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Development of the Brain in the Oegopsid Squid, *Todarodes pacificus*: An Atlas Up to the Hatching Stage

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ABSTRACT—An atlas of the developing brain up to hatching stage is established using conventional histological methods in the oegopsid squid, *Todarodes pacificus*. The brain originates from placodal thickenings in the ectoderm at the end of epiboly. The neuroblasts composing the placodes ingress in a group and accumulate into ganglia under the proximal surface of the surface epithelium. Four pairs of the ganglia, pedal, palliovisceral, cerebral, and optic, form the brain primordium. These ganglia come into contact with one another, and eventually accumulate into a ring-like cluster (circumesophageal cluster) encircling the oral ingrowth and the inner yolk around the surface of the head. The circumesophageal cluster regionally differentiates into brain lobe anlagen through formation of neuropiles and nerve tracts. The neuropiles form a ladder-like structure with two longitudinal columns situated in the ventrolateral parts of the circumesophageal mass and some axonal tracts bridging the left and right columns (ladder-like framework). The brain is quite premature at the time of hatching, especially in the supraesophageal part. Though most brain nerves are already present, many brain lobes and commissures found in the adult brain are not yet differentiated. The present results show that the morphological processes of the brain formation are essentially common among the coleoid cephalopods. The similarity of the embryonic brain in *Todarodes* to the adult brain in *Nautilus* suggests that the coleoid brain has evolved on the basic plan as seen in the nautiloid brain.

INTRODUCTION

Cephalopods possess a large multilobed brain capable of complex analyses, learning, and social behaviors (Wells, 1978; Boyle, 1986; Young, 1991, 1995; Hanlon and Messenger, 1996). Morphological studies have revealed the nervous architecture of the brain in several cephalopod species (Young, 1965, 1971, 1974, 1976, 1977a, b, 1979; Messenger, 1979; Budelmann and Young, 1993), and electrophysiological works are clarifying the correlation of the structure and function in the brain (see Abbott *et al*., 1995). In contrast to extensive anatomical studies of the adult brain, our knowledge on the developmental process of the cephalopod brain is limited (Marthy, 1987; Fioroni, 1990); most developmental studies are restricted to a part of the nervous system (Messenger, 1973; Yamamoto, 1985; Wildenburg and Fioroni, 1989; Wentworth and Muntz, 1992; Nixon and Mangold, 1996; Dickel *et al*., 1997). The outline of brain development has been reported as a part of the studies of general organogenesis in a myopsid squid, *Loligo vulgaris* (Meister, 1972; Fioroni and Meister, 1974). Details of brain formation has been described in a cirrate octopus, *Octopus vulgaris* (Marquis, 1989) and a myopsid squid, *Sepioteuthis lessoniana* (Shigeno *et al*., in preparation) using conventional histological techniques, but early sequences of the formation of nerves, axon tracts and commissures within the developing brain have not been shown. Data in cephalopod groups other than the Myopsida and the Octopoda are necessary for a comprehensive understanding of the brain development in the Cephalopoda.

Some species of the oegopsid squids such as *Illex* (Boletzky *et al*., 1973; O'Dor *et al*., 1982), *Abralia*, (Young and Harman, 1985), *Abraliopsis* (Arnold and O'Dor, 1990), *Sthenoteuthis* (Sakurai *et al*., 1995), *Todarodes* (Watanabe *et al*., 1996), and *Thysanoteuthis* (Watanabe *et al*., 1998) show a unique mode of development among cephalopods such as: a small egg size, a reduced external yolk sac, a relative delay in the formation of the gills, digestive and circulatory organs, statocysts, and two arms, and precocious hatching after a

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short embryonic period. Most oegopsid species showing this mode of development are oceanic and little is known about development of their nervous system. Development of an ommastrephid, *Todarodes pacificus*, an exceptionally coastal oegopsid species (Dunning and Wormuth, 1998) has repeatedly been studied (Hamabe, 1962; Bower and Sakurai, 1996; Ikeda *et al*, 1993; Sakurai *et al*., 1996; Watanabe *et al*., 1996) and the normal embryonic stages have been established by Watanabe *et al.* (1996). In order to provide a basic data for general as well as comparative developmental studies of the cephalopod brains, we describe fundamental processes of the brain development in *T. pacificus* using classical histological techniques. Though the eggs are difficult to obtain, we found the *T. pacificus* embryo suitable for studies of neural development because of a small size and a small cell number, and we could clarify early stages of nerve and axon tract formation in the embryonic brain to a considerable degree. The present paper deals with the brain development up to the hatching stage. Post-embryonic development of the brain in the paralarvae and juveniles will be described in the succeeding paper (Shigeno *et al.*, in preparation).

MATERIALS AND METHODS

Specimens

Adults of an ommastrephid squid, *Todarodes pacificus* (Teuthoidea, Oegopsida) were collected by jigging in the northeastern waters of the Sea of Japan. Eggs removed from just caught mature females were artificially inseminated on board with sperm stored in the female's seminal receptacles according to the method of Ikeda *et al.* (1993). The fertilized eggs were mixed with the gelatinous substance obtained from the oviducal gland. This treatment induced elevation of the chorion to form the perivitelline space (Ikeda *et al*., 1993). Embryos were reared at 20.5°C (Sakurai *et al*., 1996). Embryonic stages were identified under a stereoscopic microscope according to the normal table by Watanabe *et al.* (1996), which is summarized as follows: After maturation division (Stage 2–3; abbreviated as St 2–3) and cleavage of the blastodisc (St 4–10), the blastoderm gradually covers the egg surface from the animal to the vegetal pole (epiboly) (St 11–15). Major organ primordia appear as faint thickenings of the embryonic surface (St 16). Organ primordia become evident in the order of the shell gland (St 16), mouth and eyes (St 17), mantle, funnel, tentacle and arm 2 (St 19), arm 1 (St 20), and statocysts (St 21). Yolk is transferred from the external yolk sac to the internal yolk sac (St 24). Primordia of ventral organs such as the gills, hearts and stomach become visible as swellings on the internal yolk sac (St 25). Hatching occurs 92 hr after fertilization at 23°C (St 26).

Histology

The embryos were fixed in Bouin's solution dissolved in seawater for 12 hr and stored in 70% ethanol. The specimens were embedded in Paraplast (OXFORD). Three dimensional maps of embryonic brains were reconstructed from serial sections of 3–5 μm thick stained with Mayer's hematoxylin and eosin (HE) or Masson's trichrom stain (MS). We used semithin sections of plastic (Spurr's resin) embedded materials stained with toluidine blue in order to confirm structural details.

Terminology

The terms used in the present study are based on the definitions given for the adult nervous system of *Loligo* by Young (1974,1976, 1977b, and 1979), Messenger (1979), and Budelmann and Young (1993). Terms given by Marquis (1989) for the embryonic nervous system are also used. The structure usually called visceral ganglion in cephalopod embryology (e.g. Meister, 1972; Fioroni and Meister, 1974; Marquis, 1989) cannot be homologous to the structure called by the same name in the gastropods. To avoid confusion, we use "palliovisceral ganglion" instead of "visceral ganglion". We use "lobe anlage" for the part of the embryonic brain that we find corresponding to a future lobe in the adult brain on the basis of differentiation of specific neuropiles and/or axon tracts. We introduce new terms such as circumesophageal cluster, cerebral arch, posterior horn lateral column, and ladder-like framework.

RESULTS

Outline of the embryonic development of the brain

Late embryos of the cephalopods show arms and a tentacle at the anterior end and the mantle closes at the posterior end (cf. Fig. 7). The funnel and the collar are present in the ventral surface, and the mouth opens in the dorsal surface. Embryonic body contains a large mass of internal yolk, and the primordia of the respiratory and digestive organs occur on the ventral surface of the internal yolk sac.

The brain originates from 4 pairs of separately formed ganglia: the pedal, palliovisceral, cerebral, and optic ganglia. Each ganglion occurs as a placodal thickening of the ectoderm covering the yolk syncytium in the embryo where epiboly has just finished (St 15) (Fig. 1A). The neuroblasts composing the neurogenic placodes ingress in between the surface ectoderm and the yolk syncytium (Fig. 1B–E), and assemble into the ganglionic bodies (Fig. 1F, G). The ganglia increase in size through vigorous cell proliferation, come into contact with one another, and eventually form a ring of band encircling the internal yolk in the head region of the embryo (Fig. 2, St 19). Since the oral ingrowth (the future buccal mass and the esophagus) is also enclosed by the ring, we name the ganglionic accumulation the circumesophageal cluster. The circumesophageal cluster gradually differentiates into the brain lobes through the formation of neuropiles and axon tracts (Figs. 4–8), which are seen as pale fibrous spaces devoid of cell bodies in the HE-stained sections (Fig. 4B, C). Neuropiles form a large longitudinal column in the ventrolateral region of the circumesophageal cluster (Figs. 5 and 7). We will refer to this structure as the lateral column. Some transverse bundles of neuropiles (commissural bundles) link the left and right lateral column (Fig. 6). The lateral columns and the commissural bundles show a ladder-like configuration (Fig. 10), which we name the ladder-like framework. The brain is quite premature at the time of hatching; it retains a ring-like configuration (Fig. 9) and many lobes composing the adult brain are not yet identifiable.

