studies.

The rather primitive teleosts such as Salmonidae and Cyprinidae have naked or muscle-lacking ovaries. It was determined by electromyographic studies that the trunk muscles directly contribute to oviposition of these fishes (Uematsu *et al.*, 1980; Uematsu, 1984).

In contrast, the highly developed teleosts like most of the spiny-finned fishes have muscular hollow ovaries (*Uranoscopus*: Young, 1931, 1936; *Lophius*: Young, 1936; *Pungitius*: Yamamoto, 1938; *Oryzias*: Takano, 1968; *Mugil*: Stenger, 1959; *Gadus*: Nilsson, 1970; *Fundulus*: Brummett *et al.*, 1982; *Sarotherodon*: Uematsu, 1985). The development of the ovarian smooth musculature of these fishes appears sufficient to allow the ovary itself to squeeze out eggs, as supposed by Aronson (1957) and Hoar (1957). However, there are no studies in which contribution of the ovary to oviposition and neural control of ovarian movement in these fishes were demonstrated.

The muscular ovary of teleosts investigated so far was innervated with cholinergic excitatory nerves which arose from the abdominal sympathetic ganglia (Young, 1931, 1936; Nilsson, 1970, 1976). It remains enigmatic that acetyl-choline is the active neurotransmitter for the teleost ovary, although the innervation is solely sympathetic (Nicol, 1952). In addition, evidence for the existence of inhibitory, non-cholinergic innervation, has not been found, and morphological knowledge of the innervation is still fragmentary and superficial.

The present study was aimed at determining the autonomic innervation of the dab ovary. Here, histological and physiological investigations were carried out. First, the route of the genital nerves was traced centrally on serial sections, and the route was verified by *in situ* electric field stimulation with the aid of an intraovarian balloon connected to a pressure transducer. Second, for identifying transmitter substances involved in neurotransmission, electron microscopic and histochemical examinations of the ovarian nerve tract were made. Finally, the response of the ovary preparations to autonomic drugs were examined *in vitro* for identifying autonomic receptors.

Materials and methods

Fish used for the present study were approximately 35 mature, but not ovulated, Japanese dab, *Limanda yokohamae*, weighing 150–300 g and showing a gonad-somatic index of c.a. 20(%). The fish were kept in tanks supplied with running sea water or in circulating sea water at 5–10°C and were not fed.

Anatomical studies. Ovarian innervation was studied mainly by dissecting fresh or formalin-fixed specimens under a microscope. To confirm the results obtained from the dissection study,

serial paraffin sections were prepared from tissues to be examined in more detail and were stained with hematoxylin-eosin and/or protargol solution (Bodian, 1936).

Electron microscopy. Tissues were prefixed in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2h–3 days and then post-fixed for 1–2h in 1% osmic acid in the buffer. Next ultrathin spurr sections were stained on the grid with uranyl acetate and lead citrate and observed in a JEM-100S or Hitachi HU-11-ds electron microscope. Semithin sections stained with toluidine blue were used for orientation.

Catecholamine fluorescent histochemistry. The demonstration of catecholamine fluorescence was carried out in two ways: with the Falck-Hillarp procedure (Falck *et al.*, 1962) for serial paraffin sections of freeze-dried tissuses, and with the ALFA procedure (Ajelis *et al.*, 1979) for cryostat sections of unfixed frozen tissues. Specificity of monoaminergic fluorescence was checked by treating the sections with sodium borohydride ($NaBH_4$), which reduced the dihydro or quinoidal form of monoamines to the non-fluorescent tetrahydro form (Corrodi *et al.*, 1964).

After the fluorescence microscopy, the sections were deparaffinated in xylene and stained with hematoxylin-eosin and/or with protargol (Bodian, 1936).

Acetylcholinesterase histochemistry. Cryostat sections of 15–30 μm were picked up on cover glasses (22×22 mm) and were dried under a cool air stream for 10–30 min. The sections were fixed in 4% formaline buffered with 0.1 M phosphate buffer (pH 8.4) for 1h at 4°C. After rinsing in distilled water, the sections were immersed in Karnovsky's solution (Karnovsky, 1964) containing iso-OMPA at a concentration of 10^{-4} M for 1–3h at 4°C.

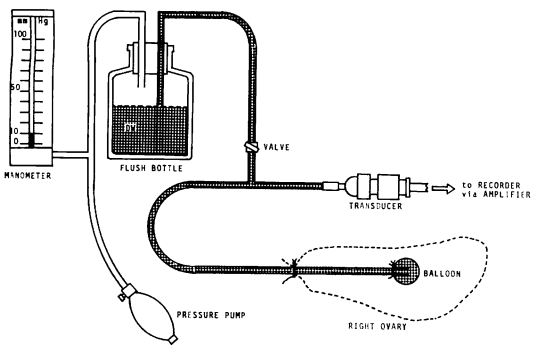


Fig. 1. Diagram showing the apparatus used for measuring the intra-ovarian pressure of the dab *in situ*. A small, rubber balloon connected to a pressure transducer via a polyethylene cannula was inserted into the right ovary from its posterior end.

***In situ* ovary preparations.** After the fish were anaesthetized in 100 ppm tricaine methanesulfonate, the roof of the cranium was removed to expose the brain and the right vagal roots. The central connection of the vagal roots to the medulla were cut to prevent antidromic spreading of vagal stimulation. The anterior portion of the spinal cord, from the posterior end of the medulla to the 10th vertebra, was exposed dorsally. The right ovary was exposed by removing the skin and muscles covering the ovary. In some experiments, the sympathetic chains were exposed for stimulation by removing the kidney; in others, the right lateral side of the anterior portion of the vertebral column was exposed to stimulate the ramus communicans.

Bipolar silver hooked or needle electrode (c.a. 2 mm apart) connected to a Nihon Koden MSE-3R stimulator via an isolator was used for stimulation. Stimuli of 1 msec pulse width, 30 Hz, and 4 V were used in all experiments, after the method of Stevenson and Grove (1977). Polarity of the stimulation current was reversed after each stimulation to avoid possible polarization. Tips of electrodes were placed on each target region, except for the right ovarian blood vessels conveying the ovarian nerve, which were mounted on hooked electrodes.

To measure the ovarian contractions as a change in intraovarian pressure, an intraovarian balloon (c.a. 1 cm^3) connected to a Nihon Koden LPU-0.1 A blood pressure transducer was used (Fig. 1). The balloon with cannula was inserted into the right ovary from its posterior end and was inflated with distilled water. The posterior part of the ovary was tied with a string to the cannula (Fig. 1). The initial pressure within the balloon was set to 10 mmHg because a preliminary test revealed that this pressure gave maximal sensitivity.

***In vitro* ovary preparations.** Fish were immobilized by transection of the medulla oblongata, and then the ovaries were carefully removed. Two or three ring preparations of 10 mm width in the relaxed condition were cut out from the middle

region of each ovary. The preparations were stored in aerated plaice Ringer (Cobb *et al.*, 1973) and then mounted horizontally in a 19 ml Ringer bath at room temperature. Tonus changes of the circular musculature were registered by a Nihon Koden TB-612T isometric tension transducer connected to a pen recorder.

The drugs used in this study were acetylcholine chloride (Wako), atropine sulfate (Wako), neostigmine bromide (Tokyo Kasei), DL-adrenaline (0.1% for injection, Sankyo), DL-isoprenaline hydrochloride (Nakarai), DL-propranolol hydrochloride (Nakarai).

The Ringer in the bath was constantly aerated for oxygenation and for mixing the Ringer and drug solutions. One ml of drug solution was added to 19 ml of Ringer in which the preparation was suspended.

Results

Anatomical and histological observation. The dab ovary consists of two lobes. The lateral view of each lobe appears like an antero-posteriorly elongated triangle. The slender, posterior portion is situated in the postero-inferior space which is enclosed with skin, hemal spines of vertebrae, and a small amount of muscle; the broad, anterior portion is in the visceral cavity. Both lobes fuse together in the antero-ventral portion connecting a very short oviduct which opens just posterior to the anus (Fig. 2).

A nerve bundle branches off the sympathetic trunk near the 8th vertebra (Fig. 4) and runs caudally through the kidney along a blood vessel which branches off the dorsal aorta (Fig. 5). The nerve gives off three branches at the posterior end of the kidney (Fig. 6). The middle one runs to the urinary bladder, and the two others run to the ovarian lobes. A bundle of ovarian nerves accompanying a paired artery and vein (Fig. 3) emerge into the visceral cavity from the ventro-posterior end of the kidney and enter the medial surface of the ipsilateral ovary (Fig. 2).

Histochemical and electronmicroscopic observations. The ovarian membrane of the dab covering the entire ovary is thick (c.a. 100 μm) in the stretched condition in a fish of c.a. 200 mm in standard length and is composed mainly of smooth muscle fibers (Fig. 7). Among the muscle fibers are collagen fibrils. In addition, nerve bundles of various thickness are also observed, although the

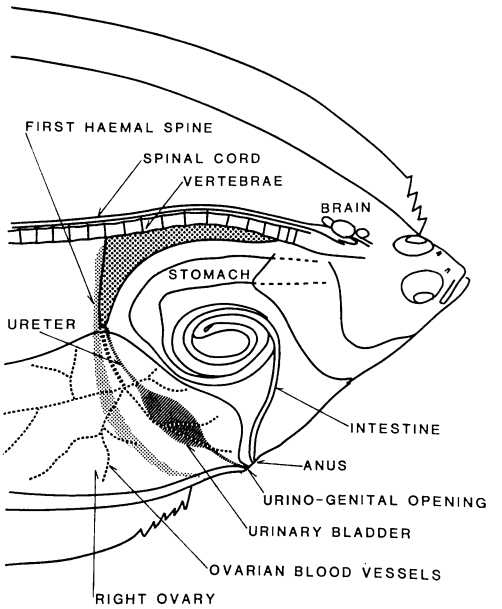


Fig. 2. A schematic diagram showing the general anatomy of the visceral organs of the dab. From the postero-ventral end of the kidney (large dots), the ureter and a pair of bundles of ovarian blood vessels emerge into the visceral cavity. The bundle enters each ovary from its proximal surface and spreads over the surface.

density of innervation is very low. Most peripherally the axons remain in bundles of less than ten, which are supported by Schwann cells (Fig. 8). Some axons only contain agranular vesicles of 40–50 nm, whereas other axons contain an additional population of granular vesicles of 60–80 nm (Fig. 8). Most nerves show acetylcholinesterase (AChe) activities (Fig. 9), but a few fibers emit adrenergic fluorescence (Figs. 10, 11). The fluorescence was reduced by $NaBH_4$ treatment.