Ingression of neuroblasts into ganglia

Since the nervous system is symmetric in structure with respect to the midplane of the embryonic body, we will describe the processes on one lateral half of the embryo unless otherwise specified. The neurogenic placodes are characterized by loosely and irregularly arranged neuroblasts with

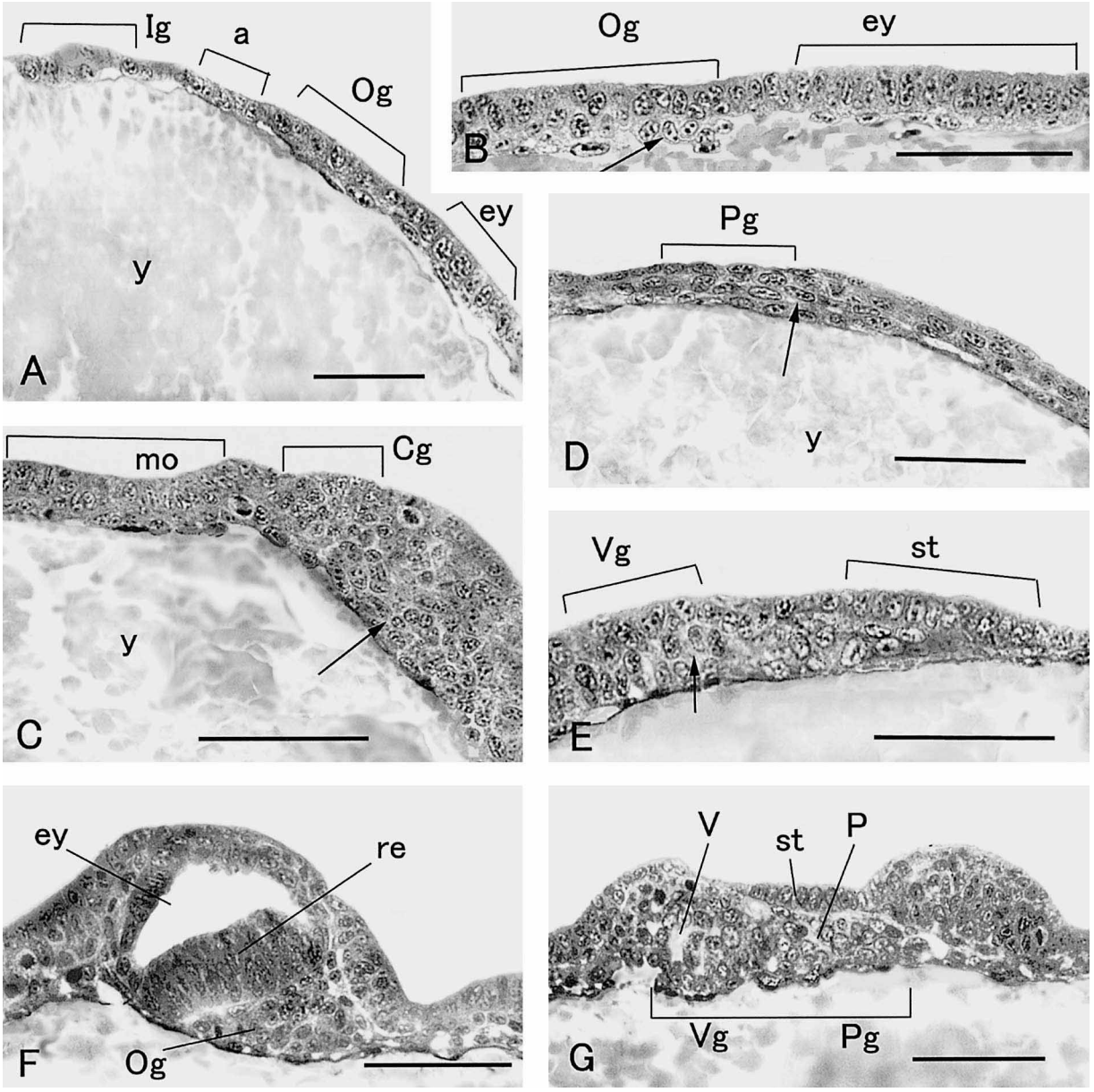

Fig. 1. Light micrographs of the early phase of brain development. (A) Occurrence of the placode of an intrabrachial ganglion (Ig), arm (a), optic ganglion (Og), and eye (ey) in the ectoderm convering the yolk (y) in the embryo where epiboly has just finished (St 15). (B) The placodes of an optic ganglion (Og) and an eye (ey) at St 16. Neuroblasts are ingressing (arrow) from the placode of the optic ganglion toward the placode of the eye. (C) The placode of a cerebral ganglion (Cg) occurring adjacent to the mouth primordium (mo) at St 17. The arrow indicates ingressing neuroblasts. (D) The placode of a pedal ganglion (Pg) and ingressing neuroblasts (arrow) at St 17. (E) the placode of a palliovisceral ganglion (Vg) occurring near the placode of a statocyst (st) at St 17. The arrow indicates ingressing neuroblasts. (F) The optic ganglion (Og) formed just under the developing retina (re) in the eye anlage (ey) at St 19. (G) A continuous mass of neuroblasts derived from the palliovisceral ganglion (Vg) and the pedal ganglion (Pg) under the primordium of a statocyst (st) at St 20. The neuropiles are visible in the neuroblastic mass from the palliovisceral ganglion (palliovisceral neuropile, V) and the pedal ganglion (posterior pedal neuropile, P). Bar, 100 μm.

nuclei lightly stained with HE (Fig. 1A–E). The surface ectoderm around the neurogenic placodes shows a simple epithelium of tightly arranged cells with a densely stained nucleus. The earliest recognizable neurogenic placode is that of the optic ganglion (St 15) (Fig. 1A). It occurs on the anterior margin of the eye placode in the lateral side of the embryo (Fig. 2). The neuroblasts ingress in a group between the eye placode and the yolk syncytium (Fig. 1B), gradually gathering into an oval-shaped ganglionic body in contact with the proximal surface of the primordial retina (Fig. 1F). Neuroblasts continue to ingress from the neurogenic placode and join the optic ganglion from St 15 through St 19. A neurogenic placode of the pedal ganglion (Fig. 1D) occurs at a region posterior to the tentacle primordium in the ventral surface of the embryo at St 16 (Fig. 2). Neuroblasts ingress in a group to form the pedal ganglion in contact with the intrabrachial ganglia. The neurogenic placode of the cerebral ganglion occurs at the lateral edge of the mouth opening in the dorsal surface