Many nerve cell bodies are scattered in the ovarian nerve bundles running in the kidney and the visceral cavity (Fig. 3). Almost all the cells are AChE-positive (Fig. 12) and non-fluorescent (Figs. 13, 14). Ultrastructurally the cells are rich in ribosomes, rough endoplasmic reticulum, and mitochondria, but have few dendritic spines and synaptic boutons (Fig. 15). Two types of synaptic boutons are found. One type contains agranular vesicles of 40–60 nm, and the second type contains granular vesicles of 70–90 nm in

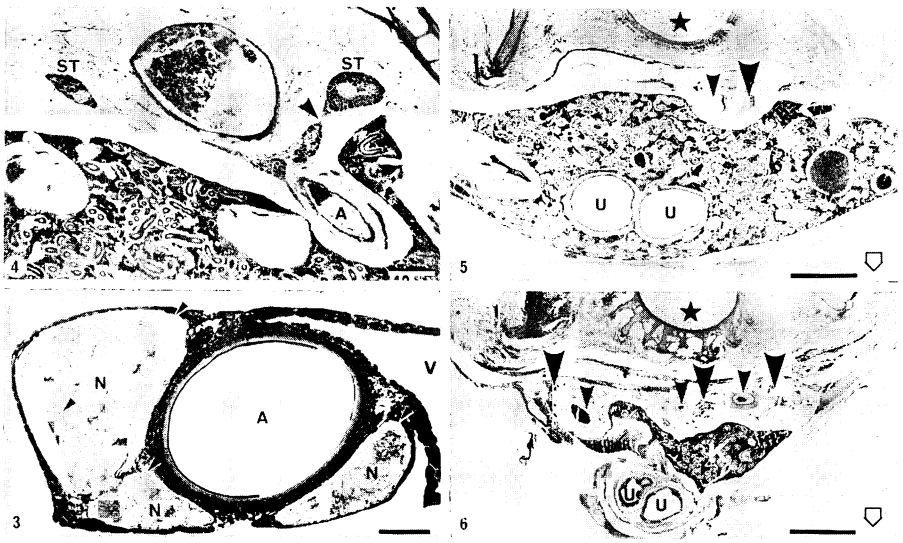


Fig. 3. Transverse sections of the dab ovarian nerves. In the visceral cavity the ovarian nerve (N) runs along a pair of blood vessels, the ovarian artery (A) and vein (V). Many nerve cell bodies (arrowheads) are seen in the nerve bundles. Spurr section stained with toluidine blue. Scale bar=100 μm.

Fig. 4. The vesicular-ovarian nerve branches off the sympathetic trunk (ST) at the point indicated with an arrowhead and runs peripherally along the vesicular-ovarian artery (A). DA, dorsal artery; K, kidney. Hematoxylin-eosin staining. Scale bar=200 μm.

Fig. 5. The vesicular-ovarian nerve (large arrowhead) runs with the artery (small arrow head) in the kidney (K), and near the postero-ventral end of the kidney, they emerge into a space between the kidney and the first haemal spine (asterisk). U, ureter. An open arrow indicates the direction of the head of the animal. Hematoxylin-eosin staining. Scale bar=500 μm.

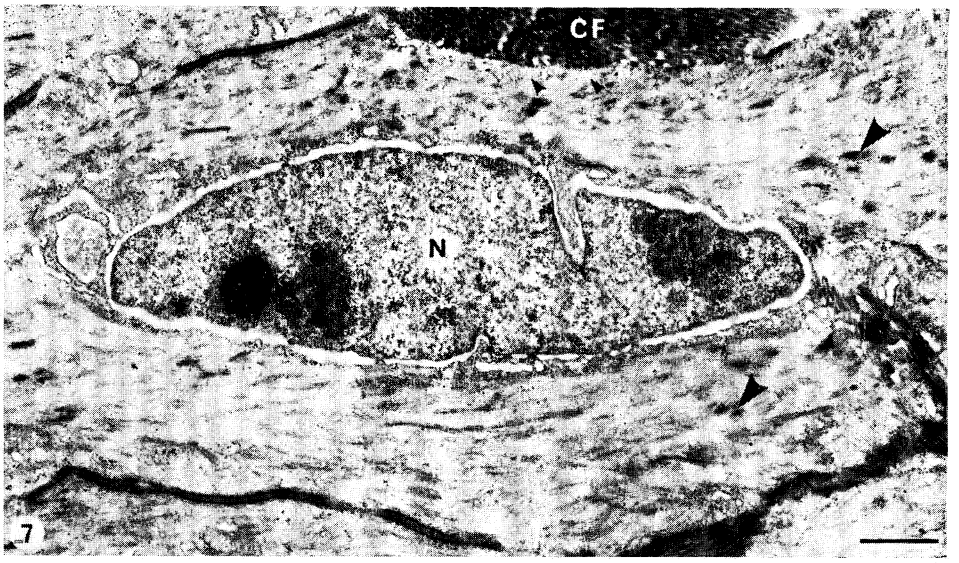
Fig. 6. The vesicular-ovarian nerve and artery breaks into three pairs of nerve (large arrowheads) and artery (small arrowheads) at the postero-ventral end of the kindey (K). The middle pair runs to the urinary bladder and the lateral pairs run to the left and to the right of the ovary, respectively. Nerve cell bodies (black dots) scattered in the nerve bundles are seen. U, ureter. Asterisk is placed in the first haemal spine. An open arrow indicates the direction of the head. Hematoxylin-eosin staining. Scale bar=500 μm.

addition to the agranular ones (Fig. 16).