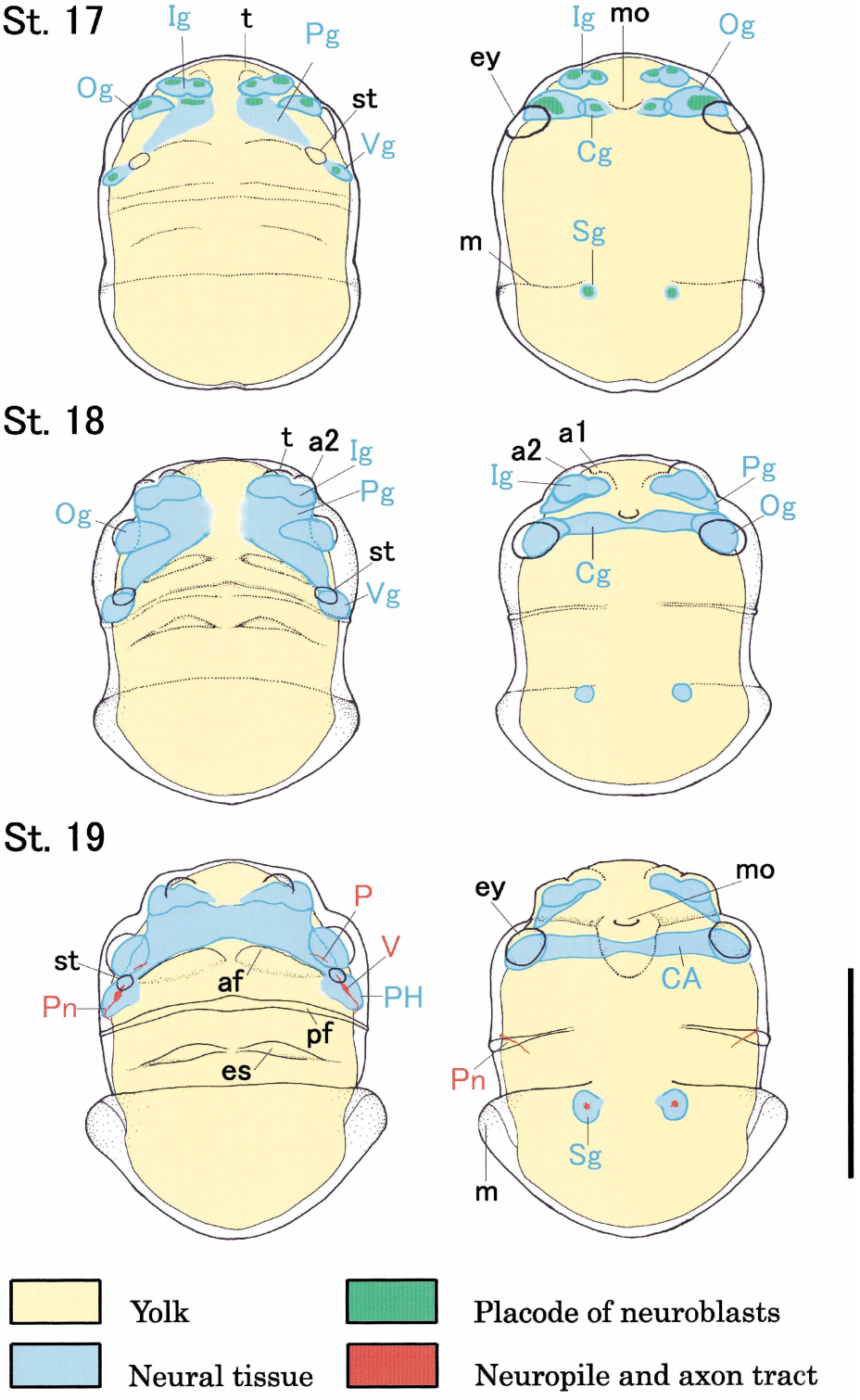

Fig. 2. Development of the nervous system in the *T. pacificus* embryos from St 17 to St 19. Left, ventral view; right, dorsal view. al, arm 1; a2, arm 2; af, anterior funnel fold; CA, cerabral arch, Cg, cerebral ganglion; es, endodermal swelling; ey, eye; Ig, intrabrachial ganglion; m, mantle; mo, opening of mouth; Og, optic ganglion; P, posterior pedal neuropile; pf, posterior funnel fold; Pg, pedal ganglion; PH, posterior horn; Pn, pallial nerve; Sg, stellate ganglion; st, statocyst; t, tentacle; V, palliovisceral neuropile; Vg, palliovisceral ganglion. Bar, 0.5 mm.

of the embryo (Fig. 1C). The internalized neuroblasts migrate in a group under the proximal surface of the ectoderm toward the ipsilateral optic ganglion (Fig. 2). The neurogenic placode of the palliovisceral ganglion is at first difficult to detect because of very small numbers of composing neuroblasts and lack of a proper landmark. Oval-shaped palliovisceral ganglion becomes discernible in the posteriolateral region of the just-formed statocyst placode in the ventrolateral part of the

embryo at St 17, extending toward the statocyst primordium (Figs. 1E and 2). Neuroblasts continue to ingress from the neurogenic placode and join the palliovisceral ganglia up to St 21. Four ganglia categorized in the peripheral nervous system (a stellate and 3 intrabrachial ganglia) become evident up to St 17 (Fig. 2). The stellate ganglion occurs near the dorsal margin of the mantle primordium. The intrabrachial ganglia occur within the primordial swellings of the tentacle, and the arms 1 and 2. (In *Todarodes*, additional two arms appear after hatching).

Accumulation of the ganglia into the circumesophageal cluster

The pedal ganglion extends in a posteriolateral direction at St 17 (Fig. 2). Laterally it comes into contact with the optic ganglion and posteriorly it reaches the palliovisceral ganglion under the proximal surface of the statocyst placode at St 18 (cf. Fig. 1G). Anteriorly the pedal ganglion extends in a dorsal direction along the posterior margin of the intrabrachial ganglia and arrives at the dorsal end of the intrabrachial ganglion in the primordium of the arm 1 at St 18 (Fig. 2). The optic ganglion keeps a spherical entity (optic lobe anlage) under the proximal surface of the retina differentiating in the eye primordium during embryonic development (Figs. 1F, 4E and 8A, B). The cerebral ganglion comes into contact with the pedal and palliovisceral ganglia in the lateral region of the head near the optic ganglion. Thus, the 4 ganglia form a continuous mass of neuroblasts in each side of the embryo. The left and right cerebral ganglia extend toward the midline of the embryo in the dorsal surface, merge with the contralateral counterpart at the posterior part of the mouth opening and form an arched bridge of neuroblasts at St 18 (Fig. 2, CA. Abbreviations are common to all figures). We name the arched bridge the cerebral arch. The dorsal roof of the cerebral arch remains to be a thin transverse belt between the surface ectoderm and the epithelium of the oral ingrowth during the embryonic period (Figs. 3 and 9). The left and right clusters of the pedal, optic, and palliovisceral ganglia are connected through the thin belt of the cerebral arch in the dorsal surface. At St 19 (Fig. 2), the left and right pedal ganglia merge with each other by a thin and broad belt of a few cell layers in the ventral surface of the embryo. Thus, the pedal, palliovisceral, optic, and cerebral ganglia form a large ring (the circumesophageal cluster) encircling the oral ingrowth and the inner yolk (Fig. 3). The ventral part of the circumesophageal cluster becomes a thick cell mass (the subesophageal mass) through a vigorous cell proliferation after St 21 (Fig. 4B). The subesophageal mass is posteriorly demarcated by the fold of the anterior funnel primordium at St 21–22. The anterior end of the subesophageal mass keeps contact with the intrabrachial ganglia throughout the embryonic period. (In the adult, the intrabrachial ganglia separate from the brain.) The neuroblasts in the pedal ganglion are stained more deeply with HE than those in the intrabrachial ganglia (Fig. 6D). The posteriolateral part of the subesophageal mass that derives from the palliovisceral ganglion protrudes posteriorly (PH in Fig. 5), which we name the

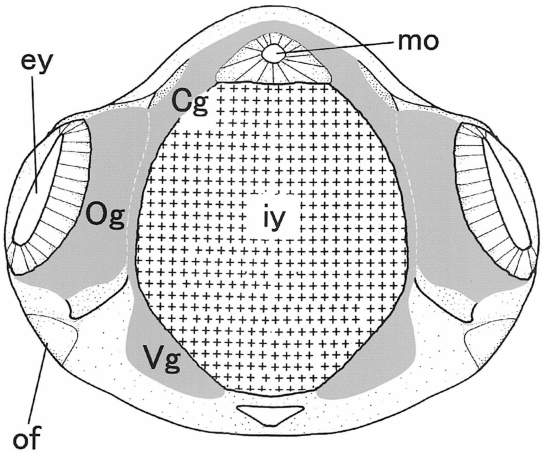

Fig. 3. Transverse section through the head of the embryo at St 22. The section shows the posterior part of the ring formed by accumulation of the ganglionic precursors of the brain. In the anterior to this section, the left and right pedal ganglion which are continuous to both the cerebral (Cg) and the palliovisceral ganglion (Vg) merge at the midplane of the embryo. Top, dorsal. ey, eye; iy, internal yolk; mo, oral ingrowth; of, olfactory orogan; Og, optic ganglion.

posterior horn. The right and left posterior horns extend toward the midline of the embryo from St 21 (Fig. 5). Deeply stained cells gradually accumulate and eventually form a transverse belt bridging the right and the left posterior horns in the ventral surface at St 24 (Fig. 7). The cerebral arch remains to be a thin belt up to St 23 (Fig. 4A), gradually changing into a thick cell mass corresponding to the supraesophageal mass in the adult brain through cell proliferation after St 24 (Figs. 6A and 8C).

Regional differentiation of the circumesophageal cluster

St 19 (Fig. 2): The earliest formed neuropiles are discernible in the posterior horn (PH) as well as in the ventrolateral region of the circumesophageal cluster. Since the posterior horn derives from the palliovisceral ganglion, we will refer to the neuropile in the posterior horn as the palliovisceral neuropile. The palliovisceral neuropile (V) is small at this stage but already gives rise to the pallial nerve (Pn) (the nerve to innervate the mantle) in a posteriodorsal direction. This is the first recognizable nerve fiber. The neuropile in the ventrolateral region of the circumesophageal cluster (P) is visible only slightly. Since the posterior pedal lobe begins to differentiate from this neuropile, we will refer to this neuropile as the posterior pedal neuropile.

St 20 (Fig. 5): The posterior pedal neuropile (P) becomes more distinct. A thin axon bundle connects the posterior pedal neuropile with the ipsilateral palliovisceral neuropile (V) (Fig. 1G). A nerve fiber (brachial nerve) (Bn) begins to elongate from the posterior pedal neuropile toward the intrabrachial ganglion (Ig) in the tentacle primordium. The pallial nerve (Pn) runs along the mesoderm of the collar primordium on the yolk syncytium and has reached the ipsilateral stellate ganglion (Sg). The visceral nerve (the nerve to innervate the visceral mass) (Vn) begins to arise from the palliovisceral neuropile

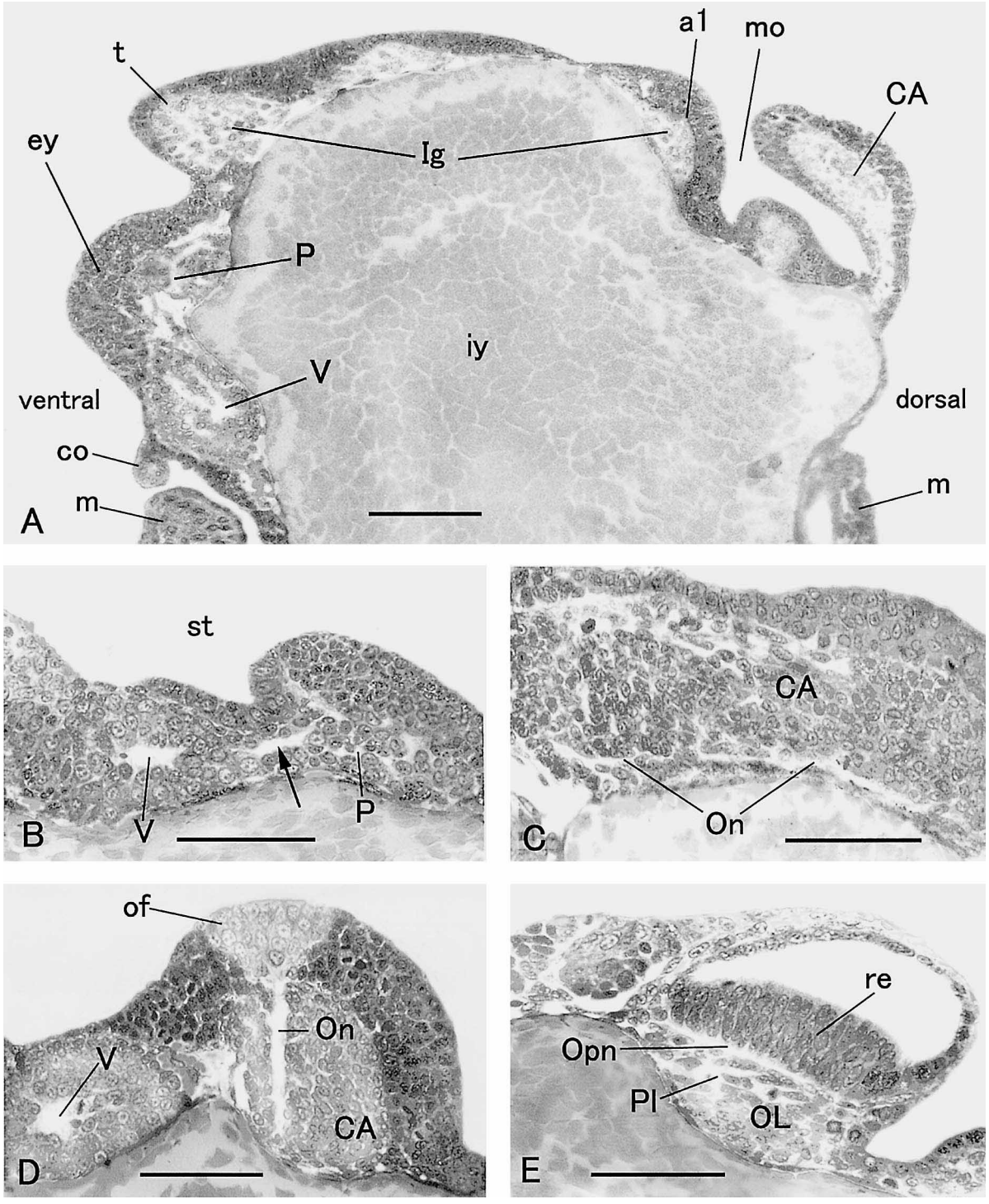

Fig. 4. Light micrographs of the developing brains in the embryos from St 21 to 22. (A) Longitudinal section of the head cut obliquely to the midplane of the embryo. St 21. (B) Longitudinal section near the lateral surface of the head. St 21. The palliovisceral (V) and posterior pedal (P) neuropiles, and the neuropile connecting them (arrow) are forming a lateral column. Left, posterior; top, ventral. (C) Longitudinal section through the foot of the cerebral arch (CA), through the posterior margin of which the olfactory nerve (On) is running. St 22. Left, ventral, Top, anterior. (D) Longitudinal section near the lateral surface of the head. St 22. Olfactory nerve (On) arising from the olfactory organ (of) enters the cerebral arch (CA). Left, posterior; top, ventral. (E) Horizontal section through the eye anlage. In the optic lobe anlage (OL), optic nerves (Opn) arising from the developing retina (re) enter the nascent plexiform layer (Pl). Left, posterior. al, arm 1; co, collar; ey, eye; Ig, intrabrachial ganglion; iy, internal yolk; m, mantle; mo, oral ingrowth; t, tentacle. Bar, 100 μm.

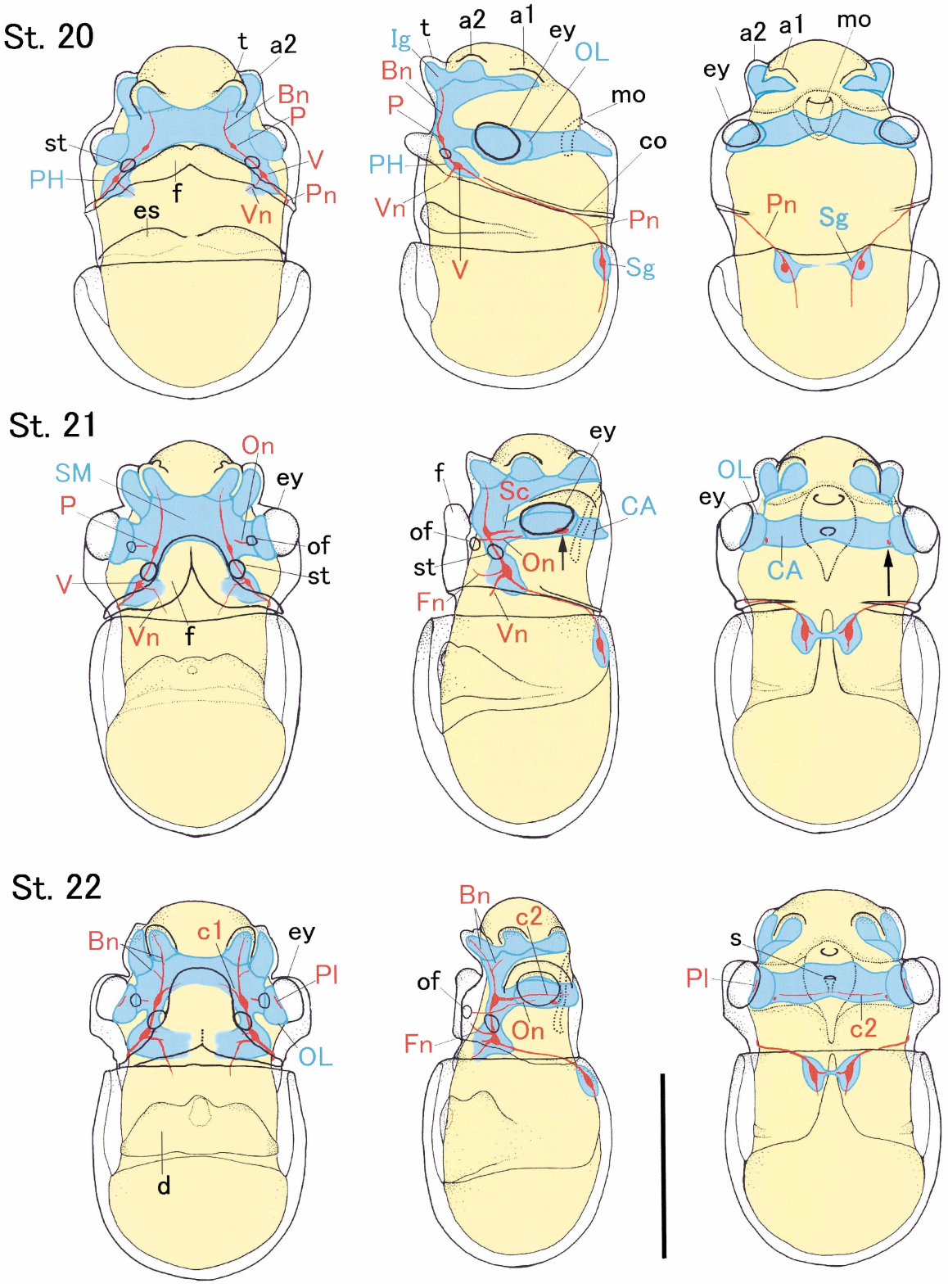

Fig. 5. Development of the nervous system in the *T. pacificus* embryos from St 20 to 22. Left, ventral view; center, left side view; right, dorsal view. The colors are used as shown in Fig. 2. a1, the first arm; a2, the second arm; Bn, brachial nerve; CA, cerabral arch, c1, commissural bundle arising from the posterior pedal neuropile; co, collar; c2, commissural bundle running through the cerebral arch; d, anlage of endodermal organs; es, endodermal swelling; ey, eye; f, funnel; Fn, posterior funnel nerve; Ig, intrabrachial ganglion; mo, oral ingrowth; of, olfactory organ; OL, optic lobe anlage; On, olfactory nerve; P, posterior pedal neuropile; PH, posterior horn; Pl, plexiform layer; Pn, pallial nerve; s, salivary duct; Sc, suprapedal commissure; Sg, stellate ganglion; SM, subesophageal mass; st, statocyst; t, tentacle; V, palliovisceral neuropile; Vn, visceral nerve; arrow in St 21, neuropile in the cerebral arch. Bar, 0.5 mm.

toward the endodermal swelling (es).

St 21 (Fig. 5). The longitudinal axon tract connecting the posterior pedal (P) and the palliovisceral (V) neuropiles becomes thick (Fig. 4A, B). The posterior funnel nerve (Fn) arises from the palliovisceral neuropile. The visceral nerve (Vn) becomes more distinct. An axon tract (Sc) arises from the posterior pedal neuropile, running in a dorsal direction in the basal region of the foot of the cerebral arch (CA). This tract is established the suprapedal commissure after hatching (Shigeno *et al*., in preparation). A small neuropile (arrow in St 21 in Fig. 5) becomes visible in the posterior region of the foot of the cerebral arch (CA). This region differentiates into the peduncle lobe and the dorso-lateral lobe anlagen in later stages. The olfactory nerve (On) begins to elongate in a dorsal direction from the olfactory organ primordium (of) located in the dorsal surface of the head (cf. Fig. 4C). The optic nerve axons occurring from visual cells of the differentiating retina in the eye primordium (ey) are recognizable in the narrow space between the retina and the optic lobe anlage (cf. Fig. 4E).