Electric stimulation studies. The balloon-pressure transducer system fitted to the ovary of anaesthetized dab could detect the ovarian contraction. When the ovary contracted, the intraovarian pressure increased usually 20 mmHg with maximal values of 35 mmHg under the condition that initial pressure was set at 10 mmHg (Fig. 17). Ovarian contractions elicited with electrical stimulation were not accompanied by peristaltic movement, but only by synchronous contractions of the ovary as a whole. The contraction did not result in oviposition in the non-ovulated fish, but caused each ovary to change from flat to cylindrical shape. However, a preliminary study in which ovulated fish were used demonstrated that the artificially induced ovarian contraction resulted in oviposition.

Obvious ovarian contractions were caused only when the following 4 sites were stimulated (Fig. 17): (1) floor of the neural canal between the 2nd and 5th vertebrae (positions b and c in Fig. 17); (2) the right lateral side of the vertebral column between the 1st and 3rd vertebrae (f–h); (3) the dorsal wall of the visceral cavity along the dorsal aorta behind the 5th vertebra (j–m); and (4) the ovarian blood vessels running in the abdominal cavity (n). When the vagal roots (i) were stimulated the ovary did not contract; however, the stomach or the intestine often responded. Stimulation of the neural canal of the 5th or 6th vertebra (a) only elicited movements of the urinary bladder.

Pharmacological studies. Administration of acetylcholine (ACh) (0.1 and 0.01 mM) caused con-



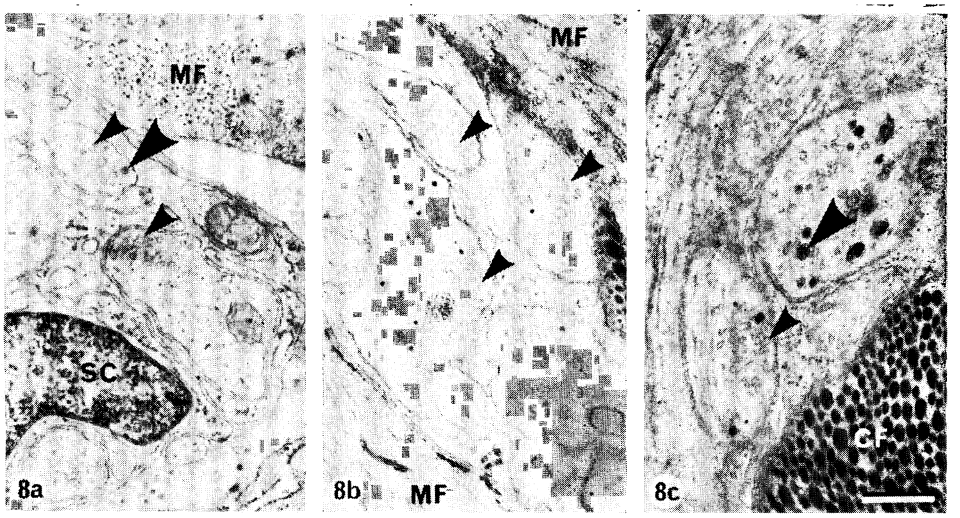


Fig. 7. Electron micrographs of the dab ovarian membrane. A smooth muscle cell in the ovarian membrane. The cell bears typical features of smooth muscle fibers. Large and small arrowheads indicate dense bodies and membrane invaginations, respectively. N, nucleus; CF, collagen fibrils. Scale bar=1 μm.

Fig. 8. Axonal profiles of nerves distributed among smooth muscle fibers (MF). Some axons in a and b contain only small agranular vesicles (small arrowheads). The others contain both small agranular and large granular (large arrowheads) vesicles. CF, collagen fibrils; SC, Schwann cell nucleus. Scale bar=500 μm.

tractions of ovarian circular muscles of the dab (Fig. 18). The contractions caused by ACh were enhanced and prolonged by neostigmine (Neo) (0.1 mM) and were readily blocked by atropine (Atr) (0.1 mM) (Fig. 18a). Both adrenaline (A) (5×10^{-6}%) and isoprenaline (Iso) ($>10^{-4}$ mM) reduced the magnitude of the contractions caused by ACh (Fig. 18b, c). After the preparations were treated with an adrenergic beta receptor antagonist, propranolol (Pro) (0.1 mM), the inhibitory action of the adrenergic drugs disappeared, and the response of the preparations to ACh became larger (Fig. 18b, c).