St 22 (Fig. 5): A commissural axon bundle (c1) begins to arise from the posterior pedal neuropile toward the midline of the embryo. The posterior funnel nerve (Fn) is quite distinct. In the optic lobe anlage (OL), the plexiform layer (Pl) becomes visible as a thin layer of a neuropile in the region facing the developing retina (Fig. 4E). Optic nerve axons are entering the nascent plexiform layer. A fine commissural bundle of axons (c2) is seen running through the cerebral arch. Olfactory nerve (On) is elongating through the posterior region of the foot of the cerebral arch (Fig. 4D).

St 23 (Fig. 7): A thin axonal tract (c3) is visible in the forming transverse belt bridging the left and right posterior horns (PH). In the posteriolateral region of the subesophageal mass, the palliovisceral and posterior pedal neuropiles and

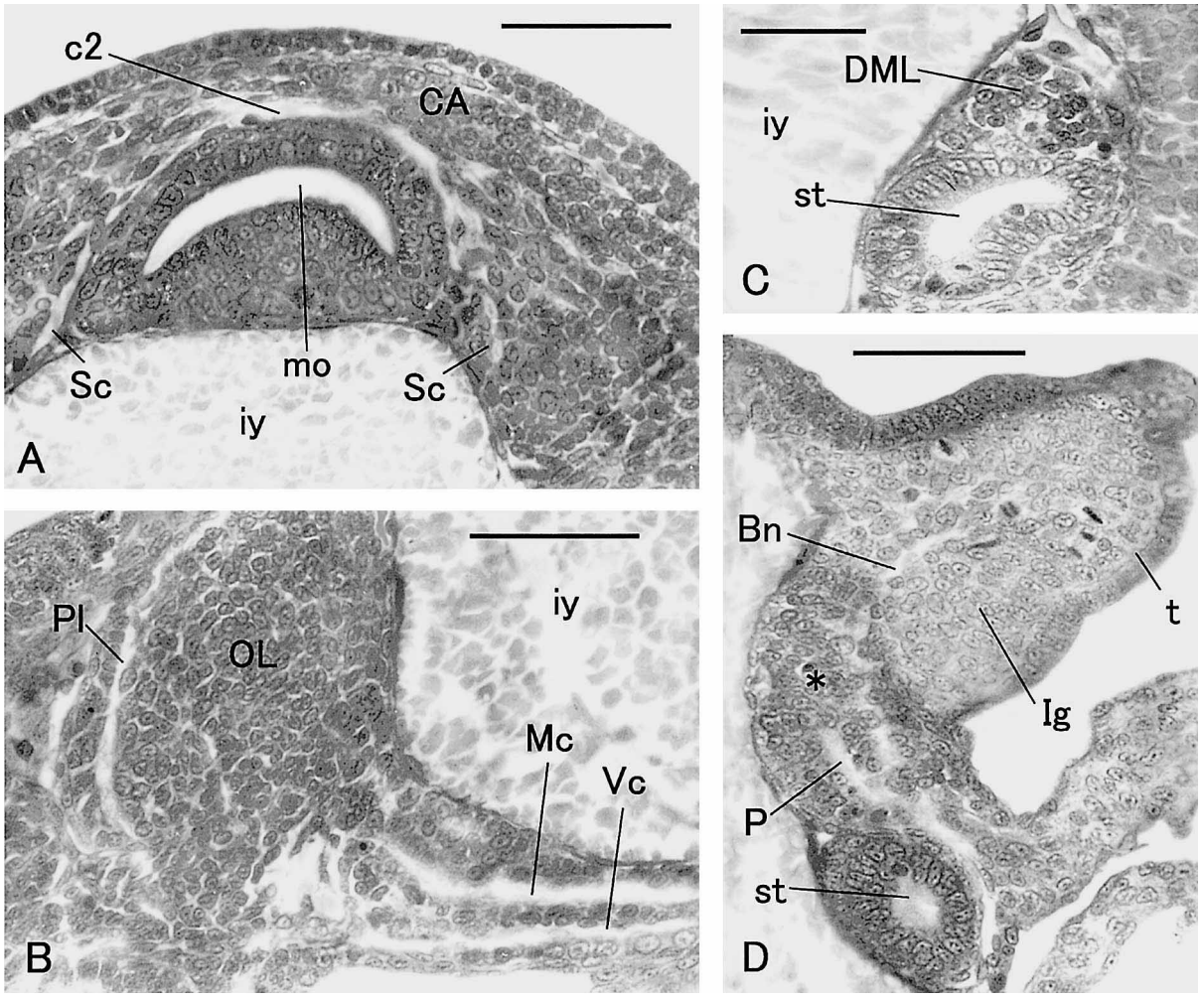

Fig. 6. Light micrographs of the developing brains at St 24. See Fig. 9 for the overall relationships among parts of the brain. (A) Transverse section through the cerebral arch. A commissural bundle (c2) basal part of which consists of the suprapedal commissure (Sc) runs along the epithelium of the oral ingrowth (mo). Top, dorsal. (B) Transverse section through the middle subesophageal mass. Median pedal (Mc) and ventral magnocellular (Vc) commissures are visible. Top, dorsal. (C) The differentiating dorsal magnocellular lobe anlage (DML) near the statocyst primordium (st). Transverse section. Top, dorsal. (D) Longitudinal section near the lateral surface of the embryo. The posterior pedal lobe anlage (∗) is in contact with the intrabrachial ganglion (Ig) in the tentacle (t). The nuclei of the neuroblasts in the former appear darker than those in the latter. Bn, brachial nerve; OL, optic lobe anlage; P, posterior pedal neuropile; Pl, plexiform layer; iy, internal yolk. Bar, 100 μm.