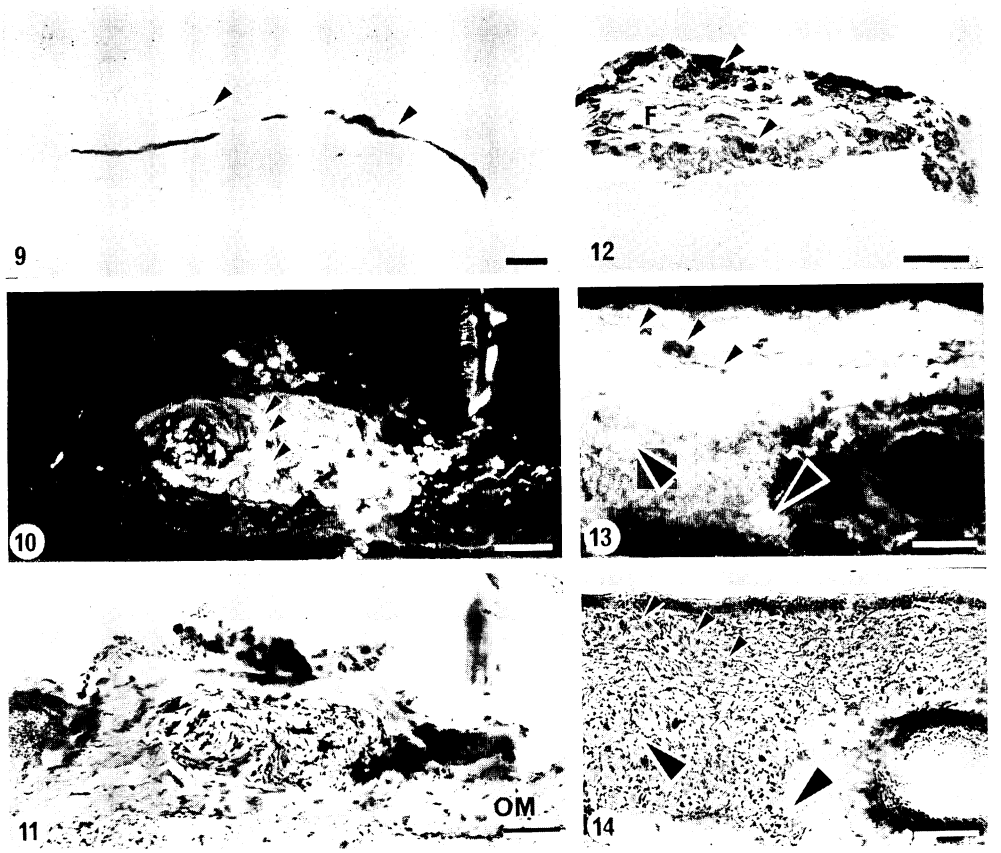


Fig. 9. Histochemistry of nerves in the dab ovarian membrane. Many AChE-positive nerves of various thicknesses (arrowheads) distributed in the membrane. Karnovsky thiocholine method. Scale bar=100 μm.

Fig. 10. Some monoaminergic fluorescent fibers (arrowheads) are seen in a thick nerve bundle running in the ovarian membrane. The other fluorescences are non-specific. Falck-Hillarp method. Scale bar=100 μm.

Fig. 11. The same section as Fig. 10. Stained with hematoxylin-eosin. OM, ovarian membrane. Scale bar=100 μm.

Fig. 12. Histochemistry of the dab ovarian nerves. Both nerve fibers (F) and nerve cell bodies (arrowheads) in an ovarian nerve bundle are AChE-positive. Karnovsky thiocholine method. Scale bar=100 μm.

Fig. 13. An adjacent section to Fig. 12 treated for demonstration of monoaminergic fluorescence. A broad fluorescent band is seen in the upper half of the picture. With this band, small, non-fluorescent areas exist (small arrowheads). In the lower half, small fluorescences (large arrowheads) are also seen. ALFA method. Scale bar=100 μm.

Fig. 14. The same section as Fig. 13 stained by Bodian's protargol procedure. Nerve fibers occupy most of the fluorescent areas in Fig. 13 (the upper half and the parts indicated with large arrowheads). The dark portions within a fluorescent area (small arrowheads) correspond to nerve cell bodies. Scale bar=100 μm.

Discussion

The innervation of muscular teleost ovaries have been studied in only three species. A recent study on *Tilapia* has revealed that the ovary possibly receives nerves of different origin from that found in the previous investigations (Uematsu, 1985). In *Uranoscopus* the ovary is innervated by the genital nerves that arise from the sympathetic trunk of the posterior abdominal regions (Young, 1931).

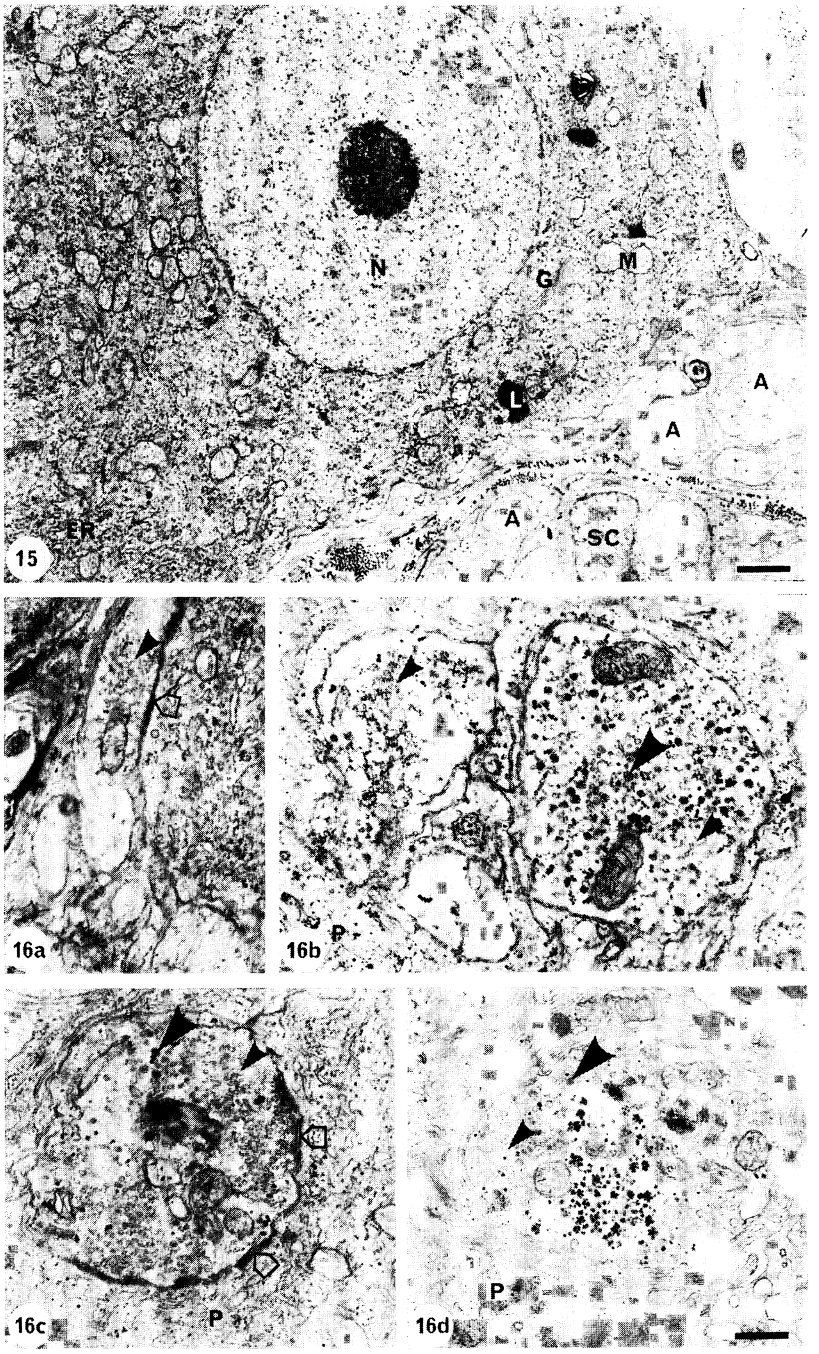


Fig. 15. Electron micrographs of a nerve cell body in the dab ovarian nerve bundle. A nerve cell body displays all the organelles found in neurons. The cytoplasm includes mitochondria (M), Golgi apparatus (G), granular endoplasmic reticulum (ER), lysosome (L) and clouds of ribosomes. The large nucleus (N) contains a large, dense nucleolus. Scale bar=1 μm.

Fig. 16. Axon terminals synapsing with nerve cell bodies. Some terminals in a and b contain small agranular vesicles (small arrowheads) alone and the others in b, c and d contain both small agranular and large granular (large arrowheads) vesicles. Open arrows indicate the synaptic junctions. A, axons; P, perikarya of nerve cell body; SC, Schwann cell nucleus. Scale bar=500 nm.

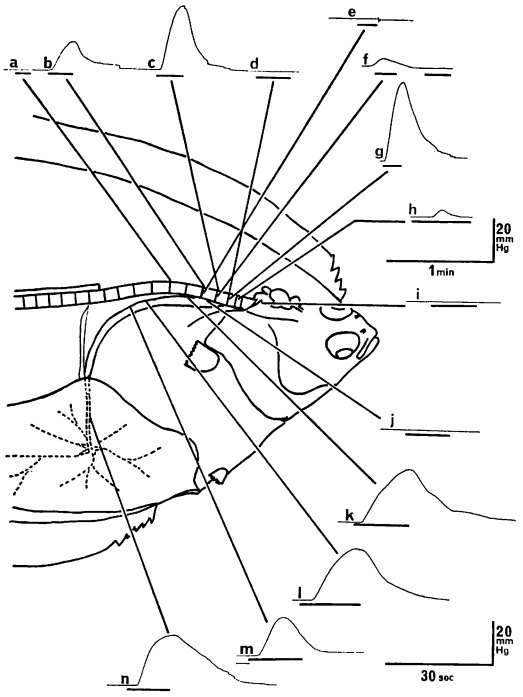


Fig. 17. A schematic drawing showing the stimulated points and the responses of the *in situ* dab ovary preparations. Bars under the response curves indicate stimulation periods. For explanation see text.