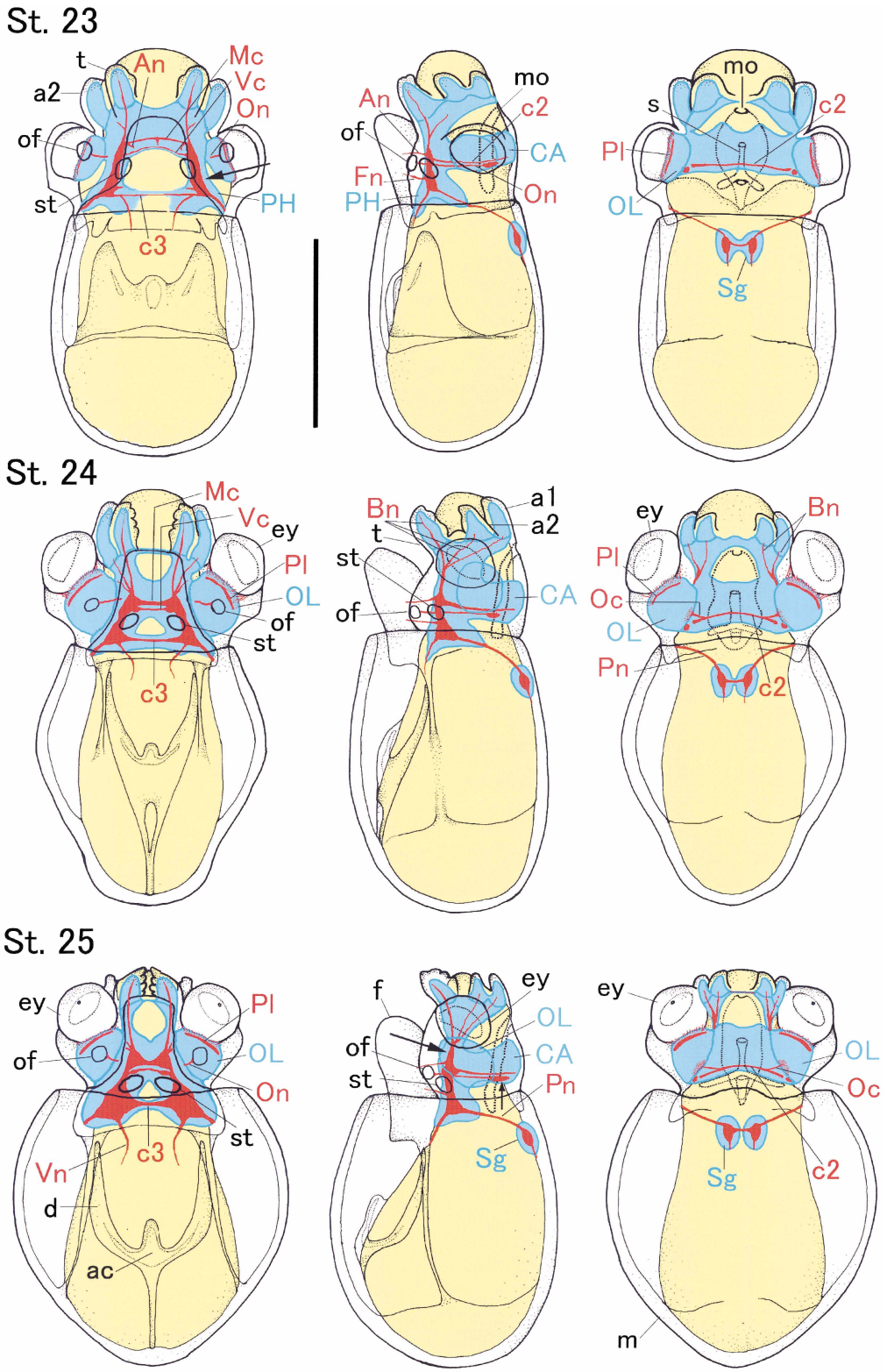

Fig. 7. Development of the nervous system in the *T. pacificus* embryos from St 23 to 25. Left, ventral view; center, left side view; right, dorsal view. The colors are used as shown in Fig. 2. ac, alimentary canal; An, anterior funnel nerve; a1, arm 1; a2, arm 2; Bn, brachial nerve; CA, cerebral arch; c2, commissural bundle running through the cerebral arch; c3, commissural bundle linking posterior horns; d, digestive gland; ey, eye; f, funnel; Fn, posterior funnel nerve; m, mantle; Mc, median pedal commissure; mo, oral ingrowth; Oc, optic commissure; of, olfactory organ; OL, optic lobe anlage; On, olfactory nerve; PH, posterior horn; Pl, plexiform layer; Pn, pallial nerve; s, salivary duct; Sg, stellate ganglion; st, statocyst; t, tentacle; Vc, ventral magnocellular commissure; Vn, visceral nerve; arrow in St 23, position of magnocellular lobe anlagen; large arrow in St 25, position of anterior and lateral posterior pedal lobe anlagen; small arrow in St. 25, position of olfactory, peduncle, and the dorso-lateral lobe anlagen. Bar, 0.5 mm.

the axon tract connecting them form a thick lateral column. The ventral, lateral and dorsal magnocellular lobe anlagen show the first sign of differentiation in the posterior to the middle part of the lateral column (arrow in St 23 in Fig. 7). In the commissural bundle linking the right and left posterior pedal neuropiles in the subesophageal mass (c1 in St 22 in Fig. 5), two commissural tracts, thick and thin, are differentiated (cf. Fig 6B). The thick one is the median pedal commissure (Mc) linking the left and right posterior pedal neuropiles, and the thin tract running posteriorly to the median pedal commissure is the ventral magnocellular commissure (Vc) linking the left and right ventral magnocellular lobe anlagen. Left and right anterior funnel nerves (An) and one median funnel nerve arise from the both ends and the midpoint of the median pedal commissure, respectively. A commissural bundle linking the right and left posterior pedal neuropiles through the cerebral arch (c2) becomes conspicuous (Fig. 6A). After hatching, the suprapedal, olfactory, peduncle and optic commissures, and some nerve fibers differentiate from this commissural bundle (Shigeno *et al.* in preparation) but they are not distinct at this stage. Only in the basal part of the foot of the cerebral arch where the commissural bundle starts from the posterior pedal neuropile, the commissural bundle consists of the suprapedal commissure alone (Sc in St 21 in Fig. 5). Olfactory nerve (On) runs in a distance with the suprapedal commissure and terminates in a cell cluster (the olfactory lobe anlage) located in the posterior region of the foot of the cerebral arch.

St 24 (Fig. 7): The transverse belt bridging the right and left posterior horns completes. The posterior horn and the transverse belt constitutes the posterior subesophageal mass. A commissural bundle (c3) runs through the transverse belt. After hatching this commissural bundle differentiates into the posterior magnocellular and the posterior chromatophore commissures, the chiasma of the first order of giant nerve fibers, and the second order giant nerve fibers (Shigeno *et al.*, in preparation) but they are not identifiable at this stage. The lateral column becomes conspicuous and the ventral, lateral, and dorsal magnocellular (Fig. 6C) lobe anlagen become more discrete in the posterior to the middle part of the lateral column. The ventral magnocellular commissure (Vc) becomes apparent (Fig. 6B). The commissural bundle running through the cerebral arch (c3) becomes thick (Fig. 6A). In the optic lobe anlage (OL), neuropile formation is evident in the plexiform layer. An axon tract (Oc) arising from the optic lobe anlage (the optic commissure) enters into the commissural bundle (c3) in the cerebral arch. The pallial nerve (Pn) becomes very thick, running on the surface of the yolk syncytium (cf. Fig. 8D). Brachial nerves (Bn) elongate from the posterior pedal neuropile toward the intrabrachial ganglia in the tentacle (Fig. 6D) as well as in the arms 1 and 2.

St 25–St 26 (Fig. 7): In the commissural bundle (c3) in the posterior subesophageal mass, a well fasciculated axon tract (the posterior magnocellular commissure) becomes evident (Fig. 8A, D). This commissure links the left and right posterior magnocellular lobe anlagen. The differentiation of the anterior pedal and the lateral pedal lobe anlagen in the posterior part of the lateral column (arrow in St 25, Fig. 7) is confirmed from the occurrence of nascent axon tracts of the post orbital and the oculomotor nerves (Fig. 8F), respectively. The post-orbital, inferior antorbital and superior antorbital nerves (Fig. 8E) arise from the posterior pedal lobe anlage (Fig. 10). A few fine nerve fibers occur toward the commissural bundle (c2) in the anterior region of the foot of the cerebral arch. The anterior basal lobe differentiates in this region after hatching (Shigeno *et al.*, in preparation) but it is not identifiable at this stage. The peduncle lobe anlage and the dorso-lateral lobe anlage are differentiating in the posterior region of the foot of the cerebral arch (arrow in St 25, Fig. 7). Some nerve fibers from the palliovisceral neuropile enter the neuropile in the peduncle lobe anlage (not shown in the Figure). In the optic lobe anlage (OL), a few nerve fibers spread over the zone central to the plexiform layer, (the radial columns and the tangential zone) but neuropiles are hardly visible there.

State of the brain at the time of hatching

The brain in the embryos at the time of hatching (Fig. 10) consists of the optic lobe anlage (OL), the subesophageal mass subdivided into the posterior (PSM), middle (MSM) and anterior subesophageal masses (Fig. 8C), and the cerebral arch (CA) (correspond to the supraesophageal mass in the adult brain)(also see Fig. 9). The brain is in a quite premature condition at the hatching stage (St 26). Many of the brain lobes found in the adult brain do not begin to differentiate. Especially in the dorsal roof of the cerebral arch, no neuropiles except for the commissural bundle (c2) are present and no brain lobes are identifiable. In the posterior region of the foot of the cerebral arch, the anlagen of brain lobes such as the olfactory, peduncle, and dorso-lateral lobes are differentiating. Brain lobe anlagen such as the anterior pedal, lateral pedal, posterior pedal, ventral magnocellular, lateral magnocellular, and dorsal magnocellular lobe anlagen are differentiating from the middle to the posterior subesophageal masses. The palliovisceral neuropile has not yet differentiated into brain lobes. The neuropiles of the brain lobe anlagen, the palliovisceral neuropiles, and the axon tracts connecting these neuropiles form a large lateral column (Fig. 8C and large arrow in Fig. 10). In the anterior subesophageal mass, neuropiles are scarcely visible. Four commissural bundles are present, two in the middle subesophageal mass, one in the posterior subesophageal mass, and one in the cerebral arch (small arrow in Fig. 10). In these commissural bundles, three commissures: median pedal (Mc), ventral magnocellular (Vc), and posterior magnocellular commissures (Pc), are established. Many commissures found in the adult brain are not yet differentiated. The right and left lateral columns together with 4 commissural bundles form a ladder-like structure that we named ladder-like framework. Most of the main brain nerves are present (Fig. 10). They are: the pallial (Pn), visceral (Vn), brachial (Bn), oculomotor (Cn), postorbital (Tn), inferior antorbital (In), superior antorbital (Sn), anterior funnel (An), median funnel, and posterior funnel nerves (Fn). The optic nerve from the retina in the eye (ey) and the olfac-

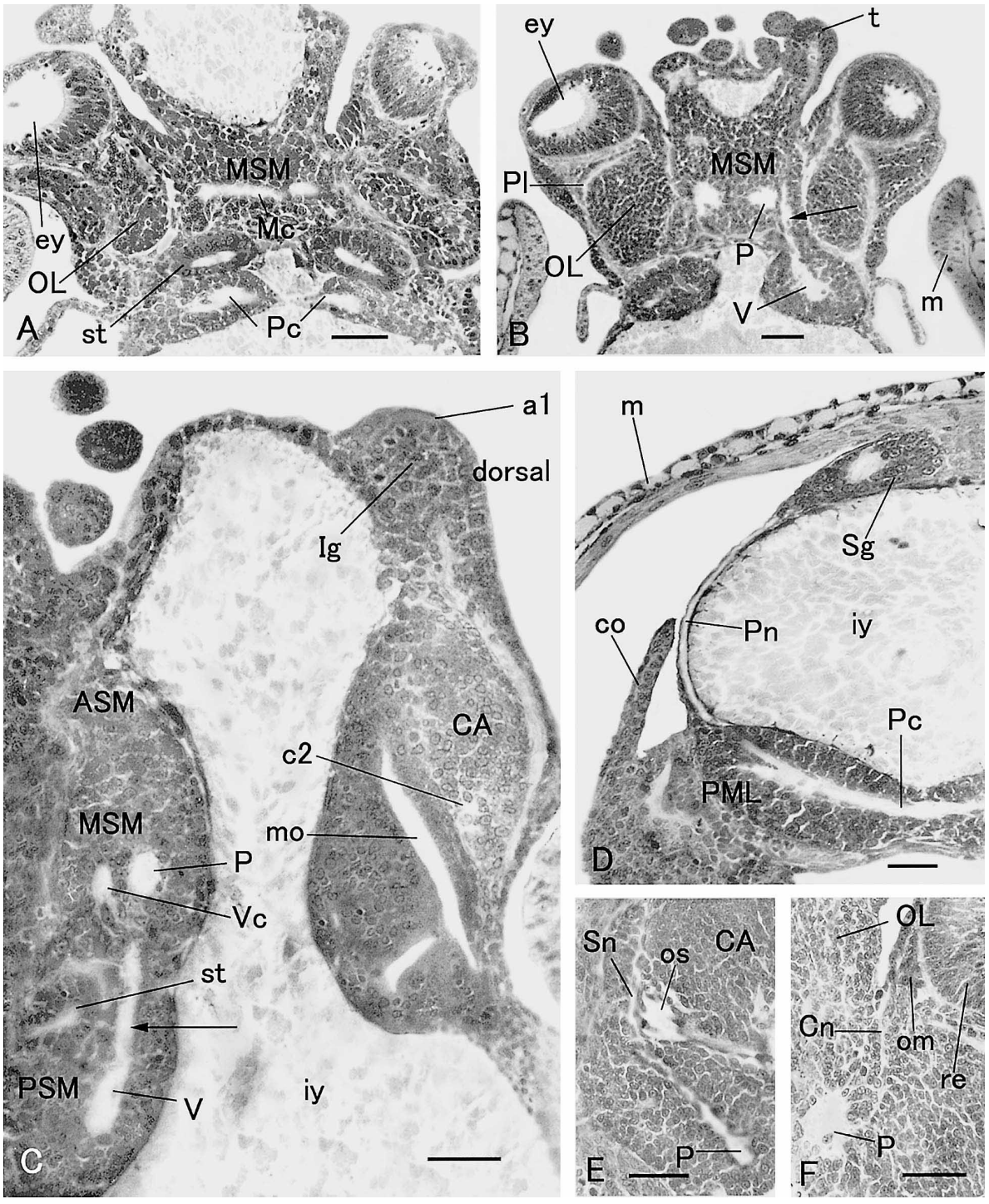

Fig. 8. Light micrographs of the developing brains in the embryos at St 25–26. (A) Horizontal section of the head through a plane near the ventral surface. Median pedal (Mc) and posterior magnocellular (Pc) commissures are visible. St 25. (B) Horizontal section of the head through a plane more dorsal to the plane in (A). The arrow indicates the neuropile forming the lateral column with the posterior pedal (P) and palliovisceral (V) neuropiles. St 26. (C) Longitudinal section of the head cut obliquely to the midplane of the embryo. The ventral part of the brain is subdivided into the anterior (ASM), middle (MSM), and the posterior (PSM) subesophageal mass. The arrow indicates the neuropile forming the lateral column with the posterior pedal (P) and palliovisceral (V) neuropiles. The lateral ends of the ventral magnocellular (Vc) and the commissural bundle (c2) through the cerebral arch (CA) show cross sectional profiles. St 26. (D) Transverse section through the posterior part of the head. The pallial nerve (Pn) arising from the palliovisceral neuropile runs along the surface of the internal yolk (iy) to the stellate ganglion (Sg). The posterior magnocellular commissure (Pc) linking the posterior magnocellular lobe anlagen (PML) is visible. St 25. (E) Horizontal section of the head showing the superior antorbital nerve (Sn) arising from the posterior pedal neuropile (P). St 26. (F) Horizontal section of the head showing the oculomotor nerve (Cn) arising from the lateral part of the posterior pedal neuropiles (P). St 26. a1, arm 1; co, collar; ey, eye; Ig, intrabrachial ganglion; iy, internal yolk; m, mantle; mo, oral ingrowth; OL, optic lobe anlage; om, oculomotor muscle; os, optic sinus; Pl, plexiform layer; re, retina; st, statocyst; t, tentacle; Bar, 50 μm.

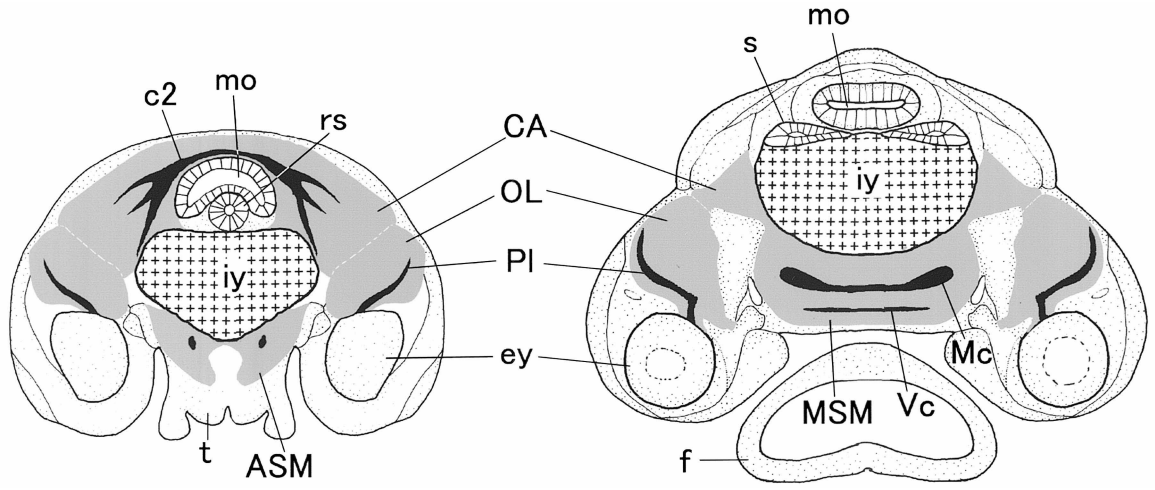

Fig. 9. Transverse sections of the head in the embryo at St 25, showing ring-like configuration of the brain anlagen encircling the oral ingrowth (mo) and the internal yolk (iy). The left section is cut through a plane posterior to the right one. Top, dorsal. ASM, anterior subesophageal mass; CA, cerebral arch; c2, commissural bundle through the cerebral arch; ey, eye; f, funnel; Mc, median pedal commissure; MSM; middle subesophageal mass; OL, optic lobe anlage; Pl, plexiform layer; rs, radular sac; s, salivary gland; t, tentacle; Vc, ventral magnocellular commissure.