Young (1936) also demonstrated in *Lophius* and *Uranoscopus* that faradic stimulation of the sympathetic chains of the abdominal regions elicited movements of the ovary and that the ovaries contracted by an acetylcholine solution. In *Gadus*, the gonad is innervated by the excitatory cholinergic nerves branching from the vesicular nerves (Nilsson, 1970), which arise from the 20th pair of the sympathetic ganglia (Nilsson, 1976). In these studies the presence of inhibitory innervation was suggested, but remained unproved. Furthermore, since the investigations by Nilsson, no detailed studies on the autonomic innervation of teleost ovaries have been carried out.

The present study on the Japanese dab revealed that electrical stimulation of the ovarian blood vessels caused ovarian contractions, indicating that the vessels carry the excitatory nerves to the ovary. Furthermore, the nerves could be traced back anatomically and also histologically to the sympathetic chain situated at the 8th vertebra. Therefore the origin of the nerves of this species must be regarded to be basically the same as in *Uranoscopus*, *Lophius* and *Gadus*. Another similarity is seen in the branching pattern. In the latter three species, the genital nerves branch off the vesicular nerves (Young, 1931; Nilsson, 1970 1976). From the results of nerve tracing with electrical stimulation, it is speculated that the excitatory ovarian nerves of the dab arise from somewhere in the spinal cord, travel in the ventral roots in the vicinity of the 3rd vertebra and reach the sympathetic chain around the 5th vertebra via ramus communicans. As for the excitatory innervation of the urinary bladder, it appears to arise from the spinal cord at a level slightly posterior to that of the ovary.

Almost all the nerves innervating the dab ovary were AChE-positive, but a few others also emitted adrenergic fluorescence. It is well known that adrenergic fibers also contain AChE (Tervo, 1977), and adrenergic and cholinergic axons run in the same Schwann cell sheath (pers. comm. S. Nilsson). Nerve terminals situated among the ovarian smooth muscle cells contained agranular vesicles alone whereas others contained a few granular ones together with the mentioned vesicles. ACh is generally assumed to be the transmitter associated with agranular vesicles (Gabella, 1981), although it is still premature to draw a general conclusion correlating synaptic function with the morphology of the synaptic vesicles (Peters *et al.*, 1976). A similar classification had been also applied to fish intestine (Watson, 1981).

The contractions elicited by acetylcholine were enhanced by neostigmine and were blocked by atropine, indicating the presence of muscarinic ACh receptors on ovarian smooth muscle cells. The inhibitory actions of adrenaline and isoprenaline were changed to excitatory ones after an application of the adrenergic beta-receptor antagonist, propranolol. This result suggests that excitatory alfa-adrenoceptors coexist in this tissue. From these experiments, it is suggested that the excitatory innervation to the ovary is mediated via the AChE-positive, non-fluorescent fibers reaching muscarinic ACh receptors on muscle cells. Although inhibitory innervation of teleost ovary has not been discovered as yet (Young, 1936; Nilsson, 1970), it is possible that the adrenergic fluorescent fibers found in the present study represent this innervation.