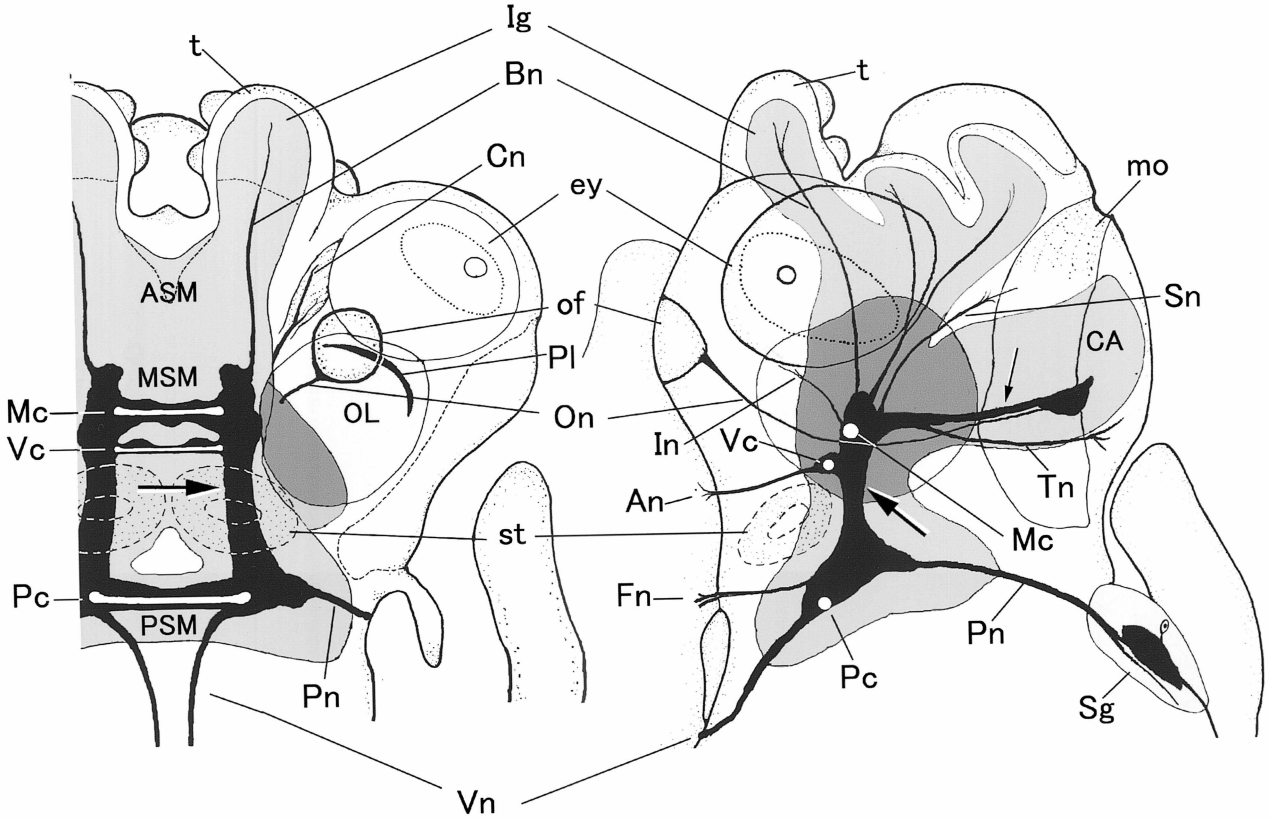

Fig. 10. The ventral (left) and the left side view (right) of the head of the hatchling (St 26), showing the neuropiles (black), commissures (white), and brain nerves. The neural tissue is showed in grey. An, anterior funnel nerve; ASM, anterior subesophageal mass; Bn, brachial nerve; CA, cerebral arch, Cn, oculomotor nerve; ey, eye; Fn, posterior funnel nerve; In, inferior antorbital nerve; Mc, median pedal commissure; mo, oral ingrowth; MSM; middle subesophageal mass; of, olfactory organ; OL, optic lobe anlage; On, olfactory nerve; Pc, posterior magnocellular commissure; Pl, plexiform layer; Pn, pallial nerve; PSM, posterior subesophageal mass; Sg, stellate ganglion; st, statocyst; Sn, superior antorbital nerve; t, tentacle; Tn, post-orbital nerve; Vc, ventral magnocellular commissure; Vn, visceral nerve; large arrow, lateral column; small arrow, commissural bundle through the cerebral arch.

tory nerve (On) from the olfactory organ (of) terminate in the optic and olfactory lobe anlagen, respectively.

DISCUSSION

In the present study, we have described the process of brain development up to the hatching stage for the first time in the oegopsid squid, *Todarodes pacificus*. The development of the brain has been described in the myopsid squids, *Loligo vulgaris* (Meister, 1972) and *Sepioteuthis lessoniana* (Shigeno *et al.*, in preparation), and in the octopus, *Octopus vulgaris*, (Marquis, 1989). We found that the morphological sequence of the brain formation in *T. pacificus* is essentially similar to that in the above 3 species. The neuroblasts occurring in placodal thickenings of the ectoderm ingress in a group to form 4 pairs of ganglia between the surface ectoderm and the yolk syncytium. The ganglia accumulate into a ring-like belt around the head. The belt is gradually consolidated into a thick cell mass, which differentiates into the brain lobes through axonogenesis following an orderly sequence. This indicates that brains of the coleoid cephalopods are conserved not only in the anatomical plan (Young, 1977a, 1988; Maddock and Young, 1987; Budelmann, 1995) but also in the morphogenetic process.

In the embryonic brain of *T. pacificus*, axonogenesis, which is recognizable as neuropile formation in the conventional histological sections, began in the posteriolateral region of the ventral surface derived from the palliovisceral ganglion (future posterior subesophageal mass), followed by the midlateral region of the ventral surface derived from the pedal ganglion (future middle subesophageal mass). Neuropiles appeared in the anterior subesophageal mass at late embryonic stages but they were not present at the time of hatching in the dorsal part of the brain (supraesophageal mass). The same orderly sequence of neuropile formation, i.e. from posterior to anterior and from ventral to dorsal, is commonly observable in the myopsid squids (Meister, 1972; Shigeno *et al.*, in preparation) and the octopus (Marquis, 1989).

A major difference in the brain development between *T. pacificus* and the above 3 species was a marked delay in the differentiation of brain lobes in the supraesophageal mass. The timing of nervous development can be compared among cephalopod species on the basis of the table by Naef (1928), where embryonic stages (shown in Roman numerals) common to most cephalopod groups are determined according to the major external features. In all 4 species examined, neuroblasts ingress at St VIII and the first neuropile forms in the posterior subesophageal region at St X. In the myopsid and the octopod species, brain lobes begin to differentiate in the supraesophageal mass at St XIV, but in *T. pacificus* neuropiles are absent there even at St XVI. This delay in the formation of the future motor center may reflect the difference in feeding behaviors; *Todarodes* larvae are thought to be suspension feeders but the larvae of the other 3 species are known to be active predators (Boletzky, 1977; Segawa, 1987).

In all the cephalopod embryos examined, the brain originates from 4 pairs of separately formed ganglia: optic, pedal, palliovisceral (referred to as visceral in the previous reports), and cerebral ganglia. This indicates that the highly consolidated cephalopod brain is formed according to the ground plan of the molluscan central nervous system (CNS), where discrete ganglia are interconnected to form a ring around the esophagus (Bullock and Horridge, 1965). In opisthobranch gastropods, the CNS showing wide range of ganglionic fusions around the esophagus begins from 3 pairs of discrete ganglia forming a circumesophageal ring in the veliger larvae (Page, 1992a, b). The cerebral, pedal, and the pleural ganglia of the veliger larva are thought to correspond to the cerebral, pedal, and palliovisceral ganglia, respectively, in the *T. pacificus* embryo from their disposition in the circumesophageal ring. The optic ganglia may develop specifically in the cephalopods as a part of their marked visual system.

The brain of the nautiloid cephalopods is considered more primitive than that of the coleoid cephalopods on the basis of the comparative anatomy (Young, 1965, 1987). The *Nautilus* brain is less centralized than the coleoid brains. It consists of a circumesophageal cluster of 3 nerve cords (cerebral, anterior subesophageal and posterior subesophageal cords), in which only several brain lobes are discriminated. Such a feature in the *Nautilus* brain is very similar to that in the embryonic brain of *Todarodes* before centralization. Thus, a comparison of the adult *Nautilus* brain with the embryonic *Todarodes* brain will be helpful to gain an insight into the brain evolution in the cephalopods. The cerebral, anterior subesophageal, and posterior subesophageal cords in the *Nautilus* brain correspond to the cerebral arch, anterior to middle subesophageal mass, and the posterior subesophageal mass in the embryonic *Todarodes* brain, respectively, according to their configuration. In the *Nautilus* brain, each brain cord contains one commissural bundle, which is not separated into individual commissures. In the embryonic brain of *Todarodes*, the cerebral arch and the posterior subesophageal mass contained one commissural bundle within which individual commissures are not yet differentiated. In the *Nautilus* brain, the 3 brain cords meet at the lateral side of the subesophageal region and the main brain nerves such as the oculomotor, ophthalmic and funnel nerves arise from a well-developed neuropile in this region. In the embryonic *Todarodes* brain, a large neuropile giving rise to main brain nerves was well developed where the cerebral arch, posterior subesophageal mass, and middle subesophageal mass met. The brain lobes found in the *Nautilus* brain are: the optic, olfactory, magnocellular, lateral cerebral, and brachial lobes. The fact that the optic, olfactory, and magnocellular lobes with homologous partners in the *Nautilus* brain occurred in the embryonic *Todarodes* brain is suggestive of a plesiomorphic character of these brain lobes. The peduncle lobe was identified in the embryonic *Todarodes* brain at the position corresponding to that of the lateral cerebral lobe of *Nautilus* brain, but at present it is not certain whether the two lobes are homologous. The basal part of the cerebral cord in *Nautilus* exhibits a complex structure with the olfactory, optic and lateral cerebral lobes. In the correspond-

ing part of the cerebral arch in *Todarodes* embryos, the neuropiles of the olfactory, optic, peduncle, and dorso-lateral lobes showed a complex interconnection. The conformal correspondence between brains of the adult *Nautilus* and the *Todarodes* embryos indicates that the coleoid brain has evolved on the basic plan as seen in the extant nautiloids.

We could clarify an outline of the early sequences of the axon tract formation in the cephalopod brain within the range of limited resolution provided by conventional histological sections. The axon tracts in the premature brain in just hatched individuals showed a simple ladder-like structure (ladder-like framework) consisting of bilaterally situated longitudinal tracts bridged by some transverse tracts. Recent histochemical and immunocytochemical methods have revealed in several species, e.g. *Drosophila* (Therianos *et al*., 1995; Nassif, 1998), zebrafish (Wilson *et al*., 1990; Chitimis and Kuwada, 1990) and mouse (Easter *et al*., 1993), that highly complex circuitry of the adult brain is conformed to a relatively simple pattern of axon tracts in the embryonic brain. The ladder-like framework in the embryonic brain of *Todarodes* is thought to function as the primary framework on which the complex neural circuitry in the adult brain is constructed. Kimmel (1993) has pointed out that gigantic neurons are working as pioneer neurons in the ventral longitudinal tracts in the embryonic brain of the zebrafish. The pallial nerves, the earliest recognizable nerves during the cephalopod embryogenesis, are known to contain the giant nerve fiber system in the adult (Young 1939). It was pointed out in *Sepioteuthis* that the giant fiber system might play a role as pioneer axons (Shigeno *et al*., in preparation). However, the present observation that the posterior subesophageal mass through which the second order giant axons enter the pallial nerves was not completed at the time of the pallial nerve formation proves that idea improbable. Observations using modern techniques will be required to know how pioneer axons determine the route of the first axon tract in the ladder-like framework and how the axons join and build up the ladder-like framework. We are undertaking immunocytochemical and ultrastructural studies of neurogenesis in cephalopods.

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