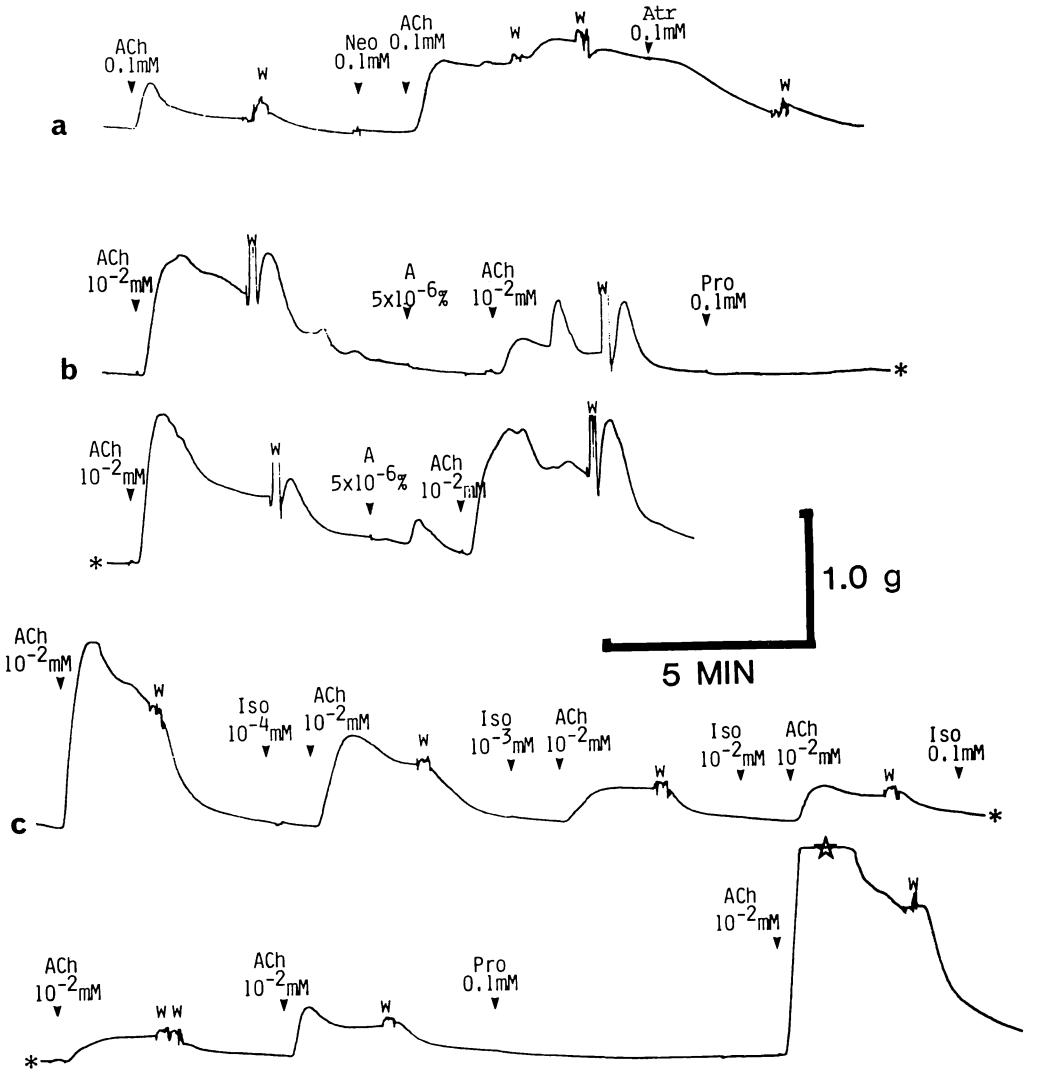


Fig. 18. Responses of the *in vitro* dab ovary preparations to drugs. a, acetylcholine (ACh) caused contraction of the preparation. The response was enhanced with neostigmine (Neo) and inhibited with atropine (Atr). b and c, both adrenaline (A) and isoprenaline (Iso) reduced the cholinergic contraction. The inhibitory effect of A and Iso was changed to one of excitation after proprenolol (Pro) treatment. (w represents a change of Ringer solution in the bath.)

In the genital nerve bundles of *Uranoscopus* (Young, 1931) and *Gadus* (Nilsson, 1976), though no ganglion cells had been previously found along the nerve, many ganglion cells were observed scattered along the length of the nerves in the dab (Fig. 3). Most of these cells were AChE-positive and non-fluorescent. Most axonal terminals of the synapses on the cells contained only agranular vesicles. For the same reasons as stated above, the synapses are possibly cholinergic.

On the basis of these results, the observed neurons are assumed to be post-ganglionic neurons of the cholinergic excitatory innervation of the dab ovary, similar to the conclusions reached by Nilsson (1970) for the vesicular ganglion cells of the urinary bladder of the cod. The preganglionic neurons of the innervation are assumed to be situated in the spinal cord, as suggested by the

results of the stimulation study. On the other hand, the adrenergic fluorescent fibers, which possibly participate in the inhibitory innervation of the ovary, may originate from sympathetic neurons in the sympathetic trunk.

It has generally been accepted that fish lack sacral parasympathetic outflow. At the same time it is suspected that the physiological properties of the fish autonomic nervous system are inconsistent with the classical divisions based on those of the system in mammals. Furthermore, in recent years, a new concept was proposed that in the fishes it is not possible to divide the autonomic nervous system into two antagonistic systems as found in mammals (Young, 1981); the vesicular nerves to the gonad and the bladder of some teleosts may represent a primordial sacral parasympathetic outflow, although they pass via the sympathetic chains (Holmgren and Nilsson, 1982). The results of the present studies support and confirm the new interpretation of the fish autonomic nervous system. But more physiological and histochemical data must be gathered before the architecture of the system can be completely established.

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(Laboratory of Fish Physiology, Faculty of Applied Biological Science, Hiroshima University, Fukuyama 720, Hiroshima, Japan)

マコガレイ卵巣の自律神経支配の組織学的・生理学的研究

植松一真

マコガレイ卵巣の自律神経支配を組織学的・生理学的に検討した．卵巣神経は第5椎骨近傍で交感神経幹から分岐した後，腎臓内を走行し，腎臓の後端から腹腔内に現れ，左右の卵巣へ至る．卵巣神経のほとんど全てはアセチルコリンエステラーゼ (AChE) 陽性であるが，僅ながらアドレナリン性螢光を発する神経繊維も含まれる．卵巣神経に電気刺激を加えると卵巣は収縮した．また，卵巣の摘出標本はアセチルコリン溶液により収縮した．このことから，マコガレイ卵巣は興奮性のアセチルコリン作動性神経の支配を受けていることが明らかとなった．上記神経に加え，卵巣神経内には AChE 陽性で非アドレナリン性である多数の神経細胞体が散在する．同細胞体表面に見られるシナプスと卵巣平滑筋細胞上の神経終末のほとんどはアセチルコリン作動性であることが電子顕微鏡観察により確認された．以上の結果より，卵巣神経内に存在する神経細胞体はアセチルコリン作動性神経支配系の節後神経であり，この軸索は平滑筋細胞に達するものと推察される．一方，卵巣の収縮はアドレナリンの β 作用によって抑制されることから，卵巣神経内のアドレナリン性螢光を発する繊維は抑制的支配に関与するものと考えられる．

(720 福山市緑町 2-17 広島大学生物生産学部水産動物生理学研究室)